Characterization of humic acids derived from rabbit manure treated by composting-vermicomposting process

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Abstract

The aim of this work was to evaluate, through different analytical techniques, the evolution of some chemical and physicochemical properties of humic acid (HA)-like fraction extracted from an organic amendment, like rabbit manure, handle with composting followed by vermicomposting process, in comparison with soil HA, in order to reach an appropriate criterion to evaluate the maturity of an organic amendment.

The raw material used was rabbit manure treated with composting followed by vermicomposting process. Soil and organic amendments were analyzed by chemical properties. The HA was extracted from the amendments samples and from an Entic Haplustol and analyzed the chemical and physicochemical characteristics by several analytical methods.

Through these analytical techniques was possible to follow the evolution of the HA-like fraction during this stabilization-maturation process

Through the composting followed by vermicomposting process the HA-like fraction are evolving toward more stable and complex macromolecule resembling native soil HA.

Keywords: organic matter, humic acids, capillary zone electrophoresis, elemental analysis.

1. Introduction

Animal wastes present health and environmental risks and should be treated accurately. Therefore, the need for more environmental sound methods for the treatment and utilization of animal manure has become imperative (Ko *et al*., 2008).

Composting and vermicomposting cannot be considered as new technologies, but these processes lead to a final stabilized product which can be used to improve and maintain soil quality and fertility (Larney and Hao, 2007; Lazcano *et al*., 2008; Caricasole *et al*., 2011)

The use of compost and/or vermicompost is an economical solution for several agricultural and environmental problems and their application is an acceptable way to improve soil fertility and plant growth (Larchevêque *et al*., 2006; Loffredo *et al*., 2008, Loffredo and Senesi, 2009)

Composting involves the accelerated degradation of organic matter by microorganisms under controlled conditions, in which the organic material undergoes a characteristic thermophilic stage that allows sanitization of the waste by the elimination of pathogenic microorganisms.

Vermicomposting involves the bio-oxidation and stabilization of organic material by the joint action of earthworms and microorganisms. The combination of composting and vermicomposting has recently been considered as a way of achieving stabilized substrates. Composting enables sanitization of the waste and elimination of toxic compounds, and the subsequent vermicomposting reduce particle size and increases nutrient availability (Lazcano *et al*., 2008).

The success in compost and/or vermicompost application is based on the use of reliable criteria for testing compost stability and maturity. Soil application of an unstable organic amendment can harm plant, by the phytotoxic compound production, defi-ciency of nitrogen and oxygen concentration, thus the plant yield would be reduced (Senesi *et al*., 2007).

A number of criteria and parameters have been proposed and used as guidelines to test the evolution and modification occurring during the composting and/or vermicomposting process and to evaluate the stability and maturity in the end-product (Smidt and Tintner, 2007; Sellami *et al*., 2008; Ponsá *et al*.,2010; Pognani *et al*., 2010; Xiaowei Li *et al*., 2011). Several indices are essential for assessing the end-product condition; these include colour, odours, temperature, respirometric test, C/N, inorganic nitrogen, cation exchange capacity (CEC), germination index (GI). Other parameters like: moisture content, total organic carbon (TOC), humic-like substances (C_{HS}, C_{HA}, C_{FA}), total nitrogen content (TN), and different methodology, have also been used to determine the state of stability and/or maturity of these materials (Lasaridi *et al*., 2006; Lhadi *et al*., 2006; Smidt and Tinter 2007; Campitelli and Ceppi 2008a; Sellami *et al*., 2008; Xiaowei Li *et al*., 2011; Pognani *et al*., 2011).

However, the amount and quality of humic like substances (HS) in an organic amendment are considered as important marker of its maturity, a guarantee for a safe impact and successful performance in soil (Xiaowei Li *et al*., 2011).

Due to the beneficial properties that would produce the HA-like fraction, the most stable fraction of the HS, their characteristics are suggested as a quality parameter (Smidt and Tinter 2007; Senesi *et al*., 2007). Therefore, a better understanding of the structures evolution of HS is necessary in order to comprehend the natural process occurring along the maturation process (Fuentes *et al.,* 2006; Romero *et al*., 2007).

Some methodologies and techniques are proposed to study HA-like fraction of the organic amendments.

The results of these studies have shown that the HA-like fraction extracted from the organic amendments differ from native soil HA.

Some of these methodologies and techniques are: elemental and functional group analyses, spectroscopic measurements such as UV-Vis, FTIR and ^{13}CNMR, capillary zone electrophoresis (CZE), potentiometrics titrations, thermal analyses, fluorescence spectroscopy, among others, (Velasco *et al*., 2004; Pietro and Castaldi 2004; Peuravuori *et al*., 2004; Ceppi *et al*., 2005; Lguirati *et al*., 2005; Peuravuori *et al*., 2005; Campitelli *et al*., 2006; Lhadi *et al*., 2006; Grube *et al*., 2006; Senesi *et al*., 2007; Sen and Chandra 2007; Smidt and Tinter 2007; Plaza *et al.,* 2008; Sellami *et al*., 2008; Campitelli and Ceppi 2008a; Fernandez *et al*., 2008; Vanifatova *et al*., 2008; Xiaowei Li *et al*., 2011; d`Orlyé and Reiller 2012).

During the maturation process, is possible to consider that the humification takes place in the stabilized organic matter (OM), and thus, the HA-like fraction suffers changes in their fraction suffers changes in their chemical and physicochemical properties chemical, physicochemical properties.

Therefore, one of the most appropriate and reliable criteria to evaluate maturity of any organic amendment should be based on the analysis of chemical and physicochemical properties of HS-like components, i.e. humic acids (HA)-like and fulvic acids (FA)-like fraction in the organic amendment, in comparison with the properties of native soil HA and FA (Senesi *et al* 2007; Droussi *et al*., 2009).

The aim of this study was to evaluate, through different analytical techniques, the evolution of some chemical and physicochemical properties of HA-like fraction extracted from an organic amendment, like rabbit manure, handle with composting followed by vermicomposting process in comparison with soil HA, in order to reach an appropriate criterion to evaluate the maturity of an organic amendment.

2. Material and methods

2.1 Soil and organic amendments analyses

The soil used to extract HA is classified as Entic Haplustol (USDA classification) located in the Province of Cordoba, Argentina.

The raw material used was rabbit manure treated with composting followed by vermicomposting process. During the composting process the thermophilic phase took place for 30 days, the process was carried out for 60 days more (maturation phase), (90 days was the final age for the composting process). The outdoor pile was turned every 15 days during the composting process in order to improve the O_2 level inside the pile. Pile moisture was controlled weekly by adding enough water to obtain moisture content not less than 50 % in composting phase. Then, the pile was spread out in ground beds (1m x 10 m) and inoculated on the first 15-20 cm depth with about 500 g m^{-2} earthworms (*Eisenia foetida*). The vermicomposting process was carried out for 120 days more (total time for composting followed by vermicomposting process was 210 days). During the vermicomposting process, the pile moisture was controlled weekly by adding enough water to obtain moisture content not less than 70 %.

The samples were collected during the composting process, at two different times: 30 days at the thermophilic phase (named **TRM**) and 90 days (named **CRM**). At each time 10 sub samples were taken at 25 cm depth in the pile and then mixed to obtain the composite sample. At the end of the vermicomposting process (210 days named **VRM**) 10 sub samples were collected at 25 cm depth in the worm bed, and then mixed to obtain the composite sample.

Soil and each organic amendment (**TRM, CRM, and VRM**) composite samples were air-dried, crushed and passed through a 2 mm sieves before all the analytical analysis.

Soil and organic amendments were analyzed for pH at a rate 1:2.5 (w:v) and 1:5 (w:v) respectively. Total Organic Carbon (TOC) was analyzed by combustion at 540 ^{0}C for 4 h (Abad, *et al*., 2002). Total nitrogen content (TN) in soil, and organic amendments were obtained by the Kjeldahl method. The carbon content of humic substances (C_{HS}), humic acids (C_{HA}) and fulvic acids (C_{FA}) were determined according to the technique proposed by Syms and Haby (1971). The carbon content of each fraction (C_{HS}, C_{HA} and C_{FA}) were calculated as percentage of the TOC, therefore, the % C_{HA} correspond to the Humification index (HI) (Roletto *et al*., 1985).

2.2 Humic Acids isolation

HA from the organic amendments and soil were extracted with NaOH 0.1 mol L^{-1}, purified with HCl:HF (1:3) and dried at low temperature until constant weight, according to the procedure recommended by Chen *et al*. (1978).

The HA samples used in this work were:
- HA extracted from an Entic Haplustol of Province of Cordoba, Argentina. Denoted as: HA-S. - HA extracted from rabbit manure at the thermophilic phase of the process. Denoted as: HA-TRM. - HA extracted from composted manure. Denoted as: HA-CRM.- HA extracted from vermicomposted manure. Denoted as: HA-VRM.

All solutions were prepared with tridistilled water and all the reagents were ACS reagent grade.

2.3 Humic Acids analyses

HA ash content was measured by heating it at 550 ^{0}C for 24 h. The elemental composition for C, H, N, S was determined by an analyzer instrument Carlo Erba 1108, using isothiourea as standard. Oxygen was cal-

culated by difference: O%= 100 - (C+H+N+S) % (ash and moisture-free basis).

To obtain the E4/E6 ratio, the absorbance at 465 nm and 665 nm was measured using Spectronic 20 Genesys Spectrophotometer on solution containing 3.0 mg of each HA in 10 mL of 0.05 mol L^{-1} NaHCO$_3$. The ratios of absorbance at 465 nm and 665 nm gave the E4/E6 ratio (Kononova, 1982).

2.4 Potentiometric titration

Potentiometric titrations were carried out according to the technique proposed by Campitelli *et al*. (2003), which is briefly: HA solution of each samples were prepared by dissolving HA ($\approx$ 50 mg) with minimum volume of NaOH solution (0.1 mol L^{-1}) and adding water up to the final volume (50 mL). An aliquot containing the desired amount of HA ($\approx$7–8 mg) was transferred to the titration flask containing 10 mL of tridistilled water. The titrant (HCl =0.05 mol L^{-1}) was added from an automatic burette (Schott Geräte T80/20) at a titrant rate of 0.1 ml/40 s. This rate was chosen taking into consideration that the variation of pH values should range between 0.02 and 0.04 pH units. The pH values were measured with an Orion Research 901 pH meter equipped with a glass-combined electrode (Orion 9103 BN). All titrations were performed in KCl 0.01 mol L^{-1} as background electrolyte. The same titration was followed in absence of HA (reference or blank titration) for each titration curve, in order to subtracts it from the raw data titration, and thus obtain the charge developed by the HA sample. Each HA solution, with the corresponding blank solution, was titrated by triplicate and the reported data representing the average values. All the reagents were ACS reagent grade.

2.5 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) experiments were performed on an Agilent Technology Capillary electrophoresis system equipped with a diode array. Operation of the instrument, data collection and analysis were controlled by Agilent ChemStation software. The polarity was negative, voltage of -10kV, temperature 25^0C, total run time 20 min (for time migration higher than 20 min no significant peak were observed). Samples were injected hydrodynamically using pressure of 5000 Pa for 60 s. The absorbance was monitored at three different wavelengths (243 nm, 214 nm and 260 nm) and 243 nm was selected to report.

Each HA electropherogram was carried out by triplicate and the reported data representing the average values.

The dimensions of the fused-silica capillary were 75 µm internal diameter; 81.7 cm total length and 73.2 cm effective length.

All the solutions and background electrolyte (BGE) were prepared from analytical (p.a. or HPLC) chemicals and ultra pure water.

BGE was buffer borate 20 mmol L^{-1} at pH=9.3, the concentration of the HA solutions were 1000 ppm.

At the beginning of daily work, the capillary was washed for 5 min with 0.1 mol L^{-1} NaOH solution, followed by 5 min washing with ultra pure water and 20 min with BGE at 25^oC and 10^4 Pa. At the end of the daily work, the capillary was rinsed with BGE for 5 min and water for 10 min, at the same temperature and pressure condition.

The capillary was treated before each sampling injection, as following, pre-condition: 2 min with NaOH 0.1 mol L^{-1} at 10^4 Pa, followed by washing with BGE for 3 min at 10^4 Pa, and finally waiting for 1 min. Post-run conditions were: 1 min with NaOH 0.1 mol L^{-1} at 10^4 Pa, followed by 5 min with water at the same pressure.

3. Results and discussion

3.1 Chemical properties and humification parameters

The pH value was similar for **TRM** and **CRM**. At the end of the vermicomposting process (**VRM**), the pH value significantly decrease; probably due to the mineralization of N and P compounds, the release of CO_2 and the presence of organic acids from microbial metabolism (Kaushik and Garg, 2004; Lazcano *et al*., 2008; Xiaowei Li *et al*., 2011). Total organic carbon (TOC) and total nitrogen (TN) in the sample at thermophilic phase (**TRM**) was high, however, both contents decreased after the vermicomposting process (Table 1). The decreasing in TOC was about 35% between the thermophilic phase and the composting process and during the vermicomposting process the loss of TOC was lower (~ 6%). Labile organic compounds, such as simple carbohydrates, fats and amino acids, are degraded in the first stages of the process; cellulose, hemicelulose, lignin and other resistant substrates, take longer time for their degradation and transformation (Bernal *et al*., 2009). The decreasing of TN was about 28% at the end of the composting process (90 days). This behaviour indicates the occurrence of a net organic matter mineralization, volatilization of ammonia N and/or lixiviation. The TN content in **VRM** was practically the same than in **CRM**, which indicate that in this period (vermicomposting) the content of N compounds was degraded at lower rate than in the

former phase of the process (composting) or probably the N contribution from the biological activity (urine and feces) of the earthworms could balance the lost trigged by the degradation (Gajalakshmi *et al*., 2005). The decreasing in C/N ratio (Table 1) during the composting process and the slightly variation in the vermicomposting phase indicate the development of the raw material towards a maturity state, suggested also through the increasing in the % C_{HS} content (Table 1)

Table 1: Chemical characteristics of soil and organic amendments at the different time of the process.

Samples	pH	TOC	TN	%C_{HS}*	%C_{HA}*	%C_{FA}*	C_{HA}/C_{FA}	C/N
Soil	6.40	28.4	2.0	35.9	17.9	18.0	0.9	14.2
TRM	9.23 a	620.5 a	20.6 a	3.05 a	5.80 b	7.25 b	0.8 a	30.1 a
CRM	9.15 a	403.7 b	14.9 b	4.02 b	6.69 c	3.72 a	1.8 c	27.0 b
VRM	8.51b	379.7 c	14.2 b	7.64 c	4.78 a	3.16 a	1.5 b	26.7 b

* % C_{HS}, C_{HA}, C_{FA} are calculated as function of TOC. TOC, TN (g kg^{-1}) Different letters (a–c), for organic amendments, in the same column indicate significant differences *(P< 0.05)* according to Tukey test (For the statistical estimation the soil was not take into consideration).

The humification parameter, the % C_{HS} , slightly increase during the composting phase (90 days), and through the vermicomposting period the C_{HS} content is about 50% greater than in composted material (**CRM**). This behaviour could be due since the vermicomposting led to homogeneous degradation of all types of C compounds, with no selective accumulation of any preferential form, and thus increase the total extractable organic C in alkaline solution (Sen and Chandra 2007; Plaza *et al*., 2008).

The %C_{HA} (humification index) increase in the first phase of the process (90 days) followed by a noticeable decrease (~28%) at the end of the vermicomposting period (Table 1). This behaviour reflects, probably, a reduction of aromatic structures, suggesting that in the final product of this vermicomposting process the organic compounds are more degradable, probably with a greater content of aliphatic structures than in the compost end-product. It seems that the earthworm produces a final product with some homogeneity between both fraction content (C_{HA} and C_{FA}) (Table 1) (Sen and Chandra 2007; Sellami *et al*., 2008). The decreasing in this fraction (%C_{HA}) could be produced, also, for the lixiviation of some hydrosolubles organic compound due to the high moisture content during this phase of the total process

C_{FA} content (Table 1) shows a continuous decrease throughout the complete time of the process (210 days), this can be explained taking into account the nature of this fraction (FA), which contain easily degradable compound such as polysaccharides which were exposed first to microbial attack (Sellami *et al*., 2007; Plaza *et al*., 2008; Xiaowei Li *et al*., 2011). The final stages of vermicomposting process would involve several changes and transformation in the chemical structure of the organic matter.

The ratio C_{HA}/C_{FA} (Table 1), also known as "degree of polymerization or polymerization index", increase during the composting process (90 days), reflecting the formation of complex molecules (C_{HA}) from simpler one (C_{FA}) and the occurrence of synthesis of organic compounds resistant to microbial degradation (Plaza *et al*., 2008). Roletto *et al*. (1985) used the ratio of C_{HA}/C_{FA} to establish the humification level of the OM of compost from different origins, including manure, indicating a value greater than 1 for a good humification

degree. However, Senesi (1989) and Iglesias-Gimenez and Perez-Garcia (1992) have suggested that a ratio C_{HA}/C_{FA} greater than 1.6 indicate a good maturity degree achieved by the organic matter.

The ratio (C_{HA}/C_{FA}) slightly decreases during the vermicomposting time, reaching a value of 1.5, suggesting a roughly reduction in the contents of molecules with a high degree of condensation and humification, which could indicate that the earthworm activities led to a homogeny degradation of all types of C compounds without selectivity, however, maturity level reached do not decrease.

Earthworms are known to digest long chains of polysaccharides, enhancing microbial colonization and thus, changing the structure of the raw material. This behaviour suggests an increase in the total aliphatic C and polysaccharides at the end of vermicomposting suggesting neosynthesis in the vermicompost (Sen and Chandra 2007).

3.2 Elemental composition of HA-like

The elemental analysis, H/C, N/C, O/C, ash content and E4/E6 ratio are shown in Table 2.

Table 2: Elemental composition (ash and moisture-free basis), H/C, N/C, O/C (atomic ratios) and E4/E6 ratio of the HA studied.

Humic acids	C (%)	H(%)	N(%)	S(%)	O(%)	H/C	N/C	O/C	Ash(%)	E4/E6
HA-S	54.6	3.3	2.8	a	39.3	0.73	0.044	0.54	1.7	4.3
HA-TRM	59.5	6.9	5.6	1.4	26.6	1.39	0.081	0.33	14.1	5.2
HA-CRM	54.2	5.8	6.1	1.7	32.2	1.28	0.095	0.45	12.0	5.8
HA-VRM	50.1	5.5	5.8	1.7	36.9	1.31	0.098	0.55	11.3	5.7

a: S content (<0.3%) detected but not quantified.

The HA-like fraction isolated throughout the process (composting followed by vermicomposting) presents C and O content similar to those HA extracted from cattle manures before and after vermicomposting by Plaza *et al*. (2008).

The HA-like fraction extracted from composted and/or vermicomposted materials usually shown a decrease in C content and an increase in the oxygen content through the process (Xiaowei *et al*., 2011); whereas, in general, the content of N, H and S are higher with respect to the soil HA (Senesi and Brunetti 1996). The atomic ratio of O/C, H/C and N/C are often used to monitor structural changes of humic substances (Adani *et al*., 2006). The increase in the O/C ratio indicates that an oxidation process take place through the composting and vermicomposting period.

The HA extracted after the composting phase (HA-CRM) shows a decrease of the ratio H/C in comparison with HA-TRM, probably due to a loss of aliphatic chains, initially present in HA-TRM. The atomic ratio H/C for HA extracted after the vermicomposting process (HA-VRM) presents a slight increase with respect to the HA-CRM, indicating less condensed or less substituted aromatics rings than HA-CRM (Chai *et al.,* 2007; Plaza *et al*., 2008). HA extracted from all organics amendments analyzed (HA-TRM; HA-CRM; HA-VRM) have H/C ratio higher than that observed for HA-S, suggesting that the humification period is not complete.

N/C ratio in all HA studied are greater than the value for HA-S, probably due to the incorporation of N-compounds present in rabbit manure, like proteina-

ceous materials, polysaccharides and compounds not degraded during both process. (Lguirati *et al*., 2005; Droussi *et al*., 2009).

The scattering of monochromatic light in a diluted solution of macromolecules or colloidal particles is closely related to weight, size and interaction of particles in solution, the value of the quotient, E4/E6, suggest the aggregation level, with higher values indicating a more aggregated humic macromolecule (Chen *et al*., 1977; Pertusatti and Prado, 2007). E4/E6 ratio provide interesting information if it is used to compare data of humic acids extracted from the same matrix, like a manure handle with a sequence of treatments (composting followed by vermicomposting). (Adani *et al*., 2006).

The HA extracted from the organic amendments analyzed have E4/E6 ratio higher than the HA-S (Table 2), indicating a more aggregated humic macromolecule, a greater aliphatic character, less molecular condensation and therefore an incomplete humification degree (Pertusatti and Prado, 2007). The HA-CRM and HA-VRM E4/E6 values are similar, suggesting that after the vermicomposting process the structural characteristic of the macromolecules do not change substantially from this point of view.

3.3 Acid base properties

The charges-pH curves (-Q versus pH) of the HA isolated, between pH 3 and 11, obtained from potentiometric titration, corrected for blank solution and fitted with sixth degree polynomial according to Machesky (1993) and Campitelli and Ceppi (2008b), are shown in the Figure 1a. This smoothing function was selected for their simplicity

The charge development of HA isolated from the different ages of the organic amendment (HA-TRM, HA-CRM and HA-VRM) are lower than that of HA-S and increase with process time.

The greater change was observed into the first 90 days of the process (from HA-TRM to HA-CRM). According to the methodology proposed by Ritchie and Perdue (2003), at pH 8, all carboxylic groups are titrated. At this pH value, the charge is greater in HA-CRM ($\approx$ 70%) than in HA-TRM. The HA-VRM charge development increase ($\approx$ 20%) in comparison with HA-CRM at pH = 8. Therefore, these charged sites (carboxylic group) present in the macromolecule, increase in the following order: HA-TRM<HA-CRM<HA-VRM. In the range between pH 8-10 Ritchie and Perdeu (2003) considered that a half of the phenolic groups are titrated. The change in the charge development in this range of pH, for the HA analyzed, was similar to that observed for the former acidic groups. In general, the total acidity increase about 70-80% during the composting period and about 20-25% in the vermicomposting phase. This tendency indicate that during this period of the process (first 90 days) occur the principal changes in this physicochemical property in the HA macromolecule, and suggest that during the composting phase take place the most important oxidative process. During the vermicomposting period, the HA-VRM charge development increases about 20-25 %, indicating different pathway in the stabilization-maturation process of the organic matter. This behaviour suggest that the HA-like fraction is under a less oxidative stage in which an homogeneous degradation of C compounds or a neosynthesis of polysaccharides could take place (Sen and Chandra 2007; Plaza *et al*., 2008; Lazcano *et al*., 2008).

Through the first derivative of the –Q versus pH curves (-dQ/dpH) obtained from the titration curves smoothed with the polynomial equation (Figure 1b), is possible: i) to obtain the apparent proton-dissociation constant (Pkaap), ii) to analyze the chemical heterogeneity of each class of acidic group present in the HA macromolecule, iii) to estimate the concentration of each set of acidic groups by calculation the area

under each peak and iv) to estimate the buffer capacity developed by each class of acidic site (Nederlof *et al*., 1994; Koopal *et al*., 2005; Campitelli *et al*., 2006; Campitelli *et al*., 2008b). In this way, is possible to follow the acid-base characteristics evolution for the principal acidic groups (carboxylic and phenolic) in comparison with those of HA-S.

The number of site classes is then equal to the number of peaks and the peak position could be used as an average of the apparent dissociation constant (pKaap).
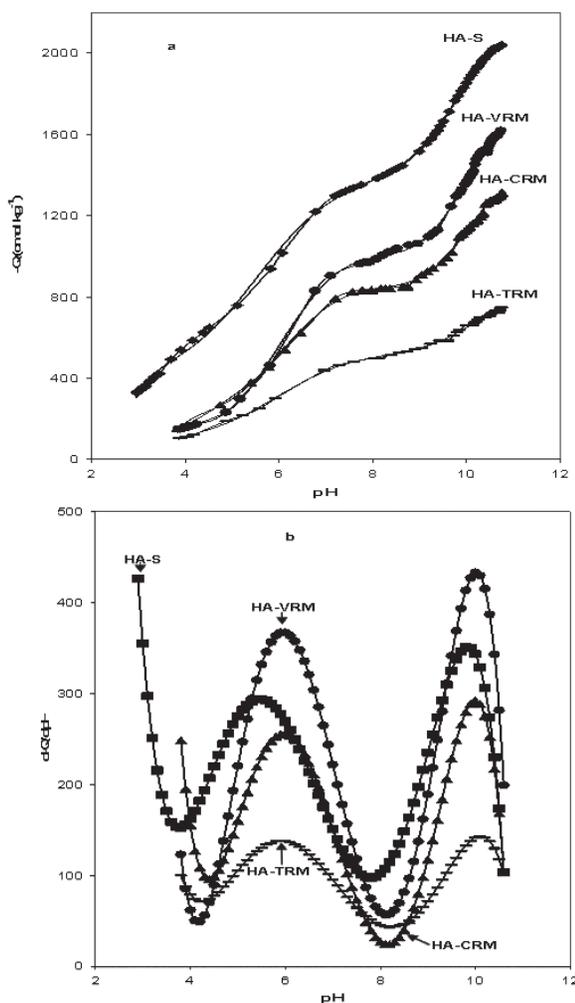
a: Charge-pH curves of humic acids extracted from rabbit manure at thermophilic phase (HA-TRM) treated with composting (HA-CRM) followed by vermicomposting process (HA-VRM) and soil (HA-S). The solid lines were calculated on the basis of the sixth polynomial equation (with R^2 values exceeding 0.999 in all cases). (Charge development were calculated taking into account the ash content)

b: Apparent proton-affinity distribution of humic acids extracted from rabbit manure at thermophilic phase (HA-TRM) treated with composting (HA-CRM) followed by vermicomposting process (HA-VRM) and soil (HA-S) obtained from the first derivatives through charge-pH curves [d(−Q)/d (pH)] smoothing with sixth degree polynomial equation through the experimental data in the range of 3–10.

The samples of HA analyzed show two main peaks, the first would be assigned to the carboxylic groups or sites (strong acidic sites) and the second to the phenolic groups (weak acidic sites). The HA showed, also, a small or developing peaks at more acidic pH values ($\leq$ 4), indicating, probably, a presence of stronger acidic sites.

HA-TRM presents two broad peaks, which have maxima at pH 5.9 and pH 10.1 respectively; the first corresponds to strong acid sites (carboxylic groups) and the second to weak acidic sites (phenolic groups). The widths of both peaks are large, indicating that in this phase of the process (30 days) the HA-like fraction is characterized by a large number of acidic sites with similar dissociation properties, and have certain overlapping between the both peaks (i.e. strong and weak acidic sites). In this way, is possible to assume that the HA have a large chemical heterogeneity or are a complex mixture of organic compounds produced during the original material decomposition. The concentration of each acidic sites and therefore, their buffer capacity contribution (height of the peaks), would be very low if the organic amendment is used at this time of the process.

The HA-CRM, present the peaks positions at pH 6 and 10, corresponding to strong and weak acidic site, respectively. Both maxima are higher and narrower than those observed for HA-TRM, indicating that the content of both acidic site increases significantly through this period of the process (90 days) and have a lower chemical heterogeneity and more diffe-

rentiation between both classes of acidic sites. This behaviour could suggest that the organic amendment at the thermophilic stage (TRM) progress towards a relatively stable humus like end-product through a bio-oxidative, mineralization and humification processes in which the unstable organic matter is partially transformed through decomposition, resynthesis and polymerization reactions.

The strength of the carboxylic groups is practically the same as the HA-TRM but the buffer capacity increases about twofold in comparison with HA-TRM.

The peak positions of both peaks for HA-VMR (6 and 10 respectively) are similar than the other amendments HA analyzed indicating that the acidic strength do not change significantly along the process. The height of the first maximum is about 35% higher than those observed in HA-CRM; while, the maximum corresponding to weak acidic sites (phenolic groups) increase about 20%, that is in agreement with that observed by Plaza *et al.*(2005; 2008) and Xiaowei Li *et al.*, (2011). The acidic sites content (table 3) (carboxylic and phenolic groups) of HA-VRM are close to that corresponding to HA-S, probably because during the vermicomposting period the organic matter reach a condition in which the complex organic compound resemble native soil humic acids (HA-S). The buffer capacity and chemical heterogeneity of HA-VRM are similar to HA-S, indicating that after the vermicomposting process the HA-like fraction of the organic amendment have similar acid-base properties that HA-S.

Table 3: Acidic functional groups content (carboxylic and phenolic) calculated by integration of the area under each maxima of the curves (d-Q/dpH) obtained through the first derivative of smoothed experimental data. pKaap values obtained by Henderson-Hasselbalch equation.

Humic acids	-COOH (cmol kg^{-1})	pKaap*	-OH (cmol kg^{-1})	pKaap*
HA-S	1033	5.19	641	9.80
HA-TRM	422	5.88	231	9.97
HA-CRM	677	5.83	409	10.18
HA-VRM	886	5.86	639	10.74

* R^2 value was 0.99.

HA isolated from rabbit manure at the thermophilic stage (HA-TRM), after the composting process (HA-CRM) and after the vermicomposting process (HA-VRM) show, for the strong and weak acidic sites (carboxylic and phenolic), pKaap values shifted toward greater values in comparison with HA-S (5.5 and 9.8 respectively), indicating that the acidic sites are slightly weaker than the corresponding groups for HA-S.

At the lowest pH measured (sites domains below 4), HA-CRM shows a developing peak (Figure 1b), indicating that very acidic sites could be present in the HA-like substances, and their content would be similar to HA-S. HA-TRM and HA-VRM present lower content of these classes of acidic sites, probably because, is the initial phase (thermophilic phase) for HA-TRM, and for HA-VRM may be due to the different biological transformation operated by vermicomposting of the raw material.

In order to verify the pKaap values obtained using the first derivative of the charge vs. pH curves (d-Q/dpH), the experimental data was fitted to a modified Henderson-Hasselbalch function describing the proton binding by two classes of binding sites, strong acidic site (carboxylic groups) and weak acidic site

(phenolic groups). The values obtained (table 3) are similar to those found through the peak position of both maxima obtained by the first derivative of the curve (d-Q/dpH), indicating that the principal acidic site classes characterizing the acid-base behaviour of the HA-like fraction of these organic amendments would be assigned to carboxylic and phenolic groups. The acidic site content obtained by the determination of the areas under each peaks of the d-Q/dpH curves are shown in table 3.

The changes observed in the acid-base properties of the HA analyzed suggest that through the composting followed by vermicomposting process the HA-like substances are transformed to complex organic compound that resembles native soil HA (Benitez *et al.*, 2005; Plaza *et al.*, 2008)

3.4 Capillary zone electrophoresis

The principal characteristics of HA-like substances are the occurrence of acidic site with different strength, the principal groups are the strong (carboxilic groups) and weak (phenolic groups) acidic site. For these HA analyzed the average Pkapp value are

around 5 – 6 for the carboxylic groups and 9 – 10 for the phenolic groups.

At the experimental condition (pH ≈ 9) almost all acidic groups of HA are deprotonated (negatively charged). The presence of negative charges permit to separate HA by electrophoresis in electrical field (+) to (-) in which the EOF (electroosmotic flow) is responsible for the movement of the analyte (Peuravouri *et al.*, 2004).

The electropherogram of HA-TRM presents five differentiable peaks with low absorbance at 243 nm (Figure 2). Between the both first peak (5.6 and 9.7 min respectively) could be distinguish numerous peaks without a correct resolution, indicating the

presence of different fractions or compounds, with a low Q/m ratio, (polysaccharides, aliphatic fractions, N compound) having close electrokinetic behaviour which cannot be separated by CZE, The most important peak can be observed at time migration ≈ 10 min with a shoulder at ≈ 11 min and a peak with a low absorbance can be observed at ≈ 16 min, indicating the presence of many fractions with different charge/mass ratio and thus, different electrokinetic mobility. This behaviour could be due to the high degree of chemical heterogeneity, due to the short period of stabilization (30 days), which is in agreement with those observed through the d-Q/dpH curve (Figure 1b).
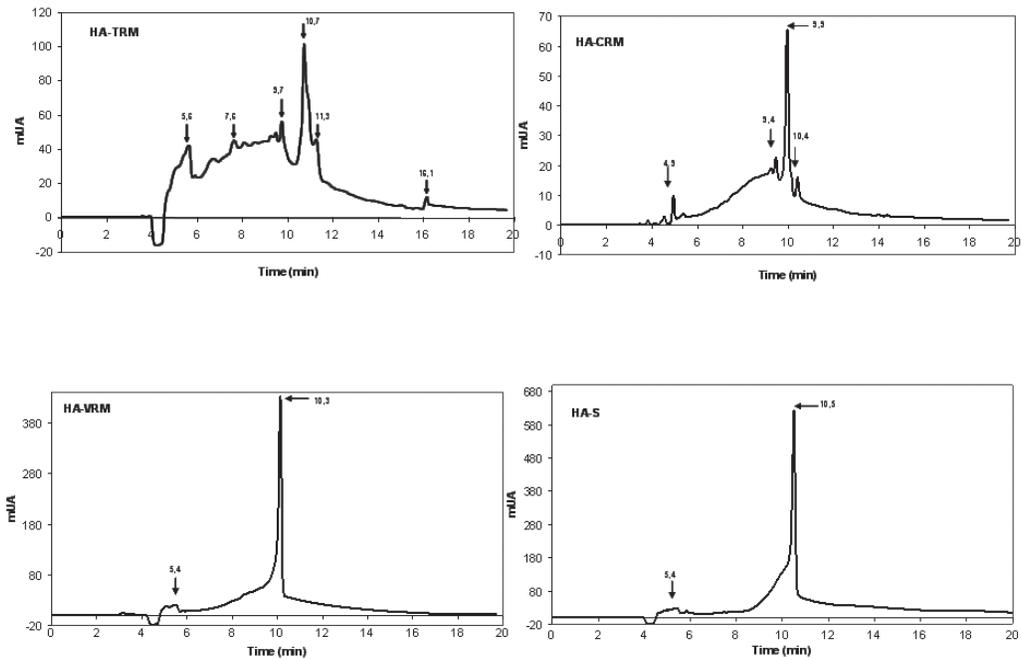

Figure 2: Electropherograms of acids extracted from rabbit manure at thermophilic phase (HA-TRM) treated with composting (HA-CRM) followed by vermicomposting process (HA-VRM) and soil (HA-S) in buffer borate 20mM (pH=9.3), the concentration of the AH solutions were 1000 ppm. CZE conditions: voltage of -10kV, injection hydrodynamic 5000 Pa for 60 s, detection at 243 nm, fused-silica capillary, 81.7 cm total length, 75 μm i. d. (effective length 73.2 cm). Total run time 20 min (for time migration higher than 20 min no significant peak were observed).

The electropherogram corresponding to HA-CRM presents a better definition than the HA-TRM (Figure 2). The electropherogram pattern (HA-CRM) shows 4 main peaks, with slightly differences in the time migration with respect to the peaks present in the HA-TRM electropherogram, and a broad peak at the time migration range 6-9 min, called usually the "humic hump", which correspond to the average electrophoretic mobility of different compounds or fractions. These changes could indicate a variation in the charge/mass ratio of each compound or fractions, and thus an evolution of the HA-like fraction toward a less chemical heterogeneity, also in agreement with that observed in d-Q/dpH curve (Figure 1b). The HA-CRM electropherogram shown similar profile to other soils HA, in similar experimental condition (He *et al.*, 2010; de Moraes and Rezende 2008), suggesting that at this phase of the process the structural transformation in HA-like fraction were significant to perform the evolution toward similar soil HA characteristics.

HA-VRM shows an electropherogram pattern in which can be observed clearly one peak at a migration time of 10 min, with a smaller absorbance than that observed for HA-S and the width peak is similar to HA-S (Figure 2). This behaviour suggests that, in the raw material used in this stabilization and maturation process, (composting followed by vermicompostig), the HA-like fraction evolves toward a similar structure of HA-S

The width of the principal peak (~ 10 min), decrease as time of process increase, indicating changes in the structure of the macromolecule, toward less chemical heterogeneity, and their absorbance increase as the time of process increase, suggesting that this fraction could be the most representative fraction in the fingerprint of the HA macromolecule. This behaviour could be used as a marker of the humification process, taking place during the stabilization-maturation process.

The smaller absorbance observed in HA-VRM in comparison with the HA-S value; indicate lower concentration of this fraction in the HA-VRM. These characteristics are in agreement with that shown by the other analytical techniques employed to study these humic acids.

The electropherograms of all HA-like fraction analyzed shown one principal peak at ≈ 10 min, suggesting that this would be a main structure which behaves as sink of the other carbon compounds present in the raw material, and in this way leads the HA-like fraction toward a supramolecular structure similar to HA extracted from natural soil.

The electropherograms revealed the evolution of the AH-like fraction since the raw materials to the final of the process, showing it through the decreasing of the number of fractions with different time migration, indicating that throughout the composting followed by vermicomposting process the HA-like fraction are evolving toward more stable supramolecular structure resembling native soil HA.

Conclusions

Through these analytical techniques was possible to follow the evolution of the HA-like fraction during this stabilization-maturation process

These techniques, potentiometric titrations and capillary zone electrophoresis (CZE) allow the estimation of the organic amendment maturity through the HA-like fraction characteristics.

The AH charge development, the acidic groups content and thus the buffer capacity, increase as the time of process increase. The acidity strength of both classes of acidic sites in the HA-like fraction of the organic amendments is slightly weaker than the acidic groups of the HA-S. The chemical heterogeneity of the HA like fraction decrease with the time of process.

The electropherograms revealed the evolution of the HA-like fraction during the composting followed by vermicomposting.

The main and more significant changes and structural transformation of the raw material take place during the composting process and could be analyzed through the evolution of the chemical and physico-chemical properties of their HA-like fraction.

Some evaluation criterion of the maturity state of HA-like fraction could be: the increasing in the charge development and buffer capacity, the decreasing in the chemical heterogeneity and the number of fraction with different electrokinetic mobility, i.e. when the HA-like fraction evolves in order to have similar characteristics as soil HA.

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