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TG–DTA, DRIFT and NMR characterisation of humic-like fractions from olive wastes and amended soil

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Abstract

The purpose of the present study is to investigate, by means of thermogravimetric analysis (TG) and differential thermal analysis (DTA), diffuse reflectance infrared Fourier transform (DRIFT) and 2D nuclear magnetic resonance (NMR) spectroscopies, the structural features of the humic-like fraction (HLF) from olive pulp (OP), its effluents originated from the fermentation processes for hydrogen (EH_2) and methane production (ECH_4) and humic acid (HA) from soil amended with each of these materials. A considerable structural modification emerged between the HLF, in particular from the ECH_4 effluent, which was characterised by a high content of polyphenolic and polypeptidic substances. The short-term amendment trial with OP and EH_2 indicated that no chemical or structural changes in soil HA appeared. In contrast, the amendment with ECH_4 substantially influenced the chemical and structural composition of soil HA. The structural interpretation performed by 2D NMR indicated the presence of aliphatic and aromatic protons while the sugar-like content and O–CH_3 groups decreased with respect to the soil control HA. It emerges from this study that olive wastes contain stabilised humic-like material that may be recycled as an amendment in areas where olive trees are cultivated.

Keywords: DRIFT; TG–DTA; 2D NMR; Soil humic acid; Humic-like fraction; Olive pulp; Olive effluents

1. Introduction

The disposal of olive mill wastes (OMW) represents a serious environmental problem in the Mediterranean basin where olive trees are mainly cropped [1], because of the high content of organic carbon (C) (cellulose, emicellulose, polyphenols and lipids) and their high electrical conductivity [2–4]. An alternative practice is the recycling of organic C and the nutrients present in OMW as an organic soil amendment, thus closing the C and nutrient cycles [5]. However, recycling of OMW in agriculture needs to minimise the phytotoxicity and negative environmental impact caused by the high content of phenols and easily decomposable organic substances [6,8].

Studies on a laboratory scale [9,10] indicated that the treatment of olive waste water (OW) under aerobic conditions leads to an increase in stable organic matter (OM) similar to soil humic substances (HS), with a drastic reduction of phytotoxicity. The amount and quality of the humic-like fraction (HLF) found in OMW during various fermentation processes is considered an important parameter of OM maturity and stability [11].

Recently, Nastri et al. [12] demonstrated that olive pulp (OP) and its effluent obtained from hydrogen (EH_2) and methane production (ECH_4) are potential soil amendments. These materials were characterised by a high amount of OM, nutrients and a negligible concentration of heavy metals. Furthermore, the results obtained indicated that only OP at high concentration delayed both seed germination and seedling growth. These effects decreased when the OP was incorporated into the soil while an enhancement of seedling growth was detected with the addition of EH_2 and ECH_4. On these basis further investigation was considered useful to evaluate if soil amended with OP and its effluents EH_2 and ECH_4 could influence the structure of native soil HS.

The HS originated from microbial decomposition of plant and animal residues represent the most important component of soil OM [13]. The structural changes that occur in HS are complex

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and therefore a physical–chemical and structural approach is needed. In addition, few studies have investigated the effects of olive wastes on the status and quality of native soil HS in short- and/or long-term amendment trials [8,14].

Among analytical techniques, thermal analysis is a method used to investigate the thermal stability of soil OM. In particular, thermogravimetry (TG), derivative thermogravimetry (DTG) and differential scanning calorimetry (DSC) have been used to study HS structure [15–19] and subsequently to monitor the composting process [20–22]. This technique involves a continuous and simultaneous measurement of weight loss (TG) and energy change (DSC) during heating of the sample [15,22]. Usually, during heating of HS a first exothermic reaction (≈300 °C) is produced by the decomposition of proteins and carboxyl groups, while the exothermic reaction at higher temperatures (≈450 °C) is originated by decomposition of refractory C such as aromatic rings and saturated aliphatic chains [23,24]. Additionally, by combining thermal analysis with spectroscopic techniques, important structural information regarding the OM transformation process can be obtained.

Particular attention was devoted to diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy as a technique for functional group analysis [19,25,26] and to liquid-state [27–29] and solid-state NMR [28,30–32] for molecular structure. The development of high-field magnets and of multi-dimensional techniques has improved the overlapped individual signals which are typical of one-dimensional liquid-state NMR spectra of HS [33]. The HS two-dimensional (2D) NMR study was introduced by Buddrus et al. [34] and more recently, detailed interpretations on the structural components of HS have been provided by other authors [33,35–38].

The aim of the present study, within the EU BIOTROLL Project, was to apply TG–DTA, DRIFT and NMR spectroscopies in order to compare OP and EH_2, ECH_4 and their HLF to understand how soil humic acid (HA) was influenced by a short-term trial amendment with these products.

2. Materials and methods

2.1. Olive waste

The OP was collected from a two-phase decanter mill and was submitted to two different fermentation processes for hydrogen production (EH_2) and for methane production (ECH_4) as described by Gavala et al. [39].

2.2. Experimental design

The surface horizon (0–20 cm) of a Typic Udifluvent soil (USDA, 1998) was sampled at the experimental farm of the University of Bologna's Agricultural Faculty (Cadriano, Bologna, Italy). The main physical–chemical characteristics of the soil were: pH (in water 1:2.5, w/v) 7.62; texture: sand 31%, silt 42% and clay 27%; electrical conductivity (EC, dS m^{-1}) 0.09; total calcium carbonate ($CaCO_3$) < 1%; total organic C (TOC) 7.6 g kg^{-1}; humic C 4.8 g kg^{-1}; total Kjeldhal nitrogen (TKN) 0.84 g kg^{-1}; C/N ratio 7.8; cation exchange capacity (CEC) 22 $cmol_c$ kg^{-1}, Olsen-P (P) 12.3 mg kg^{-1}.

Aliquots of 5 kg of dry soil were placed in plastic pots (16 cm in diameter and 25 cm in height) and each treated with an amount of OP and EH_2 and ECH_4 corresponding to 120 kg ha^{-1} of TKN, respectively. Each treatment was replicated three times and compared to unamended controls. The pots were buried into the soil up to 4/5 times their height to create a micro-environment more similar to the field conditions. After 6 months each sample was air dried and stored for subsequent spectroscopic and thermal analyses.

2.3. Chemical analysis of olive wastes and soil

The elemental characterisation and phenolic and total lipid concentration analysis of OP, EH_2 and ECH_4 have been described by Nastri et al. [12].

Total C and N in soil, HA and HLF were measured with an elemental analyser (Thermo Finnigan mod. EA 1110).

2.4. Soil HA and HLF extraction and characterisation

Each air-dried sample, about 10 g, was extracted under N_2 with 100 mL of 0.5 M NaOH and stirred for 24 h. The suspension was centrifuged at 5000 × *g* for 30 min and then filtered through a 0.45 μm filter using a Minitan S System (Millipore, Bedford, MA, USA). The solution was acidified with 5 M HCl to pH < 2 to precipitate the HA and was subsequently centrifuged at 5000 × *g* for 20 min in order to eliminate the supernatant. The HA and HLF were dissolved with NaOH 0.5 M to produce a Na-salt, and then dialysed against Millipore water, using tubing (Cellu Sep H1-USA) with a cut-off of 8000 Da, until a neutral pH was achieved. Finally, the solutions were freeze-dried.

The titrations of HA were carried out using a VIT90 Radiometer Auto titrator (Radiometer Analytical, France), whereas thermogravimetric analysis (TG) and differential thermal analysis (DTA) were carried out using a TG-DTA92 instrument (SETARAM, France) details on these procedures are described by Montecchio et al. [19]. The DRIFT spectra were recorded with a Nicolet Impact 400 FT-IR Spectrophotometer (Madison, WI) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). Peak area integration from 3000 to 2800 cm^{-1} was used to compare the CH groups in aliphatic substances between samples [19].

NMR spectra were recorded with a Bruker FT-NMR Avance 400 spectrometer (Broad Band 5 mm probe, inverse detection). Nominal frequencies were 400.13 MHz for ^1H and 100.61 MHz for ^{13}C. An internal lock on the deuterium of D_2O and DMSO-d_6 was used for all spectra. The chemical shifts at 298 K were referred to TSP. ^1H NMR data was acquired using the bipolar longitudinal eddy current delay pulse sequence (BPPLED) [40,41] and the Carr–Purcell–Meiboom–Gill sequence, commonly known respectively as "ledbpgs2s" and "cpmg1d" in the standard Bruker library. As regards the "ledbpgs2s", 4k scans were collected, implying a bipolar pulse pair ranging from 2 to 3 ms, with a diffusion time (D20) of 100–200 ms and a time

domain point of 8–16k. The gradient length and diffusion time were varied in each sample in order to achieve 95% signal suppression at maximum gradient strength. For the "cpmg1d" experiments, in order to eliminate diffusion and J effects, the echo evolution delay was set to 1 ms, the number of loops (L1) ranged from 80 to 180, scans 4k, time domain points 16–32k and a 2 s relaxation delay.

For 2D H,H-COSY (Homonuclear COrrelation SpectroscopY) and 2D H,C-HSQC (Heteronuclear Single Quantum Correlation), standard parameters were used. The phase sensitive HSQC was performed via double INEPT transfer by means of Echo/Antiecho-TPPI (Time Proportional Phase Increment) gradient selection; decoupling during acquisition was performed via trim pulses in INEPT transfer with multiplicity editing during the selection step [42].

2.5. Statistical analysis

The Student–Newman–Keuls test was used to compare significance differences within samples by using Statgraphics version 5 plus.

3. Results and discussion

3.1. Chemical characteristics

The main chemical features of OP, EH_2 and ECH_4 have previously been reported by Nastri et al. [12]. Both effluents were characterised by a significant variation in C, N and lipid contents with respect to OP (Table 1). The presence of a high N level in ECH_4 could be due to the addition of urea at the beginning of the fermentation process. Apparently, the phenolic content was not involved in either effluent, but it increased significantly with respect to that of OP. Similarly, Beccari et al. [43] found that the olive mill effluents from the methanogenic reactor still contained significant concentrations of phenolic compounds (>1000 Da), whereas the phenolic fraction <500 Da could be removed by the methanogenic process. Phenolic substances represent a problem because they can inhibit plant germination. However, the lack of phytotoxic activity [12] suggested that transformation reactions of simple phenols to HLF might occur during anaerobic fermentation. A further confirmation of the HLF formation was provided by the highest concentration of humic-like C in ECH_4 (Table 1). The presence of a higher Fe, Mg and Al concentration in ECH_4 compared to OP [12] might suggest the formation of HLF through abiotic catalysis [7].

The C and N contents in HLF isolated from OP, EH_2 and ECH_4 are shown in Table 2. C content increased only in EH_2-HLF, whereas a remarkable increase of N content appeared in ECH_4-HLF.

The C, N and COOH concentrations of soil HA extracted from the short-term amendment trial are shown in Table 3. Especially in relation to the C and N content, no statistical differences were found between the soil control at the beginning T_0, the control T_6 and the treatments with OP and EH_2 after 6 months. On the contrary, the HA isolated after 6 months from the ECH_4-treated soil, showed a significant ($P < 0.05$) increase of about 11 and 20% in C and N concentration, respectively, with respect to the control HA. This marked increase in N level would confirm the hypothesis of polymeric N forms. Similarly, Brunetti et al. [44] showed that raw olive pomace influenced the chemical properties of native HA generating an increase in C and N content and phenol substances. According to Brunetti et al. [44], these enrichments in C and N can be considered essential for maintaining high concentrations of soil OM. The lack of modification in C and N content in the OP and EH_2 amendments demonstrated that these materials are rapidly decomposed when added to soil.

Table 1
Some chemical features of olive pulp (OP) and its effluents (E) originated from acetogenic and methanogenic processes for EH_2 and ECH_4 production

	OP, average ± S.E., d.m.	EH_2, average ± S.E., d.m.	ECH_4, average ± S.E., d.m.
C (%)	53.5 ± 0.2	52.0 ± 0.5	41.7 ± 0.3
N (%)	2.5 ± 0.08	3.2 ± 0.2	4.3 ± 0.1
C/N	18.2	13.8	8.2
Humic-like C (%)[a]	13.4 ± 0.3	22.3 ± 0.6	29.8 ± 0.5
Phenolic compounds (g kg^{-1})[a]	13.7 ± 0.4	25.7 ± 0.9	32.6 ± 2.5
Total lipids (g kg^{-1})[a]	194 ± 7.1	149 ± 6.9	81.0 ± 6.6
Lignin (%)[b]	38.4	n.d.	n.d.

S.E.: standard error; n.d.: not determined; d.m.: dry matter.
[a] Nastri et al. [12].
[b] Gavala et al. [39].

Table 2
C and N content and integration area of CH stretch region of humic-like fractions (HLF) extracted from olive pulp (OP) and its EH_2 and ECH_4 effluents

HLF	C (%), average ± S.E., $n = 3$	N (%), average ± S.E., $n = 3$	C/N	3000–2800 cm^{-1}, average ± S.E., $n = 3$
OP	55 ± 0.9	2.6 ± 0.02	25	60 ± 0.2
EH_2	58 ± 1.0	1.8 ± 0.06	37	52 ± 0.1
ECH_4	55 ± 0.5	6.2 ± 0.09	10	50 ± 0.1

S.E.: standard error.

Table 3
Mean values of C and N percentages, carboxylic groups concentration and CH aliphatic area integration of soil humic acids extracted from a short-term amendment trial in pots: the control at the start (T_0) and 6 months after the start (T_6) and the treatments with olive pulp (OP) and EH_2 and ECH_4 effluents

Humic acids	C (%), average ± S.E.	N (%), average ± S.E.	COOH mequiv./100 g, average ± S.E.	CH stretch integration area, average ± S.E.
Control				
T_0	42.07 a ± 0.48	3.385 a ± 0.005	447.15 a ± 0.75	8.83 a ± 0.17
T_6	41.97 a ± 0.16	3.405 a ± 0.005	447.20 a ± 0.80	8.89 a ± 0.09
Treatments after 6 months				
OP	41.58 a ± 0.26	3.415 a ± 0.035	451.52 b ± 0.51	9.60 a ± 0.30
EH_2	40.40 a ± 0.13	3.450 a ± 0.110	499.50 c ± 0.50	9.53 a ± 0.29
ECH_4	46.60 b ± 0.28	4.135 b ± 0.015	519.50 d ± 0.50	15.56 b ± 0.27

The statistical differences between treatments were estimated *vs.* the control T_0.
S.E.: standard error. Values in the same column followed by different letters are significantly different ($P < 0.05$) according to the Student's *t*-test.

3.2. Thermal analysis (TG–DTA)

Investigating the TG–DTA of HLF (Fig. 1) three exothermic peaks in the range of about 330–555 °C (Fig. 1A–C) could be observed. In relation to the thermal decomposition steps, the peak at around 330 °C might correspond to the decomposition of proteins, carbohydrates and polyunsaturated fatty acids [45–47]. The OP-HLF showed a significant mass loss of about 51% of the first peak with respect to EH_2 and ECH_4-HLF (37.1 and 42.7%, respectively) (Table 4). The second peak might be originated from the decomposition of monounsaturated fatty acids, such as oleic acid, since it would represent about 80% of the total content of the monounsaturated fatty acids present in extra-virgin olive oil and in its wastes [45]. Feasibly, during thermal decomposition, the double bonds of unsaturated fatty acids are broken and the triglyceride molecules may be modified into a saturated structure characterised by a higher thermal stability. The mass loss of the second peak was higher in ECH_4-HLF (24.6%) than OP-HLF and H_2-HLF (23.4 and 16.4%, respectively). The third pronounced exothermic peak at about 555 °C, showed that the highest mass loss in EH_2-HLF (Table 4) and might involve the combustion of the remaining hydrocarbons and the burnout of the residual carbonaceous material of the previous steps.

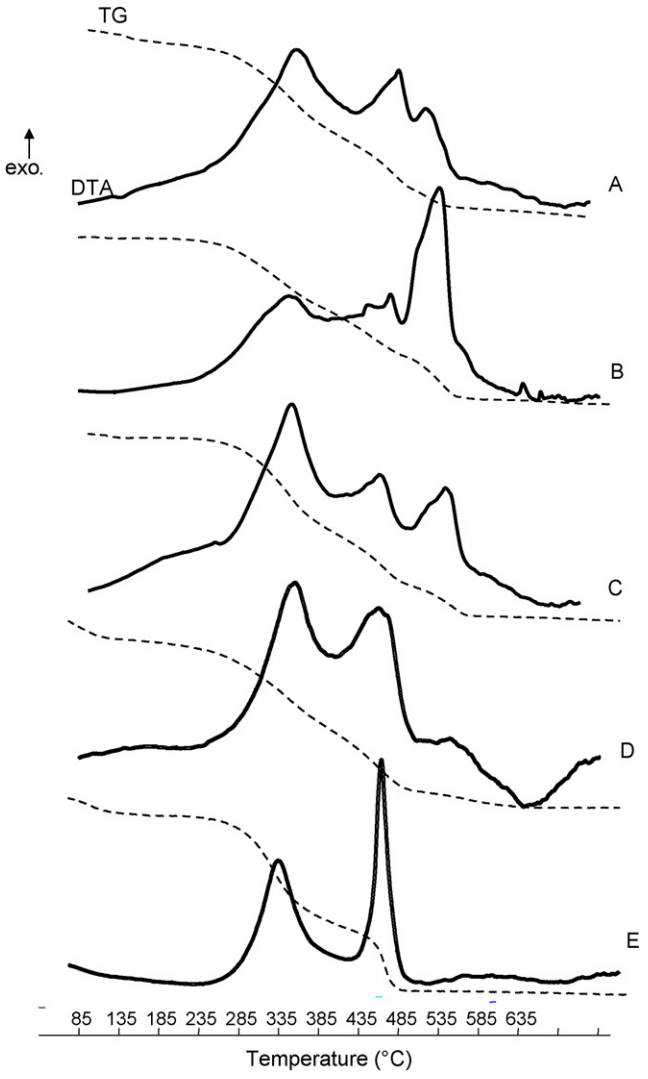

Fig. 1. DTA curves of humic-like fractions extracted from (A) OP, (B) EH_2, (C) ECH_4 and humic acids extracted from (D) soil amended with ECH_4 and (E) unamended soil.

The thermal patterns of the HA extracted from soil were characterised by two strong exothermic reactions (Fig. 1E): (i) the first peak is considered to be the result of the thermal degradation of polysaccharides, decarboxylation of acidic groups and dehydration of hydroxylate in aliphatic structures [16,23]; (ii) the second peak is related to the breakdown of aromatic structures and cleavage of the C–C bond [16]. The thermal behaviour of HA from soil amended with OP and EH_2 did not significantly change with respect to that of native HA (Table 4). The lack of modification in these samples confirmed the results of C and N concentrations (Table 3) previously discussed and spectroscopic results (see below). In contrast, the T_{max} of the first peak in ECH_4-HA (Fig. 1D) occurred at higher than that of control HA (Table 4), while the T_{max} of the second peak did not change with respect to that of control HA. In addition, the presence of a weak exothermic peak (T_{max} about 560 °C) suggested some resemblance to ECH_4-HLF (Fig. 1C).

3.3. Fourier transform infrared spectroscopy

The structural changes observed using TG–DTA analysis were supported by DRIFT spectra of HLF (Fig. 2A–C), HA from soil amended with ECH_4 (Fig. 2E) and control (Fig. 2D), whose interpretation was based on studies of HA extracted from different organic wastes [6,48,49]. The band around 3300 cm^{-1} was

Table 4
Thermogravimetry (TG) and differential thermal analysis (DTA) parameters for the humic-like fraction (HLF) from olive pulp (OP), its effluents EH_2 and ECH_4 and humic acid (HA) from soil after amendment with OP and both effluents after 6 months

	1° exo. peak		2° exo. peak		3° exo. peak	
	T_{max} (°C)	Mass loss (%)	T_{max} (°C)	Mass loss (%)	T_{max} (°C)	Mass loss (%)
HLF						
OP-HLF	336	51.1	482	23.4	520	9.1
EH_2-HLF	336	37.1	482	16.4	552	21.7
ECH_4-HLF	329	42.7	458	24.6	553	13.0
Soil HA						
OP	318	39.0	475	21.5		
EH_2	319	42.4	480	21.9		
ECH_4	345	45.0	466	34.2	568	7.2
HA	319	39.1	460	24.2		

assigned to vibration of the OH groups in alcohol, phenol or carboxylic groups. The peaks at 3006 and 3070 cm^{-1} were ascribed to C–H stretching vibration of the double C=C bond. The region between 3000 and 2800 cm^{-1} was dominated by the symmetric and asymmetric CH_2 stretching bands in aliphatic chains [50]. The intense peaks at around 1700 and 1500 cm^{-1} were assigned to vibrations of amide I (C=O), C=C in aromatic rings and the asymmetric stretching of $–COO^-$ groups. In particular the bands at 1735 cm^{-1} can be assigned to carbonyl in esters, whereas the one at around 1530 cm^{-1} might be due to amide II (C–N, NH) and aromatic vibration in phenols. The region between 1400 and 1200 cm^{-1} was assigned to methyl symmetrical bending, $–COO^-$ symmetrical stretching vibration and C–O stretching in carboxylic acids [29], respectively. Moreover, the bands between 1170 and 1000 cm^{-1} were mainly attributable to O–H stretching coupled with C–O bending of C–OH groups in carbohydrates.

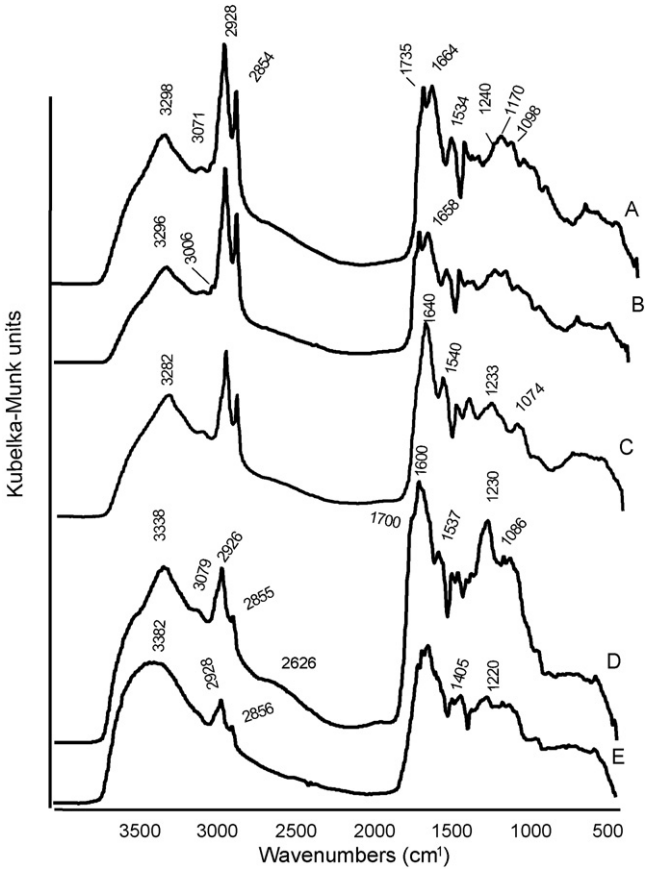

Fig. 2. DRIFT spectra of humic-like fractions extracted from (A) OP, (B) EH_2, (C) ECH_4 and humic acids extracted from (D) soil amended with ECH_4 and (E) unamended soil. All spectra are an average over 200 scans at a resolution of ±4 cm^{-1}.

The spectra of HLF extracted from OP, EH_2 and ECH_4 (Fig. 2A–C) appeared qualitatively similar due to the presence of the bands at around 3080, 1660 and 1230 cm^{-1} which are typical of fatty acids. In particular, a shift of the band at around 1660 cm^{-1} towards lower frequency in both effluent-HLF (was of approximately 24 cm^{-1} for ECH_4-HLF and 6 cm^{-1} for EH_2-HLF) indicated the presence of carbonyls joined to double bonds or aromatic rings. Additional changes in ECH_4-HLF were due to a lack of esters, the band at 1735 cm^{-1} disappeared, and a decrease in aliphatic component as determined by integration area of aliphatic region (Table 2). The decrease in this component seems to be characteristic of OM having undergone the composting process [49].

After 6 months the structural composition of HA amended with EH_2 and OP showed no difference from the control HA. On the contrary, structural modifications in HA from soil amended with ECH_4 (Fig. 2D) confirmed the results obtained using the DTA analysis. The main structural modification appeared in the aliphatic region where the integration area was higher with respect to that of control HA (Table 3). Additionally, a decrease of intensity and frequency (44 cm^{-1}) of OH stretching with respect to that of control HA might indicate the formation of intra and intermolecular hydrogen bonding between various OH groups. A new band at around 3070 cm^{-1} suggested the presence of C–H of C=C, whereas the broad band at 2626 cm^{-1} indicated the formation of intermolecular hydrogen bonding between OH groups in oxygenated compounds [50]. Furthermore, the shoulder at 1707 cm^{-1} and the strong band at 1230 cm^{-1} confirmed the presence of COOH groups [50] as also observed in the titration analysis (Table 3). The appearance of the band around 1540 cm^{-1} seems to be unequivocally related to ECH_4 added to the soil confirming the result achieved by DTA analysis.

3.4. Nuclear magnetic resonance spectroscopy

The HLF and soil HA samples were dissolved in D_2O and DMSO-d_6.

In general, all samples showed quite different 1D ^1H NMR spectra in D_2O, in which no sample was very soluble, probably due to the formation of micellar aggregates; no better resolution was obtained in the NaOD solution, even though all sample solubilities increased; higher solubility and resolution was obtained in DMSO-d_6. This solvent was preferred for the complete analysis since it can dissipate hydrogen bonds, preventing aggregation and enhancing the S/N ratio and resolution [35]. A first qualitative NMR approach for the assignment of the HLF suggests the partition of mono-dimensional proton spectra into three main regions: 3.3–0.5 ppm corresponding to aliphatic compounds, methyl and methylene groups, α and β protons with respect to carboxylic groups and aminic or amide groups; 5.3–3.3 ppm characterising hydrogen atoms bonded to carbon bearing oxygen or nitrogen; 8–6 ppm corresponding to aromatic and olefinic protons.

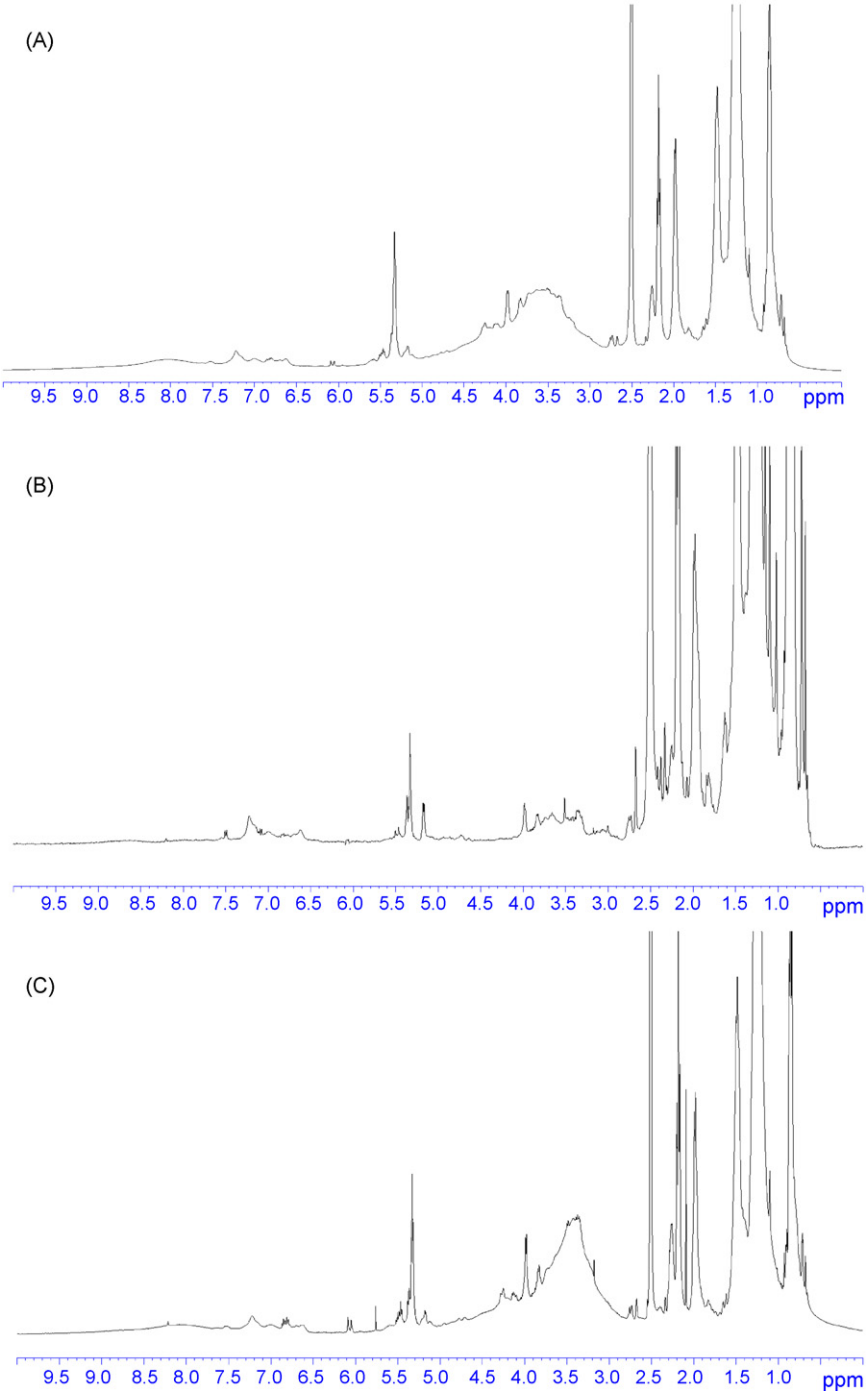

Fig. 3. ^1H NMR spectra of humic-like fractions extracted from OP (A), ECH_4 (B) and EH_2 (C) in DMSO-d_6 at 298 K.

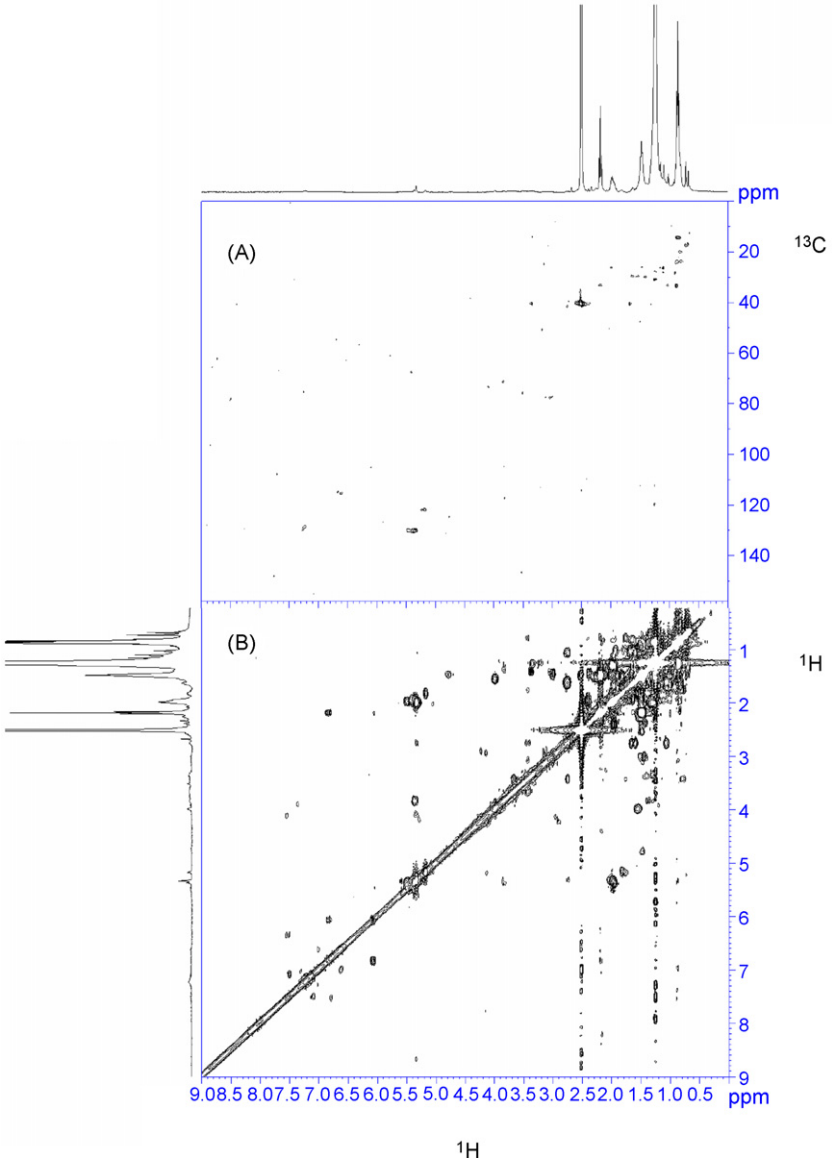

Fig. 4. ^{1}H, ^{13}C HSQC (A) and H,H-COSY (B) spectra of the humic-like fraction extracted from ECH_4 in DMSO-d_6 at 298 K.

The ECH_4-HLF in DMSO-d_6 (Fig. 3B) showed a spectral pattern that resembles, in its main features, the fatty acids profile [50–52]; the prevailing species were saturated compounds with a small percentage of unsaturated lipids. This sample can be better assigned taking into account HSQC and COSY data (Fig. 4A and B, respectively). The triplet signal at 2.15 ppm (^{13}C δ 35 ppm) is due to methylenic protons in the α position to the carbonyl group. Usually this signal does not show variations in multiplicity or chemical shift between different vegetal oils. This peak correlates with a quintuplet (^{1}H δ 1.45 ppm, ^{13}C 25 ppm) assigned to the β methylenic group; methylenic protons in position γ or further, in relation to the carbonyl group, were found in the region between 1.2 and 1.4 ppm, and the carbons directly attached to them range from 22 to 32 ppm. Finally, the methylic chemical shift was around 0.8–0.9 ppm for ^{1}H (^{13}C δ 12 ppm) and is due to the overlapping of the different triplets of methylic proton signals. The unsaturated fatty acid fraction was, on the other hand, characterised by olefinic protons (5.2–5.4 ppm) that showed a scalar coupling with protons attached to allylic carbons (^{1}H δ 2 ppm, ^{13}C δ 30 ppm). Further correlations could be found with the methylenic chain (1.2–1.4 ppm) and terminal methyl group protons. There is no evidence of polyunsaturated fatty acids. The typical signal around 2.8 ppm of protons attached to bis-allylic carbons was absent.

In addition, the ^{1}H NMR spectrum (Fig. 3B) showed the presence of a moderately intense and crowded set of signals in the region between 3 and 4 ppm which can be reasonably attributed to amino acid side chains, since homonuclear cross peaks fall at too high a field to infer the presence of sugar moieties. Signals between 6 and 8 ppm confirm both the aromatic nature of the sample, especially for polyphenolic species, and the presence of polypeptidic systems characterised by broad amidic pro-

Table 5
Percentage of integrated areas for humic-like fractions (HLF) from OP, and its effluents calculated from 1D ^{1}H NMR spectra in DMSO-d_6 at 298 K

HLF	Chemical shift range (δ in ppm)			
	2.5–0.0	5.0–2.5	5.9–5.0	10–5.9
OP	63.7	25.1	3.9	7.3
EH_2	60.1	25.8	3.9	10.2
ECH_4	52.8	30.7	3.5	13.0

tons overlapping with aromatic protons. Low molecular weight species such as caffeic and cynnamic acid were detected; typical doublets at 6 and 6.8 ppm with a *trans* $^3J_{H,H}$ of 16 Hz, due to olefinic structures in α position in relation to aromatic rings [53] were observed. The presence of polyphenolic and polypeptidic species supported the increase of N content and humic C (Table 3) determined in this sample.

A comparison between the ECH_4 (Fig. 3B) and OP-HLF (Fig. 3A) highlights, most notably, the fact that they are both characterised by a large amount of lipidic species, which makes them both soluble in DMSO. The OP-HLF spectrum (Fig. 3A), in contrast to that of ECH_4, showed a broad signal around 8 ppm due to amidic protons of peptidic structures and an intense doublet around 6 ppm, typical of unsaturation. The region between 4.5 and 5.5 ppm was almost unaltered, while a decrease in the intensity of peaks related to saccharidic systems was observed for ECH_4-HLF compared to OP, mainly due to a reduction in the complexity of the sugar moiety and therefore in the hydrogen bond network. Other than these, there were no significant discrepancies in chemical shifts. In any case, the presence of sharper signals in the ECH_4-HLF hints that "decomposition" processes have taken place giving rise to smaller molecules with higher mobility.

Similarly to that of ECH_4-HLF, the EH_2-HLF ^{1}H NMR spectrum (Fig. 3C) contained sharp peaks, probably due to the formation of low molecular weight species with high mobility during the acetogenic process. OP-HLF aromatic doublets around 7.5 and 7.1 ppm ($^3J_{HH} \sim 8$ Hz) disappeared in EH_2-HLF. A strong signal at 5.79 ppm distinguished the EH_2 from ECH_4 and OP-HLFs. The region between 5 and 3 ppm was less resolved than that of ECH_4-HLF and perhaps saccharidic aggregates are formed. Two sets of signals at 4.3 and 4.15 ppm typically discriminate EH_2-HLF from ECH_4-HLF. The origin of the broad signal may be attributed to polypeptidic species that, in acidic or neutral conditions are folded, forming a wide and strong H-bond network.

The determination of the integrated area of 1D ^{1}H NMR spectra can lead to a semi-quantitative comparison between the HLF (Table 5). As previously found by TG–DTA techniques, a considerable decrease in aliphatic components was detected for ECH_4-HLF (−10.9%) and EH_2-HLF (−3.6%) with respect to OP-HLF. An increase of integrated area between 5.0 and 2.6 ppm was evident for ECH_4-HLF (+5.6%) suggesting the presence of more oxygenated species due to humification processes. Aromatic species, in particular polyphenolic, also showed an increase in EH_2-HLF (+2.9%) and ECH_4-HLF (+5.7%) confirming the results shown in Table 1.

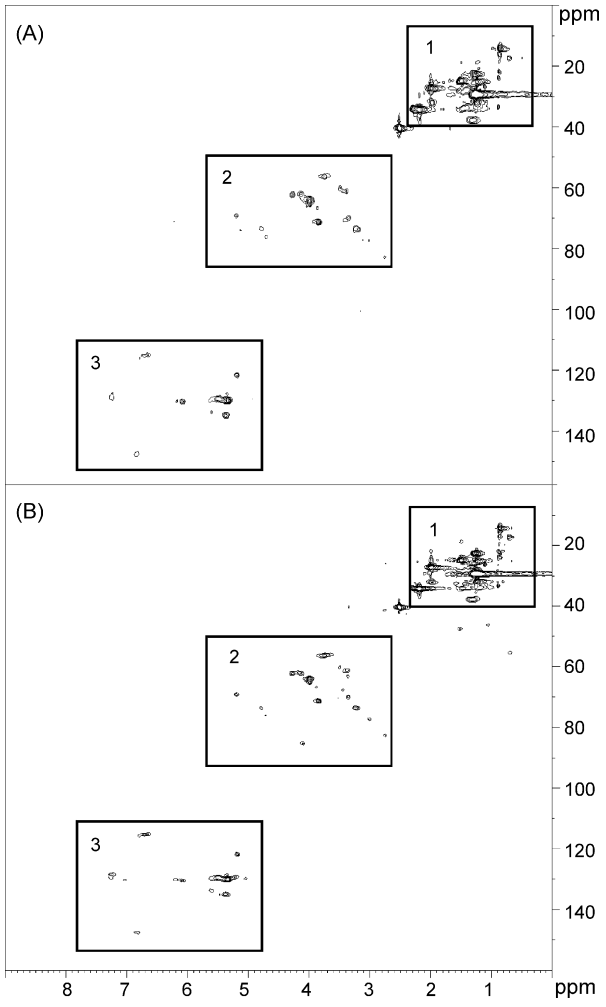

Fig. 5. Phase sensitive ^{1}H, ^{13}C HSQC spectra of humic-like fractions extracted from EH_2 (A) and OP (B)in DMSO-d_6 at 298 K.

Even though monodimensional NMR experiments may be used for quantitative determination [54], homo and heteronuclear techniques may represent a sort of real fingerprint of the analysed matrix, able to highlight uniformity in structure and composition, as proposed by Kelleher and Simpson [36]. HSQC experiments show, at first glance, an almost complete overlapping between EH_2 (Fig. 5A) and OP-HLF spectra (Fig. 5B). HSQC only detects protons directly attached to carbon, thus exchangeable protons are not observed. In any case, it is useful since the spectrum can be easily divided into three main regions corresponding to: (1) aliphatic groups and side chain residues; (2) methylenic linkers between aromatic rings, N and O heteroatoms (carbohydrates) and amino acid α protons; (3) aromatic rings. The phase sensitive HSQC differentiates CH_2 groups from CH and CH_3.

1D ^{1}H NMR spectra of HA extracted from unamended soil and ECH_4-amended soil are shown in Fig. 6. The spectra of HA from ECH_4-amended soil (Fig. 6B) showed a spectral pattern very similar to that of ECH_4-HLF (Fig. 6A) with the exception that all olefinic protons disappeared, probably owing to some kind of reduction process.

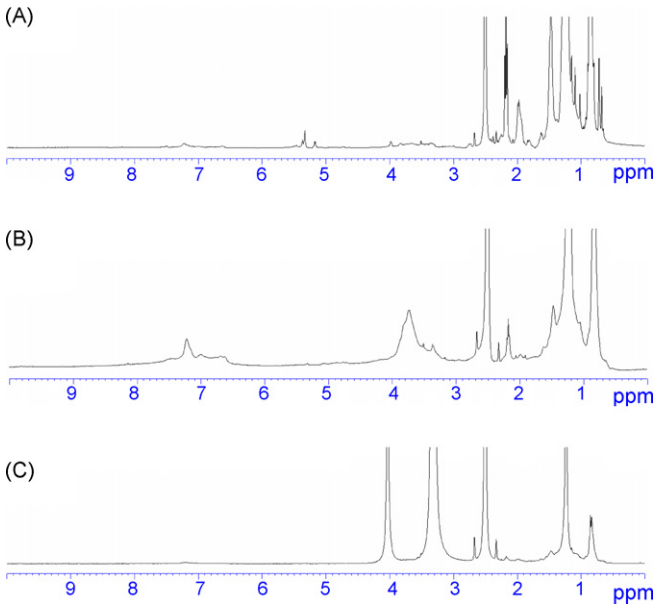

Fig. 6. ^{1}H NMR spectra of humic-like fractions extracted from ECH_4 (A), humic acids from soil amended with ECH_4 (B) and unamended soil (C) in DMSO-d_6 at 298 K.

The control HA (Fig. 6C) has poor solubility in DMSO if compared with ECH_4-HA (Fig. 6B); this behaviour may be attributed to a lower percentage of C mass, especially of lipidic nature. There was almost no hint of the presence of methylenic α and β protons in relation to carbonyl groups, suggesting the absence of carboxylic groups. A very intense and broad peak was observed around 3.3 ppm, which can be reasonably attributed to polysaccharidic units [53]. Structures like condensed THF can be excluded since there were no signals at low field (4.30–4.80 ppm). The strong peak at 4 ppm is associated to the presence of methoxyl groups, particularly abundant in lignin derivatives. In summary, after 6 months, ECH_4-HA was enriched in aliphatic and aromatic protons while the sugar-like content and O–CH_3 groups decreased with respect to the control HA.

4. Conclusions

The recycling of OP and its effluents EH_2 and ECH_4 as soil amendants represents a good way of closing organic C and natural nutrient cycles. OP and its effluents (EH_2 and ECH_4) and similarly their HLF are characterised by different chemical and physical features. In particular, the HLF from ECH_4 effluent was enriched with polyphenolic and polypeptidic species as supported by chemical and spectroscopic investigations.

The short-term amendment trial showed a different effect of these materials on organic C and N contents and the structure of native HA. The lack of chemical and structural modifications in HA, in pots amended with OP and EH_2, indicated that these materials are rapidly decomposed by soil biomass. In contrast, the amendment trial with ECH_4 enriched the native HA in C and N and some main functional groups such as COOH, aromatic and aliphatic components.

On the basis of these results we can conclude that the use and recycling of olive wastes as amendants play a fundamental role in the maintenance and enrichment of soil humic C in areas where olive trees are cultivated.

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