



minerals



Article

Fulvic Acid from Chestnut Forest as an Added Qualities to Spring Water: Isolation and Characterization from Fiuggi Waters

Enrica Allevato, Vittorio Vinciguerra, Silvia Rita Stazi, Francesco Carbone, Cristiano Zuccaccia, Giuseppe Nano and Rosita Marabottini

Topic

Past, Current and Future Processes in the Earth Critical Zone

Edited by

Dr. Gianluca Bianchini, Dr. Claudio Natali, Dr. Chiara Marchina and Dr. Valentina Brombin



<https://doi.org/10.3390/min12081019>

Article

Fulvic Acid from Chestnut Forest as an Added Qualities to Spring Water: Isolation and Characterization from Fiuggi Waters

Enrica Allevato ¹, Vittorio Vinciguerra ², Silvia Rita Stazi ^{3,*}, Francesco Carbone ², Cristiano Zuccaccia ⁴, Giuseppe Nano ⁵ and Rosita Marabottini ²

- ¹ Department of Environmental and Prevention Sciences (DiSAP), University of Ferrara, 44121 Ferrara, Italy
² Department of Innovation in Biology, Agri-Food and Forest Systems DIBAF, University of Tuscia, 01100 Viterbo, Italy
³ Department of Chemical, Pharmaceutical and Agricultural Sciences (DOCPAS), University of Ferrara, 44121 Ferrara, Italy
⁴ Department of Chemistry, Biology and Biotechnology, University of Perugia and CIRCC, 06123 Perugia, Italy
⁵ Sygnature Discovery, Discovery Building, Biocity, Pennyfoot Street, Nottingham NG1 1GR, UK
* Correspondence: silviarita.stazi@unife.it

Abstract: The aquifer of “mineral” water, historically known for its curative properties, is an identifying characteristic of the Anticolana valley. This area hosted a coppice chestnut forest for a long time. Under the forest, there is an important aquifer, historically renowned and widely recognized for preventing renal stone formation and or facilitating their expulsion. This mineral water prevents the formation of calcium oxalate and phosphate crystals in the kidney and promotes their dissolutions through soluble calcium complexes. The forest environment soil is particularly rich in humification products owing to the interaction of the rainwater–litter–soil system. The fulvic fraction is soluble in water under all pH conditions and enriches the water basin. We aimed to test these hypotheses and assess how the coppice chestnut forest is involved in fulvic acid production. Fulvic fractions isolated and purified from soil samples and mineral water (550 µg L⁻¹) were analyzed by GC-MS, FTIR, and NMR. Data from different sources were compared, showing sufficient similarities to state that the fulvic acids isolated from the water come from the processes that take place between the stems and the chestnut litter. The chestnut forest provides enrichment to water quality, which is a distinctive piece of information in defining water enhancement strategies, establishing soil management and designating sustainable forest management.

Keywords: chestnut forest; mineral spring water; fulvic acid; water quality; ecosystem service



Citation: Allevato, E.; Vinciguerra, V.; Stazi, S.R.; Carbone, F.; Zuccaccia, C.; Nano, G.; Marabottini, R. Fulvic Acid from Chestnut Forest as an Added Qualities to Spring Water: Isolation and Characterization from Fiuggi Waters. *Minerals* **2022**, *12*, 1019. <https://doi.org/10.3390/min12081019>

Academic Editor: Paul Sylvester

Received: 17 June 2022

Accepted: 11 August 2022

Published: 13 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

At the beginning of the XXI Century, the United Nations published the Millennium Ecosystem Assessment (UN, 2006). One of the objectives was—and still is—the conservation and the achievement/management of a sustainable use of ecosystems. Natural capital and ecosystem services are matrices in which preservation is a crucial objective to guarantee the continuity, quality, and quantity of ecosystem services, which are the flow of benefits that contribute to human wellbeing. The 6th Goal of the Sustainable Development Goals 2015–2030 focused on water. Water scarcity, poor water quality, and inadequate sanitation affect food safety (UN 2015). Water is a vital resource for living organisms, as well as for agriculture and other activities. A close relationship exists between water resources and woodlands; therefore, their management is strictly connected. Moreover, the forest ecosystem service for water purification and accumulation in underground aquifers is one of the most frequent experiences of payment for ecosystem services [1,2].

Mountain and forest areas are not usually affected by soil and water pollution phenomena, as the underground basins stand out for water purity and the richness of soluble

minerals. Woodland soils differ from agricultural and urban/suburban soils for their structure, functionality, and macro and micro biodiversity. Forests and woodlands develop deep and extensive root systems, and trees shed their leaves, needles, and various matter on the soil surface, resulting in a layer with relatively high levels of organic matter hosting diversified micro and macrofauna [3]. Forests and tree litter, as well as the associated macro and microfauna and flora, radically modify soils in ways crucial to watershed hydrology, water quality, and stream habitats. The velocity rainwater is slowed down by the forest canopy, reducing its power. From the leaves, the rainwater slowly falls to the ground, percolating through the organic-matter-rich layer, the soil, and then reaches the aquifer. Along the pathway from the forest canopy to the aquifer, the chemistry of natural waters is affected by storage water and by the release of chemicals by the vegetation and soluble minerals by the soil [4].

The “rainwater–forest–litter–soil” network is also responsible for providing unconventional water services of significant interest to humankind for socioeconomic and social health systems thanks to its intrinsic therapeutic properties [4,5]. The natural capital components of the Anticolana valley characterize the rainwater. The Fiuggi basin is located within the calcareous ridge of the Ernici Mountains and represents an endoreic depression elongated in the NW–SE direction at an average elevation of 580 m above sea level⁴. Agricultural areas and predominantly chestnut forests are present in this area [6].

The chestnut forest litter and volcanic soil (pyroclastic layers) improve the rainwater quality during underground flow [7]. Natural processes enrich the water in humic substances and, in particular, fulvic acids, soluble in water at all pH values.

The Anticolana valley covers a surface of 212 hectares (ha), characterized by chestnut coppice, and is part of the Municipality of Fiuggi (geographic coordinates: 41°48′ N and 13°13′ E) and Acuto (geographic coordinates: 41°47′30.94 N and 13°10′23.94 E). Fiuggi’s chestnut forests grow on soils derived from volcanic tuff, confirming the edaphic conditions typical of calciphobous and moderately acidophilic species⁴. The Fiuggi chestnut forest grows in environmental conditions where rainfall is abundant and temperatures are within the 12 °C isotherm, reflecting normal vegetation conditions [4,8].

The EU Network Natura 2000 includes this area for the relevant ecological values of chestnut habitat and other minor flora and fauna species of interest [9]. The location is also under special protection as an area of relevant public interest for its hydrogeological properties. The local chestnuts coppice forest extends close to the Fiuggi urban area, and thus is defined as an urban forest. According to this definition, it has been managed using a dedicated forest plan to ensure indefinite survival of the ecosystem. Some late 1990s studies recognized the Anticolana valley’s ecosystem as directly responsible for the enrichment of natural spring waters with organic compounds of therapeutic significance [4,7,10–12]. A direct linkage is postulated, as the extensive chestnut woodland that covers the Anticolana valley also needs the hydromineral basins of the Fiuggi spring waters. Nevertheless, no evidence thus far has been reported to back up this assumption.

The composition of spring water depends on chemical-physical processes characteristic of a specific area [12]. Anticolana spring water shows extremely low salinity (low contents of Ca, Mg, and K salts) and hardness, typical of fast-circulating waters with a short underground cycle. The chemical composition is characterized by a low mineralization level (142–200 mg dm^{−3}) and the presence of silicon dioxide (12–48 mg dm^{−3}) and humic substances (HSs) [12].

HS is a class of natural organic compounds produced by soil humification processes, thus originating from decomposition processes and biochemical transformations of molecules derived from the residues and emissions of plants and animals [13]. Humic acids are characterized by high MW. Humic acids are 1500–3000 Å in size and >3500 Da in weight, and fulvic acids are smaller (800 Å in size and MW < 3500 Da). HSs represents approximately 40%–60% of the soil organic matter. Chemically, HSs are molecular aggregates composed of sugar, fatty acids, polypeptides, aliphatic chains, and aromatic rings [14–16].

HSs can be classified into three different subtypes or fractions according to their solubility. Humin (H) is the insoluble fraction of humic substances; humic acid (HA) is the fraction soluble under alkaline conditions; and fulvic acid (FA) is the fraction soluble under both alkaline and acidic conditions [17]. This classification, based only on elementary criteria, provides no indications about their structure or chemical behavior.

Humic and fulvic acids have a wide chemical composition and are characterized by the presence of various functional groups [17]. HA acids are poorer in hydrogen and oxygen and richer in nitrogen than FA. Moreover, HA fractions are richer in phenolic groups, and FA fractions are richer in carboxylic acids [18]. Several authors, through comparative analysis of the molecular structures of HA and FA samples, have shown that FAs are enriched in oxygen-containing fragments, which explains their higher solubility in water and higher migration capacity [19,20]. HSs are formed downstream of a great variety of chemical and biological reactions occurring in the soil. Therefore, HS is not a homogeneous class of compounds and lacks unifying motifs that could help conceive a general structure. HSs are very complex and multifaceted molecules whose structure is still the subject of debate and study [21,22].

Piccolo et al. [23–26], discussing their hypotheses of macromolecular polymers, reported HSs to be large superstructures self-assembling from a relatively small number of heterogeneous molecules. Hydrophobic dispersive forces are the main interactions holding these superstructures together.

FAs are smaller molecules forming molecular aggregates with intermolecular connections or associations in solutions. These molecular aggregates are multi-charged (negative and positive) owing to phenolic, carboxylic, and amino groups [27]. The environment in which the humification processes take place strongly influences the structural composition of the HS. HS might be present in the aquifer depending on the aquifer geology and chemistry [28].

Other than the rare minerals from the soil, rainwater carries HS derived from humification processes. Owing to these qualities, spring waters in the Anticolana valley are classified as “mineral” water and further qualify as “oligomineral” because of their low TDSs (total dissolved solids). The specific composition, low salinity, and presence of natural FAs give these waters therapeutic and healing properties, as they prevent the formation of kidney stones and/or facilitate their expulsion [10,29].

This work aims to prove that chestnut soil humification processes affect the qualitative and quantitative composition of FAs in water. To prove this hypothesis, FAs isolated from spring waters and soil were studied by performing structural comparative analysis.

2. Materials and Methods

2.1. Area of Study, Water, and Soil Sampling

The area of this study is the Anticolana Valley, situated in the Lazio region of central Italy (Figure 1), where the municipality of Fiuggi is the most important town. According to the Lazio soil map, these are intermountain basins with areas of accumulation on predominantly reworked volcanic deposits and slopes with inconsistent pyroclastic deposits. Soils in this area are classified as *Eutric Cambisols* (>75%) and *Calcaric Endoskeletal Cambic Phaeozems* (<10%) [6].

The area between the Municipality of Fiuggi (41°47'52.08" N and 13°13'25.90" E) and Acuto (geographical coordinates: 41°47'30.94 N and 13°10'23.94 E) is covered by 212 hectares (ha) of chestnut coppice (Figure 1).

This area has been included in the EU Natura 2000 Network for the relevant ecological values of chestnut habitat and other minor species important for flora and fauna (Regional Council Resolution 158/2016). The Anticolana Valley is characterised by extensive chestnut woods that need the hydromineral basins of the Fiuggi waters; the presence of extensive chestnut wooded areas has allowed the waters of the hydrothermal basin to be enriched in organic substances.



Figure 1. Geographical location of the area of study.

The forest areas in the municipality of Fiuggi, above the spring’s mineral water, that are the object of this study are as follows (Figure 1):

Bosco d’Anagni (lat: 41°47.2454; long: 13°13.0799; mt altitude: 50.010266)

Bosco Le Prata (lat: 41°47.1595; long: 13°13.4982; mt altitude: 50.006155)

Bosco Le Cese (lat: 41°46.5775; long: 13°13.0414; mt altitude: 50.032194)

Three homogeneous plots (150 m × 150 m) were chosen to represent these areas. The sampled woodland areas are located above the sources of Fiuggi’s oligomineral waters, as shown in Figure 2.

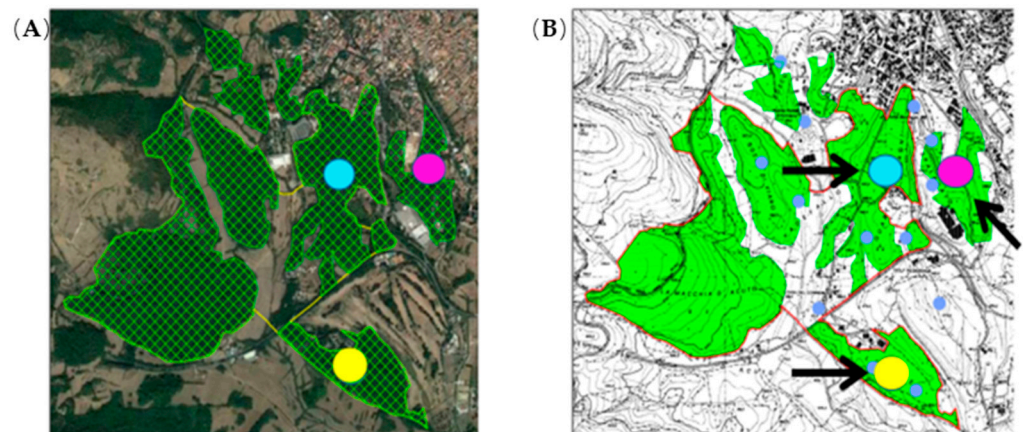


Figure 2. (A) Forest areas of the municipality of Fiuggi above the mineral water aquifers and soil sampling points: ● Quarto d’Anagni; ● Le Prata or Lagozzo; ● Le Cese. (B) Spring of Fiuggi mineral waters.

Soil samples were taken in spring 2018 with a 20 cm long sampler according to a randomized block design, taking four soil samples per plot. Each soil sample was a composite of three of these plots. All 12 samples were analyzed separately.

A total of 280 L of Fiuggi bottled mineral water supplied by the Fiuggi mineral water plant (Acqua e Terme di Fiuggi spa, Fiuggi, Lazio, Italy) was received in June 2018 and processed to extract the organic substance.

2.2. Soil Sample Preparation and Physical-Chemical Characterization

Soil samples were sieved (<2 mm) and oven-dried at a temperature below 40 °C. The textural class of the surface horizon (0–20 cm depth) fell within the sandy-loam USDA classification. Active and exchangeable acidity was measured on sieved soil suspended in a solution of deionized water (active) and in 1 N KCl (exchangeable) in a 1:2.5 ratio (*w/v*) [30]. The pH was measured in the supernatant with a pH meter (pH 211, Hanna Instruments). Soil organic carbon (SOC) and total nitrogen (TN) were determined by dry combustion using an elemental analyzer (Thermo Soil NC–Flash EA1112, Waltham, MA, USA) [31]. The cation exchange capacity (CEC) was determined after extraction with 10% *w/v* BaCl₂ solution under pH 8.1, according to Gillman [32]. The results were expressed as cmol kg^{−1} of soil. Electrical conductivity (EC; conductivity meter Orion, Germany) was measured in a 1:5 *w/v* suspension of soil and deionized water, and total lime (CaCO₃) was quantified by the volumetric method [33].

2.3. Isolation of Fulvic Acid from Soil

FAs were extracted from soil samples using the IHSS method [34,35]. Soil samples were acidified by 0.1 M HCl to pH 1–2 (1:10 *w/v* soil to solution ratio), shaken for 1 h in a shaker, and then centrifuged (10 min, 3000 rpm). The supernatant containing the first extract, named FA1, was removed from the residue by decantation. The residue was rinsed 2–3 times with distilled water by shaking and centrifugation. The remaining soil sample was neutralized with 0.1 M NaOH and extracted with 1 M NaOH (1:10 *w/v*) to pH > 12 to ensure high HA solubility. The suspension was equilibrated overnight by continuous shaking, and the supernatant containing extracted FA2 + HA was decanted. The alkaline solution was acidified with 6 M HCl to gravimetrically precipitate HA. The overall FA solution, obtained by mixing FA1 and FA2, was purified by nonionic macroporous acrylic ester resin—Supelite DAX-8. After the FA solution was added to DAX-8 resin in a glass column, unabsorbed materials were discarded, while the adsorbed FAs were eluted with a solution of 0.1 M NaOH. The collected eluate was acidified to pH 1.0 with 6 M HCl and, after 5 h, the solution was re-eluted with 0.1 M NaOH. The purified FA samples were collected and stored in a refrigerator before analysis.

2.4. Isolation of Aquatic Fulvic Acids

Water samples were isolated and purified according to the conventional isolation IHSS method of aquatic humic and fulvic acids [36,37]. A total of 280 L of mineral water was adjusted to pH 2 using HCl and passed through DAX-8 resin (Supelite DAX-8, Sigma-Aldrich Co., St. Louis, MO, USA). The aquatic humic substance (AHS) was eluted from the resin using 0.1 M aqueous NaOH, and HA and FA were then separated by adjusting the solution to pH 1. The FA (supernatant) was converted to the acidic form by passing through an H⁺-type cation-exchanger (Amberlite IR120-B, Organo Co., Tokyo, Japan). Finally, a powdered FA sample was obtained by freeze-drying.

The FA fractions from soil and water were both subjected to pyrolysis gas chromatography mass spectrometry (Py-GCMS), nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR) characterization, and elemental analysis.

2.5. Pyrolysis-GC-MS Analysis

Approximately 4 mg of freeze-dried fulvic acid was mixed with 10 µL tetramethylammonium hydroxide (TMAH) dissolved in methanol (50% *w/v*) (Sigma Aldrich). They

were then placed in a spiral plunger of a special solid injector, and the solvent was evaporated at 40 °C for 5 min; approximately 2 mg of solid sample was introduced into a Pyrojector II (SGE) pyrolyzer placed at 600 °C and directly connected to the split/splitless injector (280 °C) of the gas chromatograph. GC separations were performed on a Shimadzu QP5050 gas chromatograph using a BPX capillary column (SGE) (30 m × 0.25 mm). The temperature of the column was programmed from 60 to 300 °C at a rate of 5 °C min⁻¹ and held for 30 min at 300 °C. The main peaks of the eluted compounds were identified by unequivocal matches with spectra of the National Institute of Standards and Technology (Nist 107 and Nist 21) mass spectral library. The compounds were assigned only when the spectral similarity was greater than 90%, and the fragmentation pattern was consistent with their structure.

2.6. NMR Analysis

¹H and ¹³C NMR spectra were recorded on Bruker AVANCE III HD 400 spectrometers equipped with a 5 mm smart probe. TMS (tetramethylsilane) was used as an external reference. Sample preparation as well as the acquisition and processing of NMR spectra were performed as previously described [38]. Specifically, ¹H NMR spectra were recorded using the standard pulse sequence available on Bruker for selective saturation of the HDO resonance. The pre-irradiation time was set to 10 s, the spectral width was set to 18 ppm (7212 Hz), the FIDs were acquired using 32 K data points, the acquisition time was 2.3 s, a 45° flip pulse angle was used, and 64 scans were averaged for a total acquisition time of 15 min. A line broadening of 0.1 Hz and zero-filling to 64 K digital points were applied prior to Fourier transformation. ¹³C{¹H} NMR spectra were recorded using the inverse-gated decoupling scheme to avoid nuclear Overhauser enhancement (NOE). The pre-scan delay was set to 2 s, the spectral width was set to 282 ppm (28,410 Hz), the FIDs were acquired using 128 K data points, the acquisition time was 2.3 s, a 45° flip pulse angle was used, and 10 K scans were averaged for a total acquisition time of about 12 h. ¹H NMR spectral integration was performed by dividing the spectrum into six regions [38]: 0–1.7 ppm for CH, CH₂, and CH₃ moieties, at least two carbon atoms away from polar functional groups or aromatic rings; 1.7–3.0 ppm for CH, CH₂, and CH₃ moieties in the α position with respect to carboxylic acids or aromatics; 3.0–5.0 ppm for CH, CH₂, and CH₃ moieties in the β position with respect to oxygen groups (polysaccharides or carbohydrates); 5.0–6.5 ppm for olefinic protons; 6.5–9.0 ppm for aromatic protons; and 9.0–12.0 ppm for protons of aldehydes. ¹³C NMR spectral integration was performed by dividing the spectrum into five regions [38]: 0–50 ppm for aliphatic carbons, 50–110 ppm for carbon atoms directly bonded to oxygen, 110–165 for aromatic carbons, 165–190 ppm for carboxylic carbons, and 190–210 ppm for carbonylic carbons.

2.7. FTIR Analysis

The FTIR spectra of fulvic acid isolated from soil and water were recorded on an FTIR-4100 spectrometer (Jasco Corporation, Easton, MD, USA). Samples were prepared by the KBr disc method. FA samples were mixed with KBr at 2% *w/w*, finely ground in an agate mortar, and then pressed to a disc using a handheld laboratory hydraulic press, Specac Mini-Pellets-Press (Specac Inc., Nanomaterials 2019, 9, 281, 4 of 18, Fort Washington, MD, USA). The spectra were acquired in absorption mode in the range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ versus a pure KBr background.

3. Results

With a declared objective to highlight the importance of the contribution of the chestnut woodland ecosystem, this research studies the humification products (fulvic acid, FA) originating in the soils above the springs of Fiuggi mineral waters. With the aim of identifying the origin of the fulvic acid present in the chestnut grove waters, this research proceeds with a comparison of the fulvic components isolated from soil and water by

means of instrumental characterizations obtained with the aid of pyrolysis-GC-MS, NMR, and FT-IR.

3.1. Physico-Chemical Characteristics of Soils

The samples taken in the study areas were characterised to define their physical-chemical characteristics. Table 1 shows the parameters for each area.

Table 1. Physical-chemical soil characteristics.

Parameters	Quarto d'Anagni	Quarto Le Prata	Le Cese
Clay (%)	28	30	42
Silt (%)	40	38	36
Sand (%)	32	32	22
Texture class (USDA)	Clay loam	Clay loam	Clay
Electrical conductivity ($\mu\text{s Cm}^{-1}$)	858.75 ± 5.047	995.00 ± 20.21	545.00 ± 55.15
Total limestone (%)	<LD	<LD	<LD
pH (H ₂ O)	5.8 ± 0.3	5.2 ± 0.1	4.9 ± 0.1
pH (KCl)	5.4 ± 0.3	4.6 ± 0.1	4.1 ± 0.1
TOC (g kg ⁻¹)	46.5 ± 0.87	39.2 ± 0.26	37.2 ± 0.39
TN (g kg ⁻¹)	3.61 ± 0.36	3.02 ± 0.18	3.13 ± 0.53
C/N ratio	12.80 ± 1.14	13.00 ± 0.10	11.96 ± 0.76
C.E.C. (Cmol ₍₊₎ kg ⁻¹)	44.69 ± 0.57	34.22 ± 0.78	39.38 ± 1.31

Table 2 shows the amounts of fulvic acid, expressed in mg kg⁻¹, obtained from the soils under study according to the IHSS method. A total of 280 L of Fiuggi water processed as described in the previous section led to the isolation of 154 mg of fulvic acid, corresponding to an average concentration of 0.55 mg L⁻¹.

Table 2. Quantities of FA, expressed in g kg⁻¹, extracted from the soil samples taken at the study sites (data are averages of four samples, analyzed in triplicate; $n = 12$).

Soil	FA (g kg ⁻¹)
Quarto d'Anagni	5.2 ± 0.02
Quarto le Prata	6.2 ± 0.04
Le Cese	5.6 ± 0.04

3.2. FTIR

Figure 3 shows the FTIR spectra of fulvic acids extracted from soil samples of the chestnut forests (A) and the mineral water (B).

The fulvic acid spectra showed a few main distinct peaks [39–41]. The broad band from 3400 to 3300 cm⁻¹ is associated with H-bonded oxhidryl groups. The band from 2940 to 2920 cm⁻¹ and the peak at 2850 cm⁻¹ are assigned to the antisymmetric and symmetric aliphatic C-H stretching of methylene groups, respectively. An intense peak at 1725–1710 cm⁻¹ is due to the carboxylic group. Furthermore, the bands between 1628 and 1650 cm⁻¹ belong to the skeletal aromatic vibrations and the quinones' carbonyl stretching.

The peak at 1450–1456 cm⁻¹ is related to the aliphatic C-H stretching. Alcoholic hydroxyls' deformation and phenolic C-O stretching are also assigned to a band at 1420–1411 cm⁻¹. The peak from 1384 to 1376 cm⁻¹ can be attributed to the skeletal aliphatic deformations and/or antisymmetric stretching of the carboxyl groups. The band at 1227–1224 cm⁻¹ is assigned to the stretching of the carboxyl groups and to the C-O stretching of aryl ethers and phenols. A peak at 1130–1126 cm⁻¹ is attributed to the C-O stretching of various alcoholic and ether groups. The band at approximately 1040–1030 cm⁻¹ is caused by the C-O stretching of polysaccharides and polysaccharide-like substances and/or by the Si-O stretching of silicate impurities.

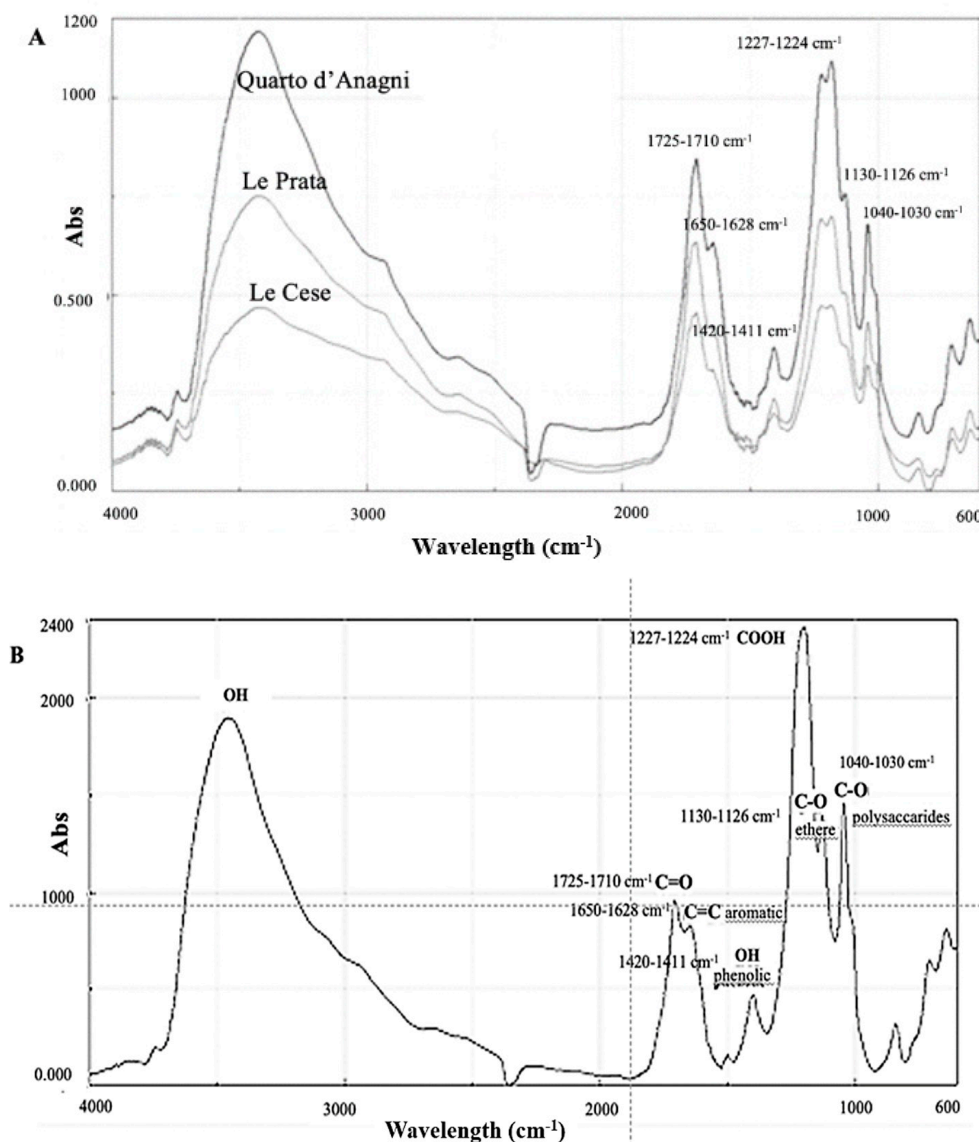


Figure 3. FTIR spectra of fulvic acids extracted from the soil samples of chestnut forests (A) and mineral water (B).

3.3. NMR

Figures 4 and 5 show the ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of FA samples extracted from soil (A) and water (B), respectively. The relative distribution (%) of hydrogen and carbon atoms belonging to different functional groups is reported in Tables 3 and 4, respectively.

Aromatics appear somewhat more abundant in the water than in the soil sample, whereas a larger quantity of carboxylic and aliphatic moieties appears to be present in the soil sample. It may be worth noticing that, with respect to typical FA samples [42,43], relatively sharp NMR resonances are observed in both water and soil samples, suggesting that a substantial fraction of low to medium-low MW species is present in both samples. A single soil spectrum representative of the areas under study is reported, as the different samples were very similar to each other.

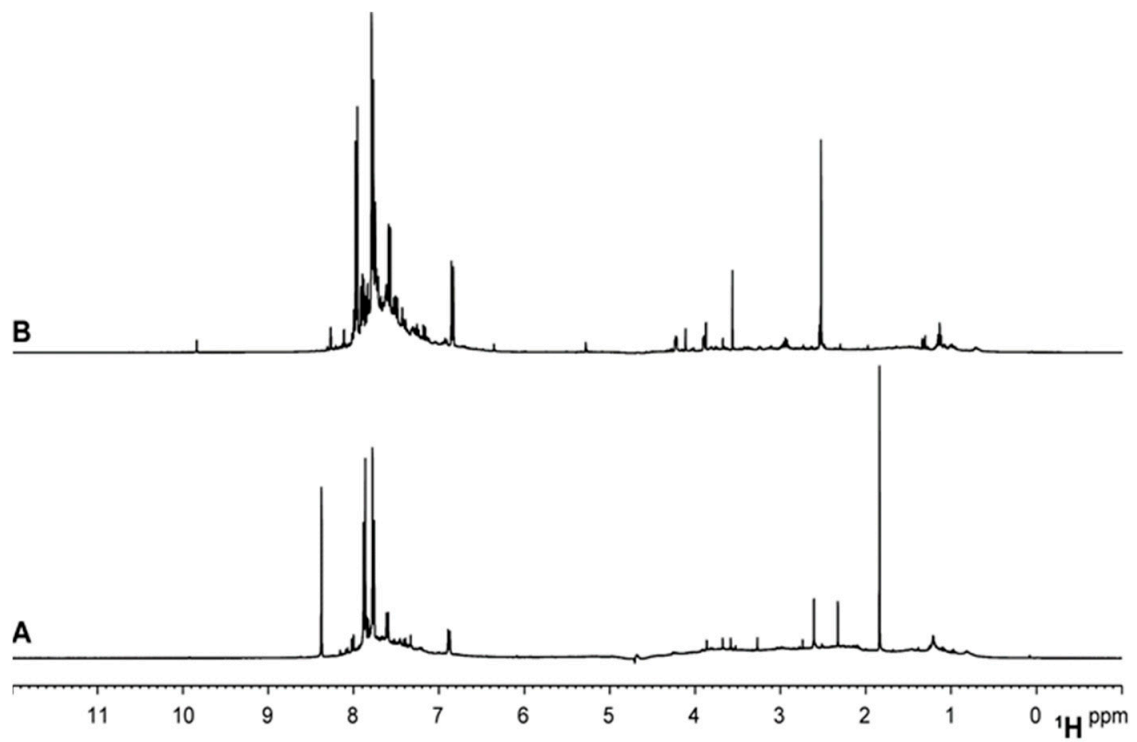


Figure 4. ^1H NMR spectra of FA samples extracted from soil (A) and water (B).

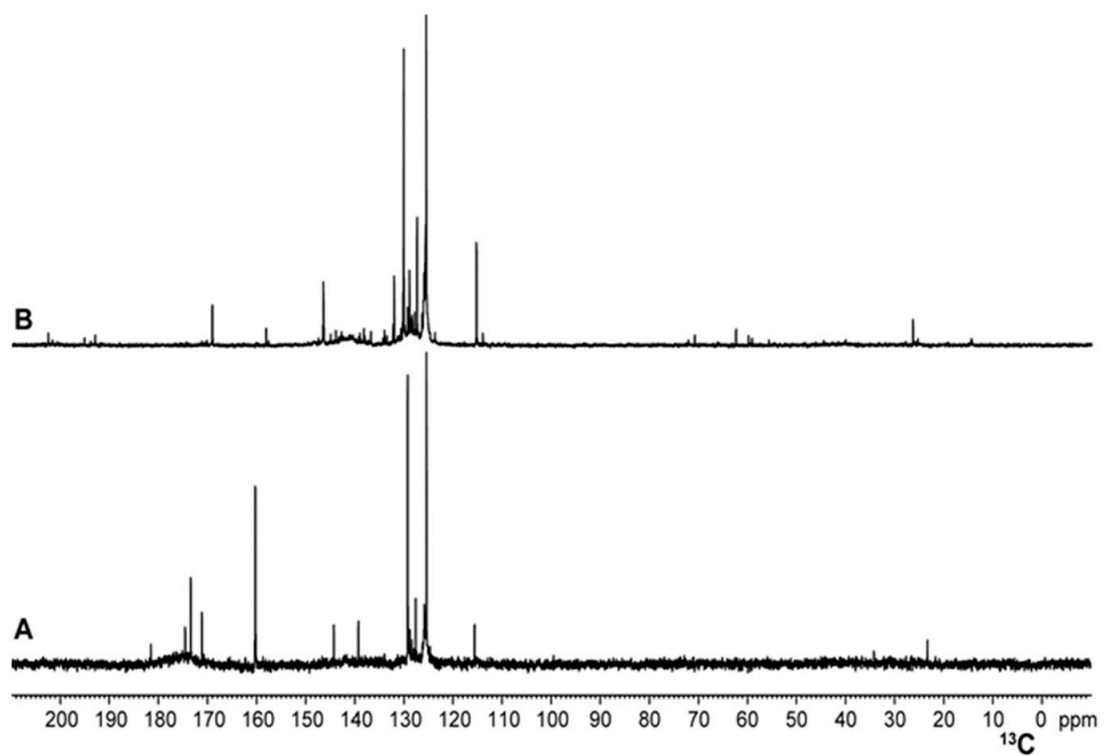


Figure 5. ^{13}C NMR spectra of FA samples extracted from soil (A) and water (B).

Table 3. Proton distribution (%) estimated from the integration of ¹H NMR spectra [38].

	0–1.7 ppm	1.7–3.0 ppm	3.0–5.0 ppm	5.0–6.5 ppm	6.5–9.0 ppm	9.0–12.0 ppm
sample	Alkyl-H	Alkyl-H	Carbohydrate-H	Olefin-H	Aromatic-H	Aldehyde-H
water	8	10	8	1.5	72	0.5
soil	13.5	25	18.5	3	39.5	0.5

Table 4. Carbon distribution (%) estimated from the integration of ¹³C{¹H} NMR spectra [38].

	0–50 ppm	50–110 ppm	110–165 ppm	165–190 ppm	190–210 ppm
sample	Aliphatic-C	O-Alkyl-C	Aromatic-C	Carboxylic-C	Ketonic-C Aldehydic-C
water	8	3	84	2	3
soil	6	4	63	26	1

3.4. Pyrolysis-GC-MS

Figures 6 and 7 show the pyrograms obtained from soil (A) and Fiuggi water (B) after samples pre-treated with TMHA were considered to be thermally assisted hydrolysis and methylation reactions. Owing to the high similarity between the different samples, a single soil pyrogram representative of the areas under study is shown.

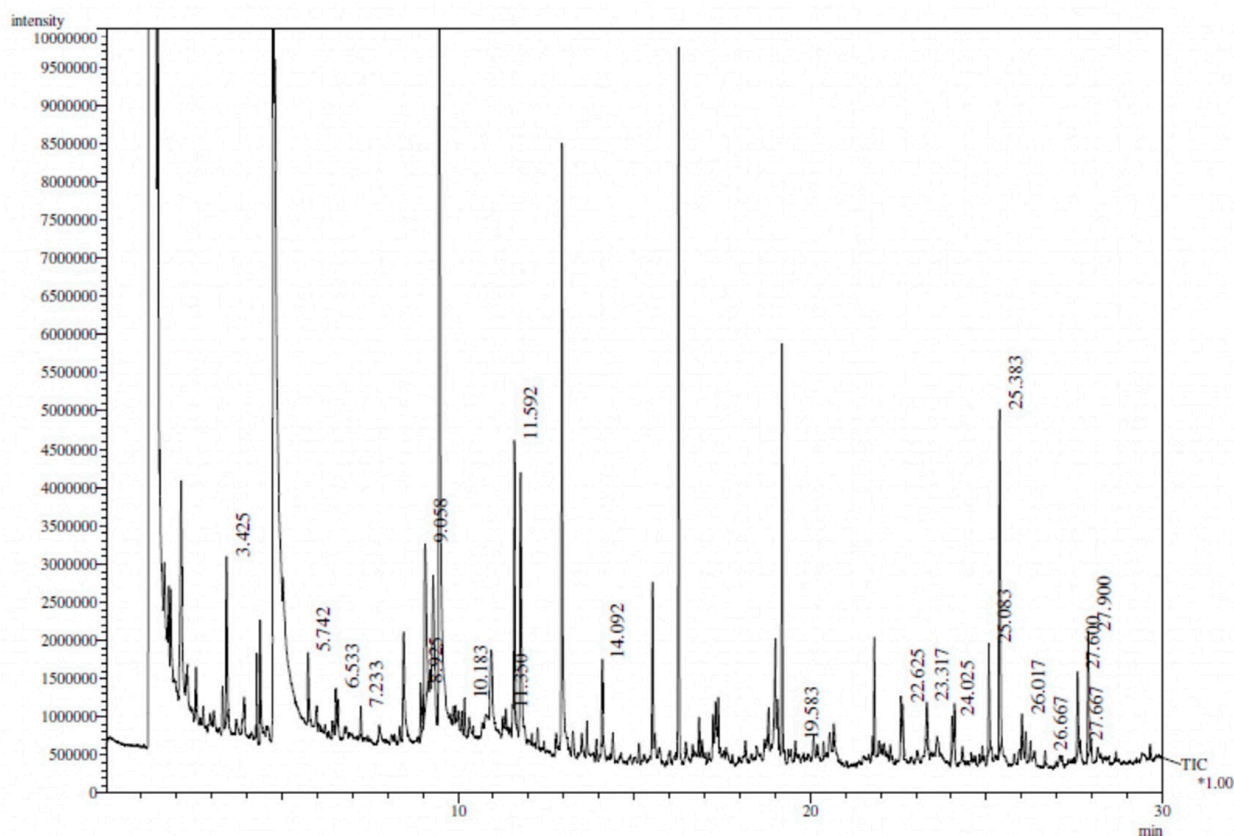


Figure 6. Pyrogram obtained by the Py-GC-MS process of FA extracted from the soil.

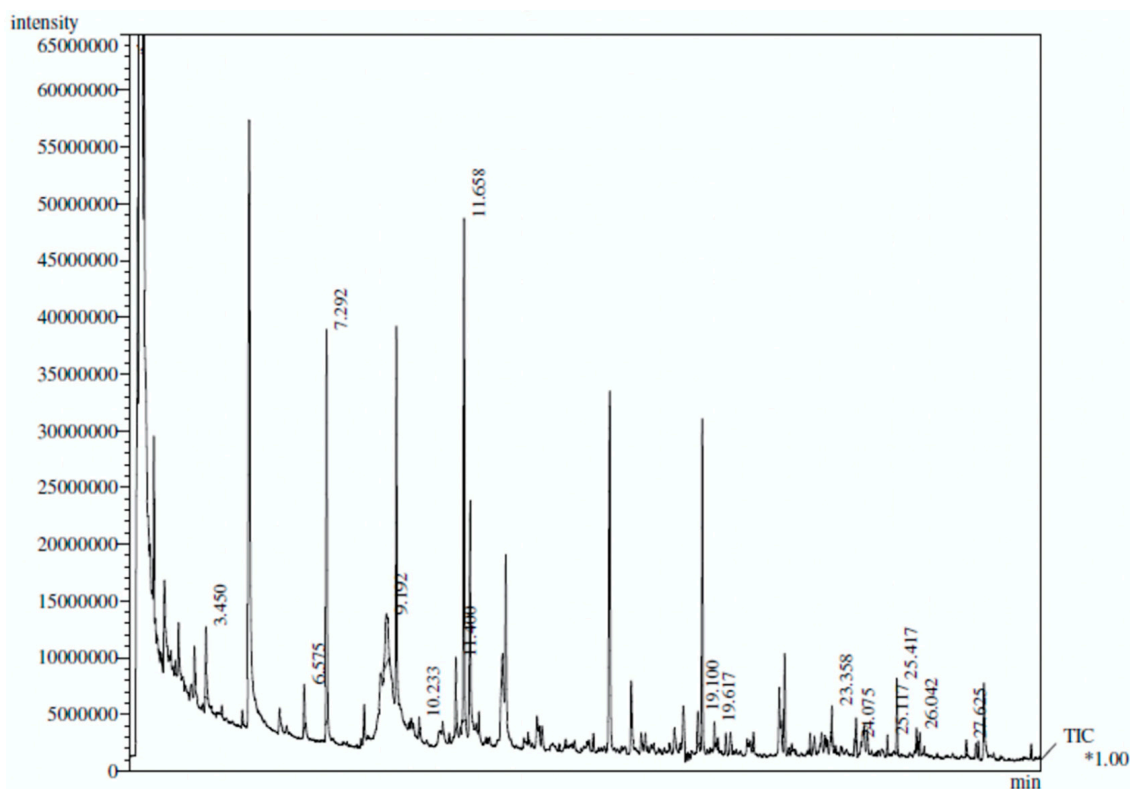


Figure 7. Pyrogram obtained from the Py-GC-MS process of FA extracted from Fiuggi water.

The analyzed samples were characterised (Table 5) by the presence of aliphatic compounds, methyl esters of C7–C18 monocarboxylic acids (both saturated and unsaturated), C4–C9 dicarboxylic acids (a small amount of which were unsaturated), phenol methyl ethers, sulphur, and nitrogen compounds of the same type as those found in the aqueous fractions.

Table 5. Compounds identified by Py-GC-MS.

Retention Time (min)	Name	Class of Compound
3.367	Toluene	aromatic
5.772	Ethylbenzene	aromatic
6.559	Styrene	aromatic
7.261	Benzene, methoxy-	aromatic
8.917	Hexahydro-1,3,5-Trimethyl-s-triazine	heterocycl
9.101	Phenol	aromatic
10.111	2-Ethyl-4-methylpentanol	alcohol
10.191	Butanedioic acid, dimethyl ester	dicarboxylic acid
11.358	Benzene, (methylthio)-	aromatic
11.608	Benzoic acid, methyl ester	aromatic carboxylic acid
11.809	Methanethioamide	Thioamide
14.096	Benzoic acid, 4-methyl-, methyl ester	aromatic carboxylic acid
19.580	Dodecanoic acid, methyl ester	carboxylic acid
20.690	Benzoic acid, 3,4-dimethoxy-, methyl ester	aromatic carboxylic acid
22.333	10-Undecenoic acid, methyl ester	monounsaturated carboxylic acid
22.628	Methyl tetradecanoate	carboxylic acid
23.326	2-Propenoic acid	carboxylic acid
23.644	Octadecanoic acid, methyl ester	carboxylic acid
24.040	Pentadecanoic acid, methyl ester	carboxylic acid
25.088	9-Hexadecenoic acid, methyl ester,	carboxylic acid
25.391	Hexadecanoic acid, methyl ester	carboxylic acid
26.018	13-Docosenoic acid, methyl ester	carboxylic acid

Table 5 shows some of the compounds detected in the pyrolysed samples.

4. Discussion

In this study, FA of humic substances was isolated and purified from Fiuggi mineral water, the springs of which are located underneath chestnut forests. These FAs were then compared with FAs isolated and characterized from the chestnut forest soils under study. FAs isolated from soil samples and Fiuggi mineral water were subjected to pyrolysis, GCMS, FTIR, and ^1H and ^{13}C -NMR.

The fractionated soils were subjected to quantitative determination of fulvic acid (FA). The results showed a significantly different amount of FA between the three sites under study: Quarto le Prata had the highest amount, followed by Le Cese and Quarto d'Anagni.

Fiuggi water, processed as described in the previous section, resulted in the isolation of 154 mg of FA from 280 L of water, corresponding to a concentration of 0.55 mg L^{-1} .

The FA concentration detected in Fiuggi mineral water is within the parameters set by Italian legislation for water intended for human consumption; the Italian legislation in force (D.lgs. 31/01), which transposes European legislation, states that, for surface water and water intended for human consumption or domestic use, the concentration of TOC should be in the order of $0.1\text{--}10\text{ mg L}^{-1}$.

The FTIR absorption spectra obtained from FA extracted from soil from the sites examined showed spectral similarity between them and a similarity superimposed with the spectrum obtained from FA extracted from water. The analysis of the FTIR spectral data made it possible to assign peaks characteristic of the observed spectral zone to functional groups, as reported by Wu et al. (2016) [44].

The absorbance distributions obtained from the FAs did not show significant differences from the literature data [14], with an intense band at 1720 cm^{-1} due to the presence of a high amount of carboxyl groups. Peaks at $1720\text{--}1630\text{--}1120\text{ cm}^{-1}$ were clearly visible in FAs extracted from both soil and mineral water. A very intense band was present in the range of $1227\text{--}1224\text{ cm}^{-1}$ (C-O elongation and O-H deformation of COOH groups), indicating the presence of an important acid component in the FA structure. The spectral absorbances acquired for FA showed no significant differences from those reported in the literature [14], with an intense band at 1720 cm^{-1} due to the presence of a high amount of carboxyl groups. No main differences were found between the samples.

Polar hydrophilic groups (COOH and OH) and aromatic components constitute the functional groups mostly present in the extracted FA. The main absorption bands found were 3400 cm^{-1} (O-H bond stretching of phenols, H_2O , OH), 2920 cm^{-1} (due to stretching of the C-H bond of CH_2 groups), 1720 and 1220 cm^{-1} (due to stretching of the -C=O bond and stretching of the -C-O bond, as well as deformation of the O-H bond of COOH groups, respectively), 1620 cm^{-1} (due to stretching of the C=C bond of aromatic rings as well as primary amides and secondary amides, and again due to stretching of the C=C bond of aromatic rings), 1400 cm^{-1} (due to phenolic OH and/or OH of COOH), and 1050 cm^{-1} (due to stretching of the C-O bond of polysaccharides and/or vibration of the Si-O bond of mineral compounds (ashes)).

Much more intense absorption peaks at the same wavelengths were observed in the spectra of FA samples extracted from water. This result attested to the presence of a much richer functional component in this structure than in that extracted from the soil. In particular, an intense broad band at 3400 cm^{-1} due to H_2O O-H bond stretching, a very intense band in the range of $1227\text{--}1224\text{ cm}^{-1}$ due to C-O stretching and O-H deformation of COOH groups, and an intense peak related to C-O bond stretching of polysaccharides at 1040 cm^{-1} were identified. The trend toward the similarity of the structures of FA extracted from soils and water was also evident from the results of py-GC-MS analysis.

While FTIR analysis made it possible to identify which functional groups were characteristic of the FA molecule, the use of py-GC-MS proved useful in obtaining detailed structural information on the components of organic molecules. Pyrolysis with in situ methylation in the presence of TMAH enabled structural analysis of macromolecules. The

method was based on the simultaneous thermal decomposition and methylation of humic macromolecules into a variety of fragments, which were then separated by gas chromatography and identified by MS according to their characteristic fragmentations. In this process, methyl esters and methyl ethers were produced. The presence of tetramethylammonium hydroxide also prevented the decarboxylation of aromatic acids and the dehydration of alcohol products, thus preventing the loss of important structural information: polar compounds such as phenols and carboxylic acids in HS are difficult to detect; therefore, using tetramethylammonium hydroxide (TMAH), methyl derivatives of acids and phenols were separated and identified.

The FA extracted from soil samples of the different chestnut groves presented a pyrolytic profile similar to the corresponding fraction extracted from Fiuggi water; that is, they were characterised by the presence of aliphatic compounds, methyl esters of C7–C18 (both saturated and unsaturated) and dicarboxylic acids (C4–C9, a small proportion of which are unsaturated), aromatic, sulfur, and nitrogen compounds of the same type as those found in the aqueous fractions.

The pyrolysis products identified included several classes of compounds, in good agreement with previous reports [45–51].

The TMAH-GC-MS chromatograms of fulvic acids (FAs) isolated from different soils are shown in Figure 6 in the previous section. In general, the distribution of the products was found to be similar to that obtained by Hatcher and Clifford [52]; the first portion of the chromatogram contained mainly peaks of aromatic compounds. The second portion consisted of peaks corresponding to aliphatic compounds, mostly fatty acid methyl esters [52,53].

The four samples considered were typically found to contain a high fraction of aliphatic compounds; that is, methyl esters of monocarboxylic acids (C7–C18, both saturated and unsaturated) and dicarboxylic acids (C4–C9, of which a small proportion are unsaturated), alkene and alkane pairs (C12–C23), and short-chain alkenes. Some of the pyrolysis products were identified as aromatic compounds, such as alkylbenzenes, phenols and alkylphenols, benzoic acids, mono- and di-methoxybenzoic acids, polyaromatic hydrocarbons, nitrogen-containing compounds, amino acids, heterocyclic amines (imidazoles, pyrroles, pyrimidines, pyridines, and pyrrolidines), and other nitrogen-containing aromatic compounds (indoles, benzonitriles, benzendiamines, and quinolines) and their derivatives.

The results showed that various aromatic compounds were thermally released from FA and were mainly composed of methyl esters of mono-, di-, and tri-methoxy (alkyl)-benzenecarboxylic acids. The aromatic compounds identified in the pyrograms of the analyzed fractions showed the contribution of all lignin block constituents, and these entities appeared to present oxidative degradation products derived from lignin [54].

It is precisely the oxidative degradation of lignin that suggested the possibility of an explanation for the presence of compounds derived from this relatively insoluble polymer in dissolved humic substances [55]; in this case, in FA extracted from water, even if modified, these humic substances retained an incorporated lignin signature sufficient to qualitatively indicate the vascular plant material of origin. Another group of products identified in the pyrograms of the analyzed fractions are nitrogen-containing compounds. Pyrroles, pyridines, indoles, benzonitriles, and other N-heterocyclic compounds, rather than pyrolytic degradation products of other molecules, have been regarded as constituent components of soil organic matter [56]. These compounds were assumed to be derived from amino acids, proteins, and other polypeptides [56,57]. Benzenes and phenols, the dominant groups in the samples tested, were rather nonspecific pyrolysis products that might have had multiple sources, including lignin and proteins. The distribution of the aliphatic products identified in the various humic samples examined here reflected the contribution from higher plants. The aliphatic products of pyrolysis (n-alkanes, n-alkenes, n-methyl ketones, and n-fatty acids) might originate from leaf waxes; that is, biopolymers such as cutin/cutans, suberin/suberanes, and algeenan. Cutin is associated with most tissues in the aerial parts (leaves, flowers, and fruits) of higher plants and is found in both

the outer and inner layers of plant cuticles and in the lamellae that support the epicuticular protective layer made of wax [58,59]. Suberin, on the other hand, is a complex polymer network that forms the protective layer of bark, woody trunks, and underground parts of plants such as roots [59,60]. Although these biopolyesters were minor components of the original biomass, their contribution was so high because they are more refractory to biodegradation than other major plant components, including lignin [45].

Information from the NMR data of FA samples extracted from the soils and water of Fiuggi appear consistent with the results of the other analytical techniques. In agreement with the literature and with the other data from FTIR and py-GCMS, phenolic, carboxyl, and aliphatic groups were detected. Although rather similar, a detectable difference can be observed between the two samples. Aromatics seem slightly more abundant in FA from the water sample, whereas aliphatics and carboxylic derivatives appear to predominate in FA extracted from the soil sample.

From the results obtained, it is possible to make generic structural assignments between the spectra obtained and the components of the SOM from which the FAs originated. In general, it is possible to associate the presence of aromatic and carboxylic Cs in the structure of FAs in ^{13}C -NMR spectra with SOM components such as lignin, suberin, amino acids/proteins, fatty acids, and esterified lipids [61].

5. Conclusions

The analytical results obtained showed that fulvic acids in the Fiuggi mineral water presented spectral similarities and qualitative similarities in terms of pyrolytic profiles to fulvic acid isolated from the soil above the aquifer, which is covered by a chestnut forest.

The enrichment that the topsoils confer to the waters, passing through the volcanic soil that hosts the chestnut forest, is extremely valuable for the purposes of defining strategies aimed at the valorisation of the waters, the correct management of the soil, and the sustainable management of the natural forest capital involved, ultimately ensuring the continuous availability over time of this important resource for human survival. The maintenance and promotion of soil quality, the adoption of silvicultural modules and schemes, and forest management aimed at achieving greater biological efficiency of the stands are thus fundamental requirements to guarantee the sustainability of the ecosystem and allow the promotion of multiple ecosystem functions, particularly with regard to water.

Author Contributions: E.A., chemical analysis, data analysis, drafting; F.C., material sampling, data discussion, drafting; R.M., experimental design, chemical analysis, data analysis; G.N., data analysis, drafting; S.R.S. experimental design, sampling, data analysis, drafting; V.V., chemical analysis, data analysis; C.Z., nuclear magnetic resonance analysis, data discussion. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Giovanni Ludovici and his staff for the research support and collaboration in the operational phases. The authors thank the University of Perugia and MIUR (“Progetto AMIS-Dipartimenti di Eccellenza”) for NMR instrument time. The authors thank the mineral water plant of Fiuggi for their kind availability.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Leonardi, A.; Masiero, M.; Meier, L. Forests and Water, Valuation and payments for forest ecosystem services. In *UNECE/FAO Forestry and Timber Section*; United Nations Publication: Geneva, Switzerland, 2018.
2. Bellver-Domingo, A.; Hernández-Sancho, F.; Molinos-Senante, M. A review of Payment for Ecosystem Services for the economic internalization of environmental externalities: A water perspective. *Geoforum* **2016**, *70*, 115–118. [[CrossRef](#)]
3. Neary, D.G.; Ice, G.G.; Jackson, C.R. Linkages between forest soils and water quality and quantity. *For. Ecol. Manag.* **2009**, *258*, 2269–2281. [[CrossRef](#)]
4. Prestininzi, A.; Ludovici, G. Ecosystem of the Anticolana Valley. *Nephron* **1999**, *81*, 82–92. [[CrossRef](#)] [[PubMed](#)]

5. Jenkins, M.; Schaap, B. Forest ecosystem services. Background study prepared for the thirteenth session of the United Nations Forum on Forests. In *United Nations Forum on Forests*; UN Press: New York, NY, USA, 2018.
6. Carta dei Suoli del Lazio—Scala 1:250.000—v. 2019. Tutela Risorse e Vigilanza sulle Produzioni di Qualità. Available online: <http://dati.lazio.it/catalog/it/dataset/carta-dei-suoli-del-lazio-1-250-000-ed-2019/resource/b27732c7-05cc-4447-8dac-386d1fbcf829> (accessed on 27 July 2019).
7. Calace, N.; Petronio, B.M.; de Angelis Curtis, S.; Fraioli, A.; Delfini, M.; D’Ascenzo, G. Chemical analysis of water of the Anticolana Valley: Isolation of humic compounds. *Nephron* **1999**, *81*, 93–97. [[CrossRef](#)] [[PubMed](#)]
8. Freitas, T.R.; Santos, J.A.; Silva, A.P.; Fraga, H. Influence of Climate Change on Chestnut Trees: A Review. *Plants* **2021**, *10*, 1463, Erratum in *Plants* **2022**, *11*, 11. [[CrossRef](#)] [[PubMed](#)]
9. Regional Council Resolution 158/2016. In *Adozione delle Misure di Conservazione finalizzate alla designazione delle zone speciali di conservazione (ZSC) ai sensi della Direttiva 92/43/CEE (Habitat) e del DPR 357/97 e s.m.i.—codice IT 60500*; Regione Lazio: Frosinone, Italy.
10. Fraioli, A.; De Angelis Curtis, S.; Ricciuti, G.; Serio, A.; D’Ascenzo, G. Effect of water of Anticolana Valley on urinary sediment of renal stone formers. *La Clin. Ter.* **2001**, *152*, 347–351. [[PubMed](#)]
11. Di Silverio, F.; Ricciuti, G.P.; D’Angelo, A.R.; Fraioli, A.; Simeoni, G. Stone recurrence after lithotripsy in patients with recurrent idiopathic calcium urolithiasis: Efficacy of treatment with fiuggi water. *Eur. Urol.* **2000**, *37*, 145–148. [[CrossRef](#)] [[PubMed](#)]
12. Davydova, N.K.; Sergeev, V.N.; Girbul, E. The role of humous acids in acqua di Fiuggi mineral water in degrading stones formed in the urinary tract (Review). *Pharm. Chem. J.* **2014**, *48*, 587–592. [[CrossRef](#)]
13. MacCarthy, P. The principles of humic substances. *Soil Sci.* **2001**, *166*, 738–751. [[CrossRef](#)]
14. Rashad, M.; Hafez, M.; Popov, A.I. Humic substances composition and properties as an environmentally sustainable system: A review and way forward to soil conservation. *J. Plant Nutr.* **2022**, *45*, 1072–1122. [[CrossRef](#)]
15. Simpson, A.J.; Kingery, W.L.; Hayes, M.H.B.; Spraul, M.; Humpfer, E.; Dvorsak, P.; Kerssebaum, R.; Godejohann, M.; Hofmann, M. Molecular structures and associations of humic substances in the terrestrial environment. *Naturwissenschaften* **2002**, *89*, 84–88. [[CrossRef](#)] [[PubMed](#)]
16. Guimarães, D.V.; Gonzaga, M.I.S.; da Silva, T.O.; da Silva, T.L.; Dias, N.D.S.; Matias, M.I.S. Soil organic matter pools and carbon fractions in soil under different land uses. *Soil Tillage Res.* **2013**, *126*, 177–182. [[CrossRef](#)]
17. Sutton, R.; Sposito, G. Molecular structure in soil humic substances: The New View. *Environ. Sci. Technol.* **2005**, *39*, 9009–9015. [[CrossRef](#)] [[PubMed](#)]
18. Pettit, R.E. Organic matter, humus, humate, humic acid, fulvic acid and humin: Their importance in soil fertility and plant health. *CTI Res.* **2004**, *10*, 1–7.
19. Orlov, D.S. *Humic Substances of Soils and General Theory of Humification*; Taylor & Francis: London, UK, 1995.
20. Lodygin, E.D.; Beznosikov, V.A. The molecular structure and elemental composition of humic substances from Albeluvisols. *Chem. Ecol.* **2010**, *26*, 87–95. [[CrossRef](#)]
21. Lehmann, J.; Kleber, M. The contentious nature of soil organic matter. *Nature* **2015**, *528*, 60–68. [[CrossRef](#)] [[PubMed](#)]
22. Gerzabek, M.H.; Aquino AJ, A.; Balboa YI, E.; Galicia-Andrés, E.; Grančič, P.; Oostenbrink, C.; Tunega, D. A contribution of molecular modeling to supramolecular structures in soil organic matter#. *J. Plant Nutr. Soil Sci.* **2020**, *185*, 44–59.
23. Piccolo, A.; Nardi, S.; Cancheri, G. Macromolecular changes of soil humic substances induced by interactions with organic acids. *Eur. J. Soil Sci.* **1996**, *47*, 319–328. [[CrossRef](#)]
24. Piccolo, A.; Nardi, S.; Cancheri, G. Micelle like conformation of humic substances as revealed by size-exclusion chromatography. *Chemosphere* **1996**, *33*, 595–600. [[CrossRef](#)]
25. Piccolo, A. The supramolecular structure of humic substances. *Soil Sci.* **2001**, *166*, 810–832. [[CrossRef](#)]
26. Piccolo, A. The supramolecular structure of humic substances. A novel understanding of humus chemistry and implications in soil science. *Adv. Agron.* **2002**, *75*, 57–134.
27. Ukalska-Jaruga, A.; Debaene, G.; Smreczak, B. Particle and structure characterization of fulvic acids from agricultural soils. *J. Soils Sediments* **2018**, *18*, 2833–2843. [[CrossRef](#)]
28. Thurman, E.M. Isolation, Characterization, and Geochemical Significance of Humic Substances from Ground Waters. Ph.D. Thesis, University of Colorado, Boulder, CO, USA, 1979.
29. Albertini, M.C.; Dachà, M.; Teodori, L.; Conti, M.E. Drinking mineral waters: Biochemical effects and health implications - The state-of-the-art. *Int. J. Environ. Health* **2007**, *1*, 153–169. [[CrossRef](#)]
30. Marabottini, R.; Stazi, S.R.; Papp, R.; Grego, S.; Moscatelli, M.C. Mobility and distribution of arsenic in contaminated mine soils and its effects on the microbial pool. *Ecotoxicol. Environ. Saf.* **2013**, *96*, 147–153. [[CrossRef](#)] [[PubMed](#)]
31. Stazi, S.R.; Mancinelli, R.; Marabottini, R.; Alleinato, E.; Radicetti, E.; Campiglia, E.; Marinari, S. Influence of organic management on As bioavailability: Soil quality and tomato As uptake. *Chemosphere* **2018**, *211*, 352–359. [[CrossRef](#)] [[PubMed](#)]
32. Gillman, G.P. A proposed method for the measurement of exchange properties of highly weathered soils. *Aust. J. Soil Res.* **1979**, *17*, 129–139. [[CrossRef](#)]
33. Loeppert, R.H.; Suarez, D.L. Carbonate and Gypsum. In *Methods of Soil Analysis*; Sparks, D., Page, A., Helmke, P., Loeppert, R., Soltanpour, P.N., Tabatabai, M.A., Johnston, C.T., Sumner, M.E., Eds.; Soil Science Society of America Book Series, no. 5; Soil Science Society of America: Madison, WI, USA, 1996. [[CrossRef](#)]

34. Hayes, M.B.H. Extraction of Humic Substances from Soil. In *Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolation, and Characterization*; Aiken, G.R., McKnight, D.M., Wershaw, R.L., MacCarthy, P., Eds.; Wiley-Interscience: New York, NY, USA, 1985; pp. 329–362.
35. Swift, R.S. Fractionation of Soil Humic Substances. In *Humic Substances in Soil, Sediment and Water*; Aiken, G.R., McKnight, D.M., Wershaw, R.L., MacCarthy, P., Eds.; Wiley: New York, NY, USA, 1985; pp. 387–408.
36. Aiken, G.R. Isolation and Concentration Techniques for Aquatic Humic Substances. In *Humic Substances in Soil, Sediment and Water: Geochemistry and Isolation*; Aiken, G.R., McKnight, D.M., Wershaw, R.L., MacCarthy, P., Eds.; Wiley-Interscience: New York, NY, USA, 1985.
37. Thurman, E.M.; Malcolm, R.L. Preparative isolation of aquatic humic substances. *Environ. Sci. Technol.* **1981**, *15*, 463–466. [[CrossRef](#)]
38. Gigliotti, G.; Macchioni, A.; Zuccaccia, C.; Giusquiani, P.L.; Businelli, D. A spectroscopic study of soil fulvic acid composition after six-year applications of urban waste compost. *Agronomie* **2003**, *23*, 719–724. [[CrossRef](#)]
39. Stevenson, F.J. *Humus Chemistry: Genesis, Composition, and Reactions*; John Wiley and Sons: New York, NY, USA, 1994.
40. Moraes, G.M.; Xavier, F.A.S.; Mendonça, E.S.; Araújo Filho, J.A.; Oliveira, T.S. Chemical and structural characterization of soil humic substances under agroforestry and conventional systems. *Rev. Bras. Cien. Solo.* **2011**, *35*, 1597–1608. [[CrossRef](#)]
41. Machado, W.; Franchini, J.C.; de Fátima Guimarães, M.; Filho, J.T. Spectroscopic characterization of humic and fulvic acids in soil aggregates, Brazil. *Heliyon* **2020**, *6*, e04078. [[CrossRef](#)] [[PubMed](#)]
42. Haiber, S.; Herzog, H.; Burba, P.; Goscinia, B.; Lambert, J. Two-dimensional NMR studies of size fractionated Suwannee River fulvic and humic acid reference. *Environ. Sci. Technol.* **2001**, *35*, 4289–4294. [[CrossRef](#)] [[PubMed](#)]
43. Albers, C.N.; Hansen, P.E. ¹³C-NMR chemical shift databases as a quick tool to evaluate structural models of humic substances. *Open Magn. Reson. J.* **2010**, *3*, 96–105. [[CrossRef](#)]
44. Wu, M.; Song, M.; Liu, M.; Jiang, C.; Li, Z. Fungicidal activities of soil humic/fulvic acids as related to their chemical structures in greenhouse vegetable fields with cultivation chronosequence. *Sci. Rep.* **2016**, *6*, 32858. [[CrossRef](#)] [[PubMed](#)]
45. Martín, F.; del Rio, J.C.; González-Vila, F.J.; Verdejo, T. Pyrolysis derivatization of humic substances II: Pyrolysis of soil humic acids in the presence of tetramethylammonium hydroxide. *J. Anal. Appl. Pyrolysis* **1995**, *31*, 75–83. [[CrossRef](#)]
46. Martín, F.; González-Vila, F.J.; del Rio, J.C.; Verdejo, T. Pyrolysis derivatization of humic substances I: Pyrolysis of fulvic acids in the presence of tetramethylammonium hydroxide. *J. Anal. Appl. Pyrolysis* **1994**, *28*, 71–80. [[CrossRef](#)]
47. Del Rio, J.C.; Hatcher, P.G. Analysis of aliphatic biopolymers using thermochemolysis with tetramethylammonium hydroxide (TMAH) and gas chromatography—Mass spectrometry. *Org. Geochem.* **1998**, *29*, 1441–1451. [[CrossRef](#)]
48. Santos Bento, M.F.; Pereira, H.; Cunha, M.Á.; Moutinho, A.M.C.; van den Berg, K.J.; Boon, J.J. A study of variability of suberin composition in cork from *Quercus suber* L. using thermally assisted transmethylation GC—MS. *J. Anal. Appl. Pyrolysis* **2001**, *57*, 45–55. [[CrossRef](#)]
49. Nierop, K.G.J. Origin of aliphatic compounds in a forest soil. *Org. Geochem.* **1998**, *29*, 1009–1016. [[CrossRef](#)]
50. Nierop, K.G.J.; Pulleman, M.M.; Marinissen, J.C.Y. Management induced organic matter differentiation in grassland and arable soil: A study using pyrolysis techniques. *Soil Biol. Biochem.* **2001**, *33*, 755–764. [[CrossRef](#)]
51. Quéneá, K.; Derenne, S.; Largeau, C.; Rumpel, C.; Mariotti, A. Spectroscopic and pyrolytic features and abundance of the macromolecular refractory fraction in a sandy acid forest soil (Landes de Gascogne, France). *Org. Geochem.* **2005**, *36*, 349–362. [[CrossRef](#)]
52. Hatcher, P.G.; Clifford, D.J. Flash pyrolysis and in situ methylation of humic acids from soil. *Org. Geochem.* **1994**, *21*, 1081–1092. [[CrossRef](#)]
53. Amir, S.; Hafidi, M.; Lemee, L.; Bailly, J.-R.; Merlina, G.; Kaemmerer, M.; Revel, J.-C.; Amble, A. Structural characterization of fulvic acids, extracted from sewage sludge during composting, by thermochemolysis—gas chromatography—mass spectrometry. *J. Anal. Appl. Pyrolysis* **2006**, *77*, 149–158. [[CrossRef](#)]
54. Saiz-Jimenez, C. Origin and Chemical Nature of Soil Organic Matter. Ph.D. Thesis, Delft University Press, Delft, The Netherlands, 1988.
55. Guggenberger, G.; Zech, W. Dissolved organic carbon in forest floor leachates: Simple degradation products or humic substances? *Sci. Total Environ.* **1994**, *152*, 37–47. [[CrossRef](#)]
56. Schnitzer, M.; Schulten, H.R. New Ideas on the Chemical Make-up of Soil Humic and Fulvic Acids. In *Future Prospects for Soil Chemistry*; Soil Science Society of America: Madison, WI, USA, 1998; Volume 55, pp. 153–177.
57. van Bergen, P.F.; Flannery, M.B.; Poulton, P.R.; Evershed, R.P. Organic Geochemical Studies of Soils from the Rothamsted Classical Experiments. III: Nitrogen-Containing Macromolecular Moieties in Soil Organic Matter from Geescroft Wilderness. In *Nitrogen-Containing Macromolecules in the Bio and Geosphere*; Stankiewicz, B.A., van Bergen, P.F., Eds.; ACS Symposium Series, 707; Oxford University Press: New York, NY, USA, 1988; pp. 321–338.
58. Tulloch, A.P. Chemistry of Waxes of Higher Plants. In *Chemistry and Biochemistry of Natural Waxes*; Chemistry and Biochemistry of Natural Waxes; Kolattukudy, P.E., Ed.; Elsevier: Amsterdam, The Netherlands; New York, NY, USA, 1976; pp. 235–287.
59. Walton, T.J. Waxes, Cutin and Suberin. In *Lipids, Membranes and Aspects of Photobiology*; Methods in plant biochemistry; Harwood, J.L., Bowyer, J.R., Eds.; Academic Press: New York, NY, USA, 1990; Volume 4, pp. 105–158.

-
60. Kolattukudy, P. Biochemistry and function of cutin and suberin. *Can. J. Bot.* **1984**, *62*, 2918–2933. [[CrossRef](#)]
 61. González-Vila, F.J.; Lankes, U.; Lüdemann, H.D. Comparison of the information gained by pyrolytic techniques and NMR spectroscopy on the structural features of aquatic humic substances. *J. Anal. Appl. Pyrolysis* **2001**, *58*, 349–359. [[CrossRef](#)]