SJSS
SPANISH JOURNAL OF SOIL SCIENCE

Chemical diversity and molecular signature of soil humic fractions used as proxies of soil quality under contrasted tillage management

Diversidad química y patrón molecular de las fracciones húmicas como indicadoras de la calidad del suelo bajo distintos sistemas de laboreo

Diversidade química e assinatura molecular das frações húmicas como indicadoras da qualidade do solo em diferentes sistemas de cultivo

AUTHORS

Panettieri M.[1, 2,@]
marco.panettieri@csic.es

Jiménez-González M.A.[1]

De Sosa L.L.[3]

Almendros G.[1]

Madejón E.[3]

[@] Corresponding Author

[1]Museo Nacional de Ciencias Naturales (MNCN-CSIC). C/ Serrano 115-B. 28006, Madrid, Spain.

[2]Instituto de Ciencias Agrarias (ICA-CSIC). C/ Serrano 115-B. 28006, Madrid, Spain.

[3]Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC). Avenida Reina Mercedes 10. 41080, Sevilla, Spain.

Received: 18.08.2020 | **Revised:** 08.02.2021 | **Accepted:** 11.02.2021

ABSTRACT

Increasing food production while avoiding the progressive degradation of agricultural soils has become one of the major challenges at a global level. In consequence, the development of sustainable tillage methodologies or cultivation strategies is an important subject of current research. In fact, it has been observed that the implementation of reduced tillage (RT) vs. traditional tillage (TT) in the long term not only improves soil physicochemical properties but also global soil quality in terms of soil health. In particular, the increase of the soil organic carbon (SOC) content under RT conditions is one of the most important factors, but there is little information about the chemical composition and humification level of this carbon, and thus about its persistence at long-term. This is of particular importance considering the policies of carbon sequestration and climate change mitigation, such as the "4 per 1000" initiative. In this study, molecular-level characterization of the humic acid (HA) and fulvic acid (FA) fractions isolated from a soil after 19 years under RT and TT practices was carried out. This study would provide objective descriptors of the impact of these two tillage practices in the chemical composition of the resulting SOC. With this purpose, the potential of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) for assessing changes in the molecular assemblages released from the humic fractions (HA and FA) was examined. The results showed enhanced diversity and chemical richness (expressed as number of molecular formulas) in the composition of SOC under RT. Different tillage-specific compound classes were associated with both tillage practices. As a whole, the humic fraction showed a higher proportion of molecular formulas for lipid and hydroaromatic families in the case of RT compared to TT, while the same fraction under TT showed a greater richness of oxidized protein-derived formulas than RT. In the case of FAs, a similar pattern was observed for hydroaromatic and protein-derived formulas, but the proportion of molecular formulas assigned to unsaturated lipids was higher in TT than in RT. In addition, increased number of formulas for aromatic and condensed aromatic compounds was observed in FAs under TT respect to RT.

DOI: 10.3232/SJSS.2021.V11.N1.04

SJSS
SPANISH JOURNAL OF SOIL SCIENCE

RESUMEN

El incremento en la producción de alimentos evitando los procesos de degradación de los suelos agrícolas se ha convertido en uno de los mayores retos a nivel global. Por esta razón, el desarrollo de nuevas técnicas de laboreo o estrategias de cultivo sostenible es uno de los principales campos de investigación en agricultura. La implementación de estrategias como el laboreo reducido (RT) frente al laboreo tradicional (TT) mejoran ciertas propiedades físico-químicas del suelo. Una de estas propiedades de especial relevancia es el contenido de carbono orgánico del suelo (SOC), que se ve favorecido en términos de cantidad bajo condiciones de RT. Sin embargo, se tiene poca información acerca de la composición química de este carbono y su persistencia a largo plazo. Esta información es particularmente importante considerando las políticas de secuestro de carbono y mitigación del cambio climático, como la iniciativa 4 por 1000. En este estudio, se realizó una caracterización química comparativa de las fracciones de ácidos húmicos y fúlvicos aisladas de la materia orgánica de un suelo proveniente de un experimento de campo en el que se han comparado dos tipos de laboreo (RT y TT) durante 19 años. Para la caracterización química se empleó la espectrometría de resonancia ciclotrónica por transformada de Fourier (FTICR-MS). Los resultados mostraron una mayor diversidad y riqueza química en la SOC en el tratamiento RT. La fracción húmica mostró una mayor proporción de compuestos de naturaleza lipídica e hidroaromática en el caso de RT, mientras que la misma fracción en suelos bajo TT mostró una mayor riqueza en compuestos más oxidados de naturaleza proteica. En el caso de los ácidos fúlvicos, se observó una tendencia similar a la de los húmicos; sin embargo, en este caso la proporción de lípidos insaturados fue mayor en los suelos bajo TT que en los suelos RT. Además, se observó un ligero aumento en el número de compuestos con carácter aromático y aromático condensado en los ácidos fúlvicos extraídos en suelos bajo TT en comparación con aquellos extraídos en los suelos bajo RT.

RESUMO

O aumento da produção de alimentos, evitando os processos de degradação progressiva dos solos agrícolas, tornou-se um dos maiores desafios a nível global. Consequentemente, o desenvolvimento de técnicas ou de estratégias de cultivo sustentável é um dos principais campos de investigação em agricultura. A implementação, a longo prazo, de estratégias como a mobilização mínima (RT) vs cultivo tradicional (TT) melhoram não apenas determinadas propriedades físico-químicas, mas também a qualidade global do solo. Uma das propriedades de especial relevância é o conteúdo em carbono orgânico do solo (SOC), o qual aumenta em condições RT. Porém, há pouca informação acerca da composição química e nível de humificação deste carbono e, portanto, a sua persistência a longo prazo. Esta informação é particularmente importante considerando as políticas de sequestro de carbono e mitigação das alterações climáticas, como a iniciativa 4 por 1000. Neste estudo, realizou-se uma caracterização química comparativa das frações de ácidos húmicos (HA) e ácidos fúlvicos (FA) isoladas da matéria orgânica de um solo proveniente de um ensaio de campo em que se compararam os dois tipos de cultivo (RT e TT) durante 19 anos. Para a caracterização química foi usada a espectrometria de massa de ressonância ciclotrónica de ião com transformada de Fourier (FTICR-MS). Os resultados mostraram maior diversidade e riqueza química (expressa em número de fórmulas moleculares) na SOC em condições de RT. A fração húmica apresentou maior proporção de compostos de natureza lipídica e hidroaromática no caso de RT, enquanto a mesma fração nos solos em TT apresentou maior riqueza em compostos mais oxidados de natureza proteica. Os ácidos fúlvicos apresentaram um padrão semelhante ao dos ácidos húmicos; contudo, a proporção de lípidos insaturados foi maior nos solos em TT do que nos solos em RT.

KEY WORDS
Soil management, soil organic carbon, humic acids, fulvic acids, high resolution mass spectrometry.

PALABRAS CLAVE
Manejo del suelo, carbono orgánico del suelo, ácidos húmicos, ácidos fúlvicos, espectrometría de masas de alta resolución.

PALAVRAS-CHAVE
Gestão do solo, carbono orgânico do solo, ácidos húmicos, ácidos fúlvicos, espetrometria de massa de alta resolução.

1. Introduction

In a global context of a constantly growing population, increasing food production while minimizing environmental impacts has become a major challenge for sustainable agriculture and policymakers. The use of targeted tillages is one of the policies aiming to increase sustainable agriculture management (Gajri et al. 2002). Intensive tillage often involves progressive loss of soil productivity and quality paralleling soil organic carbon (SOC) depletion

(Lal 2008; Minasny et al. 2017; Chenu et al. 2019). Concern has been raised about the negative impact of mouldboard ploughing in certain farms (Lal et al. 1989; Panettieri et al. 2013), especially in areas subjected to potential risks of erosion and desertification (Lal 2003; Melero et al. 2011). However, mouldboard is still a valid option in other edaphic and climatic scenarios (Dang et al. 2015). Aside from ploughing, the correct management of crop residues is crucial to increase SOC stocks and maintain soil fertility levels. Gajri et al. (2002) defined as conservation tillage those types of tillage that avoid soil inversion and left an adequate amount of crop residues as soil cover, practices that have been proposed as sustainable managements for most of agricultural lands (Panettieri et al. 2014; Dignac et al. 2017). Among the conservation tillage systems, reduced tillage (RT) decreases soil disturbance and decomposition rates, therefore improving SOC stocks and soil fertility with a postulated key role in mitigating climate change, as proposed by the recent 4 per 1000 initiative by the United Nations (Minasny et al. 2017). These aspects have been related with an overall increase of soil quality associated to high levels of enzymatic activities, unveiling a more efficient cycle of C and nutrients in soils under conservation tillage (Panettieri et al. 2014, 2015). Under similar edaphic and climatic conditions, the pattern that transforms fresh organic matter, i.e. crop residues, into more simple or complex forms of SOC is governed by the type and distribution of C inputs, soil physical properties, and soil microbial communities (Dignac et al. 2017). The combination of all those factors that are affected by tillage may lead to intermediate states of crop residues degradation and/or SOC oxidation. Therefore, tillage not only affects the quantity of C stored, but also its quality (Panettieri et al. 2014). These changes are difficult to detect as only certain pools of SOC are affected by tillage on a timespan of few years, while a large background of SOC remains unaffected (Leifeld and Kögel-Knabner 2005). To monitor those changes, researchers need to overcome the issues generated by the complexity of the soil matrix. The main strategies include extractions of selected pools of SOC and the use of high-end analytical techniques (Almendros et al. 2003; Leifeld and Kögel-Knabner 2005; Plaza et al. 2012).

For this study, we have focused on two alkali-extracted pools of SOC (i.e. HA and FA) analysed by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) (Sleighter and Hatcher 2007). Alkali extraction has been extensively used to isolate SOC before its chemical characterization. Despite the recent hermeneutic debate about the role, native macromolecular structure and biological functions in soils of the humic-type laboratory preparations (Lehmann and Kleber 2015; Baveye and Wander 2019; Kleber and Lehmann 2019), the interest in humic substances as a source of environmental information reflected in its molecular structure is supported by wide and well-referenced record in the scientific literature (Olk et al. 2019; De Nobili et al. 2020; Hayes and Swift 2020; Jiménez-González et al. 2020).

The concept of chemical diversity of soil organic matter has been recently revisited in terms of soil biochemical functionality (Jiménez-González et al. 2018). In fact, high SOC levels have been correlated with high biodiversity of overlapping soil microbial communities and plant species (Banerjee et al. 2016; Wagg et al. 2019), leading quite logically into a high diversity of organic compounds in soil. Plants are the largest contributor of C inputs to soil, whereas soil microbial communities are the most important degraders of these inputs. For this reason, enhanced chemical richness is expected with an increase in the variability of both the C inputs and the metabolic pathways available for its transformation. Thus, the study of chemical diversity in soils can become a key tool for understanding how different soil management can affect the evolution and composition of the SOM stocks.

The objectives of this work are: (i) to identify new indexes of soil quality related to the chemical diversity of soil organic carbon (ii) to relate those indexes to biogeochemical cycle of soil organic carbon, and (iii) to extrapolate the soil organic carbon persistence under specific land-uses in a global change scenario.

The working hypothesis of this study assumes that, compared to traditional tillage, reduced tillage would result into an enhanced organic biogeochemical complexity which would be reflected in the molecular signature of composition of the SOC forms. In particular, the structural components of the humic matter represent a molecular signature depending on the different SOC storage processes, in some cases including specific biomarkers responsive of the preferential activity of the of the organisms involved in SOC turnover. To test this hypothesis, the chemical diversity indexes obtained by analysing HAs and FAs extracted from soil plots cultivated under reduced and traditional tillage were compared. To our knowledge, such innovative approach has never been proposed before.

2. Material and Methods

2.1. Experimental area, crop rotation and tillage operations

Soil samples were collected at “La Hampa” dryland experimental farm located in Coria del Río (Seville, Spain, 37°17′ N, 6°3′ W) belonging to the “Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC)”. The soil is classified as Typic Xerofluvent (Soil Survey Staff 2014). Edaphic characteristics are summarized in **Table 1**.

The climatic conditions recorded by a meteorological station located at the farm were typically Mediterranean with mild rainy winters (496 mm mean annual rainfall) and very hot and dry summers. The mean annual daily temperature at the experimental site is around 19 °C, with maximum and minimum mean monthly temperatures of 33.5 °C and 5.2 °C registered in July and January, respectively.

The experiment was established in an area of approximately 2500 m^2, cropped with wheat under rainfed conditions until 1991. In June 1992, after crop harvesting, the area was divided into 6 plots of 22 × 14 m and two treatments were established in a completely randomized experimental design with three replicates per treatment.

One of the two treatment was a traditional tillage (TT) consisting of a 30-cm deep mouldboard plough inducing topsoil/subsoil inversion followed by two cultivator passes and a disc harrowing each at 15-20 cm depth. Crop residues were burned from 1991 to 2005, then this practice was forbidden and crop residues were partially removed for commercial purposes and partially buried into the soil by the mouldboard plough. The second treatment, reduced tillage (RT), was defined by reduction of the number of tillage operations, retaining a chisel pass at 15-20 cm depth followed by disc harrowing at 5-7 cm depth every year. To match conservation agriculture principles (Moreno et al. 1997; Plaster 1992), crop residues were left covering ~60% of the soil surface.

Initially, a wheat (*Triticum aestivum* L.) – sunflower (*Helianthus annus* L.) crop rotation was established for both treatments. In 2004, a fodder pea crop (*Pisum sativum* L.) was included in the rotation.

Table 1. Mean and standard error of the physical and chemical characteristics of the soil sampled in 2012 at a 0-5 cm depth (RT, reduced tillage; TT, traditional tillage). Significant differences between treatments ($P < 0.05$) are marked with an asterisk

Soil characteristic	Unit	Mean values	
		RT	TT
pH		8.0 ± 0.1	7.9 ± 0.02
$CaCO_3$	g kg^{-1}	285 ± 12	
Sand	g kg^{-1}	549 ± 13	
Silt	g kg^{-1}	212 ± 5	
Clay	g kg^{-1}	239 ± 8	
SOC	g kg^{-1}	13.9 ± 0.2 *	10.8 ± 0.3
N-Kjeldahl	g kg^{-1}	1.0 ± 0.04	0.9 ± 0.03
P-Olsen	mg kg^{-1}	24 ± 1.7	25 ± 1.4
K-available	mg kg^{-1}	423 ± 19	409 ± 30

For TT plots, tillage operations were sufficient to control weeds proliferation and no herbicides were applied. Weed control under RT was carried out using trifluraline (18%) at a dose of 2 L ha^{-1} (only for sunflower crop) and glyphosate (18%) at a dose of 4 L ha^{-1} for the three crops of the rotation.

Fertilization was applied to soil only during the wheat campaign, using a dose of 400 kg ha^{-1} of complex fertilizer (15N-15P_2O_5-15K_2O) before sowing, followed by a top dressing with urea (46% N) at a 200 kg ha^{-1} dose.

2.2. Soil sampling and sample preparation

Soil samples were collected in January 2011 at a 0-5 cm depth, right before fodder pea sowing. Five sampling points per plot were merged to obtain a composite sample. The moist field soil was air-dried and sieved with a 2 mm mesh sieve before alkali extractions. The extraction of HAs and FAs was conducted by placing 20 g of soil sieved in a 250-mL centrifuge bottle and adding 1M NaOH free of CO_2 following the procedure described by Wander (2004). The soil particles were dispersed within the suspension using an ultrasonic probe for 5 minutes, then centrifuged for 5 min at 3500 rpm using a fixed angle rotor with minimum and maximum radiuses of 32 and 137 mm, respectively. The supernatant was recovered and stored in vessels and the extraction operations were repeated 5 times. The pH of the solution containing humic substances was lowered to 1 adding HCl (3 M) under N_2 flow, then left in the dark for 8 h to allow the precipitate to settle. This operation separates the insoluble humic acids (HAs) from the soluble FAs. The suspension was centrifuged again (5 min, 3500 rpm using a fixed angle rotor) recovering the supernatant solution of FAs and the flocculated HAs. Both fractions were placed into two cylinders made with dialysis membrane closed with cellophane ends (cut off 10-12 kDa) and placed into milliQ water. The water was changed daily and the dialysis was continued until the conductivity of the milliQ water remained constant and lower than 50 µS cm^{-1}. In total, we obtained a composite sample of HAs and a composite sample of FAs per treatment, all freeze-dried and stored until further analysis. More information about the chemical composition of humic substances assessed by ^{13}C nuclear magnetic resonance and detailed values about soil chemical and biochemical variables can be found in a previous study (Panettieri et al. 2014).

2.3. Chemical composition of HAs and FAs assessed by FTICR-MS analysis

For the ultrahigh resolution MS analysis, 0.5 mg of each HAs and FAs extract were firstly dissolved in NH_4OH (1%) then diluted in a 1:1 v:v solution of methanol:water to improve the ionization efficiency. The solutions were injected into a Bruker 12 Tesla Apex-Qe FTICR-MS instrument hosted at the Old Dominion University College of Sciences Major Instrumentation Cluster (ODU-COSMIC, Norfolk, Virginia, USA). The Apollo II electrospray ionization (ESI) source was operating in negative ionization mode, the injection flow rate was 120 µL•h^{-1}, nebulizer and drying gas pressures were 20 psi and 15 psi, respectively. One blank sample was analysed under the same conditions, peaks found in the blank were not included in the list of identified compounds.

The empirical molecular formulas were assigned in the range from 200 to 800*m/z*, using an in-house Matlab code (The MathWorks, Inc., Natick, MA) according the following criteria: $^{12}C_{2–50}$, $^{1}H_{5–100}$, $^{14}N_{0–6}$, $^{16}O_{1–30}$, $^{32}S_{0–2}$ and $^{32}P_{0–2}$ within an error of 1 ppm, and using the rules outlined by the number of double bond equivalents (DBE), which represent the number of double bond in a structure and is calculated according:

$$DBE = \frac{1}{2}(2C + N - H + 2) \qquad (1)$$

The modified aromaticity index (AI_{mod}) was used to identify the aromatic structures (AI_{mod} > 0.5) condensed structures (AI_{mod} > 0.67) (Koch and Dittmar 2006), these two values of AI_{mod} are represented by diagonal lines in the **Figure 1**.

$$AI_{mod} = \frac{1 + C - 0.5O - S - 0.5H}{C - 0.5O - S - N - P} \qquad (2)$$

Formulas definitions followed the criteria established by Sleighter and Hatcher (2007) taking into account C, O, N and H ratios and aromaticity index (AI). Fatty acids were used for the mass calibration of the instrument, then the identified compounds were expressed as percentage of the total intensities for each sample (Sleighter and Hatcher 2007).

Identified formulas were represented using classic two-dimensional van Krevelen diagrams based on the H/C and O/C ratios (van Krevelen 1950). The van Krevelen diagrams were divided into areas corresponding to the composition of standard molecules viz. lipids, proteins, carbohydrates, unsaturated hydrocarbons, tannins, lignins, aromatic and condensed aromatic compounds (DiDonato et al. 2016; Jiménez-González et al. 2020).

Three series of compounds were represented with different colours: the common compounds present in RT and TT and the specific compounds present for each treatment.

The three-dimensional data representation developed by Almendros et al. (2018) was used to depict by an agglomerative manner the relative abundance (as % of the total intensity) of the compounds on the *z* axis of a 50 × 50 matrix derived from the van Krevelen plots. When several compounds overlapped in the same cell described by H/C and O/C ranges their abundances were aggregated e.g., the case of olefins. From this matrix, an interpolated surface is obtained by applying the moving average algorithm (i.e., averaging each cell value with those of its 4 orthogonal neighbour cells).

In order to facilitate comparisons between the molecular assemblages, the subtraction plots between the proportions of the compounds in homologous FA and HA samples of different treatments were generated from the same matrix used for the three-dimensional representation. Subtraction values for each cell were represented as positive/negative heatmap to evidence the differential characteristics between HAs and FAs from plots under the two different tillages.

2.4. Statistical analyses

The diversity indexes "compounds richness", "Shannon (H')", "Simpson (D)", and "Pielou J Evenness" were calculated with R software version 3.6.1 (R Core Team, http://www.r-project.org/) using the "vegan" package (Oksanen et al. 2018). The Simpson λ index estimates the probability that two randomly sampled formulas from the dataset correspond to the same compound type (Simpson 1949), in this case Simpson D index is expressed as the reciprocal D = 1-λ, being higher at higher diversity. The Pielou J evenness index estimate the equitability of the formula distribution (Pielou 1966).

3. Results

Since both treatments were implemented under similar crop rotations, edaphic and climatic conditions, the similarity found for some of the indexes was somehow expected (**Table 2**). This was the case of Simpson D index, which showed no difference for any of the two fractions studied, and for Pielou J evenness, which showed only a small increase for HAs from TT. However, other indexes were more sensitive: the RT samples had a higher Shannon *H'* index compared to TT samples, and samples extracted from soils under RT presented higher richness of identified formulas if compared to the corresponding samples from TT (**Table 2**). The number of formulas for HAs and FAs increased by 10.7% and 16%, respectively, under RT.

Table 2. Diversity indexes calculated for the identified formulas of humic and fulvic acids extracted from soils under traditional tillage (TT) and reduced tillage (RT)

Sample	Treatment	Compound richness	Shannon *H'*	Simpson D	Pielou J evenness
Humic acids	TT	1059	6.12	0.99	0.88
	RT	1173 (+ 10.7%)	6.17	0.99	0.87
Fulvic acids	TT	1526	6.29	0.99	0.86
	RT	1771 (+ 16.0%)	6.40	0.99	0.86

The distribution of the identified formulas on the van Krevelen diagrams of the HAs and FAs is shown in **Figure 1**. Details on the number of formulas and their relative contribution to the total number of compounds identified and to the total measured intensity are given in **Table 3**.

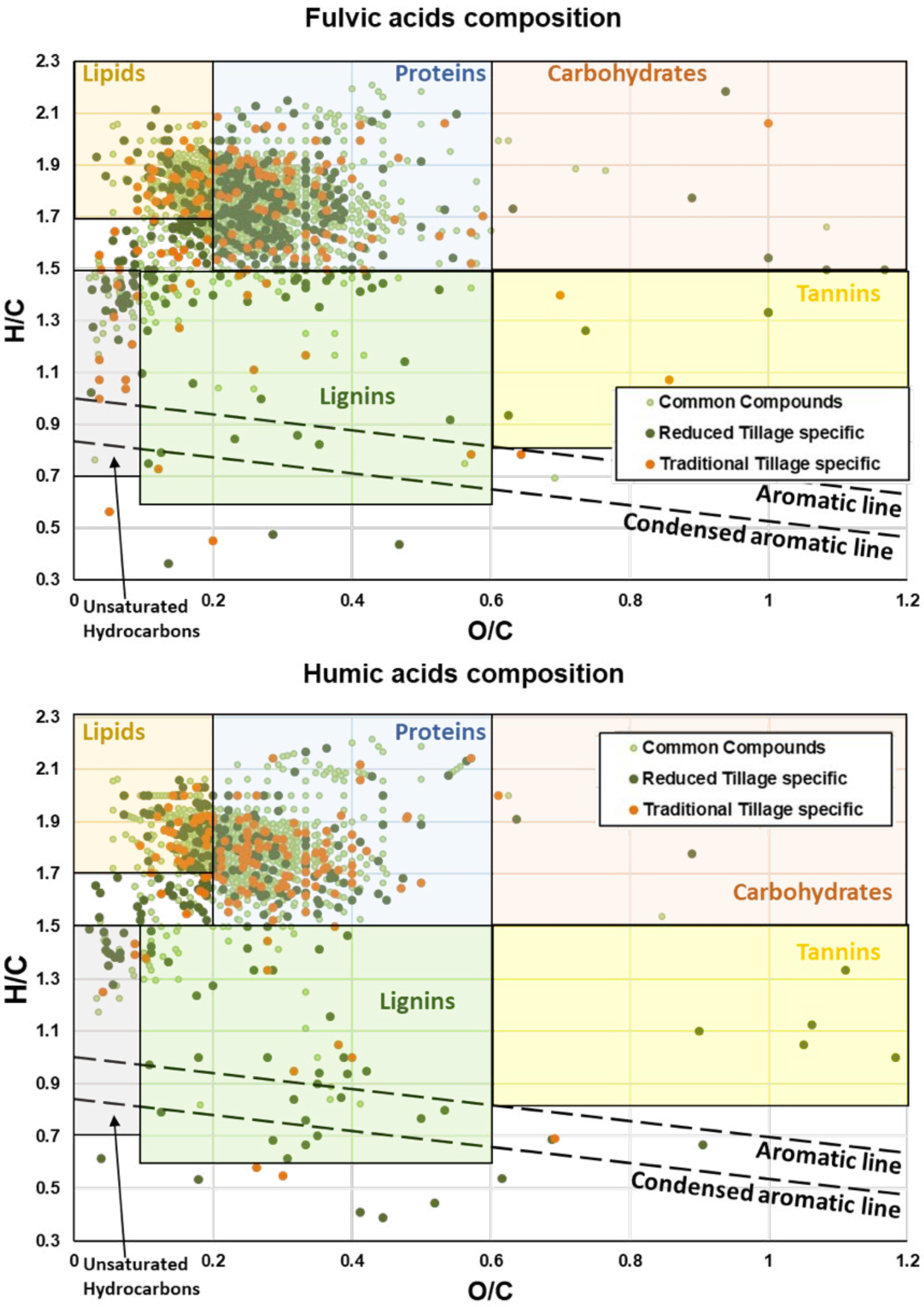


Figure 1. Van Krevelen plots showing the distribution of identified compounds of humic and fulvic acids extracted from soil samples under reduced tillage and traditional tillage. Colour codes for the compounds indicate common formulas for both tillage types and tillage-specific compounds.

Table 3. Formula assignation to compound classes defined by atomic ratio range, aromaticity index (AI), quantification and relative contribution to total number of formulas and total measured intensity for humic and fulvic acids extracted from soils under traditional tillage (TT) and reduced tillage (RT). Differences (Diff.) are expressed as percentage increases referred to TT values

Class	Definition	Quantitative data	Fulvic acids			Humic acids		
			RT	TT	Diff.	RT	TT	Diff.
Lipid	O/C 0.0-0.2 H/C 1.7-2.2	Number of formulas	354	322	**+9.9 %**	260	222	**+17.1%**
		% Formulas	20%	21%		22%	21%	
		% Intensity	16%	17%		18%	16%	
Protein	O/C 0.2-0.6 H/C 1.5-2.2 N/C > 0.05	Number of formulas	651	587	**+10.9%**	422	423	**-0.2%**
		% Formulas	37%	38%		36%	40%	
		% Intensity	38%	39%		37%	40%	
Carbohydrates	O/C 0.6-1.2 H/C 1.5-2.2	Number of formulas	10	7	**+42.9%**	4	3	**+33.3%**
		% Formulas	0.6%	0.5%		0.3%	0.3%	
		% Intensity	0.2%	0.1%		0.1%	0.1%	
Lignin	O/C 0.1-0.6 H/C 0.6-1.7 AI < 0.67	Number of formulas	337	263	**+28.1%**	190	150	**+26.7%**
		% Formulas	19%	17%		16%	14%	
		% Intensity	9%	8%		8%	7%	
Tannins	O/C 0.6-1.2 H/C 0.5-1.5 AI < 0.67	Number of formulas	7	2	**+250%**	7	0	**--**
		% Formulas	0.4%	0.13%		0.6%	0%	
		% Intensity	0.1%	0.03%		0.2%	0%	
Unsaturated Hydro-carbons	O/C 0.0-0.1 H/C 0.7-1.5	Number of formulas	48	37	**+29.7%**	24	14	**+71.4%**
		% Formulas	3%	2%		2%	1%	
		% Intensity	7%	7%		8%	7%	
Condensed Aromatics	O/C 0.0-1.0 H/C 0.3-0.7 AI > 0.67	Number of formulas	4	3	**+33.3%**	7	3	**+133.3%**
		% Formulas	0.2%	0.2%		0.6%	0.3%	
		% Intensity	0.1%	0.1%		0.2%	0.1%	
Compounds that do not fit into any of the above categories		Number of formulas	360	305	**+18.0%**	259	244	**+6.1%**
		% Formulas	20%	20%		22%	23%	
		% Intensity	29%	28%		29%	29%	
Total number of formulas			1771	1526	**+16.0%**	1173	1059	**+10.8%**

Intensities for compound classes were similar for both treatments, but RT samples showed a higher number of formulas identified within each compound class. The higher contribution to total formulas was given by protein-derived compounds, followed by lipids and lignin-derived compounds.

The averaged O/C, H/C, C/N ratios calculated on the total formulas and tillage-specific compounds for each sample are reported in **Table 4**. Around 70% of the identified formulas included N. Differences between RT and TT formulas were calculated as % of increase with respect to TT formulas. Minor differences were detected for total compounds, in which TT showed a higher C/N and a lower H/C compared to RT.

Table 4. Averaged atomic ratios calculated for the total and tillage-specific compounds of humic and fulvic acids extracted from soils under traditional tillage (TT) and reduced tillage (RT)

Treatment	Sample	Total compounds			Specific compounds		
		O/C	H/C	C/N	O/C	H/C	C/N
Humic	TT	0.252	1.746	19.200	0.258	1.625	25.099
	RT	0.253	1.772	18.096	0.268	1.724	19.281
Fulvic	TT	0.254	1.741	18.738	0.258	1.674	23.903
	RT	0.254	1.751	17.897	0.259	1.662	22.007

In the case of RT, specific compounds found on HAs were more oxidized and more unsaturated, as shown by their higher O/C and H/C ratios compared to TT. On the other hand, TT specific compounds showed higher C/N ratios for both HAs and FAs compared to RT.

The three-dimensional representation of the proportions of the compounds in the van Krevelen diagrams showed that both fulvic and humic acids from the two treatments presented the highest intensity peaks in the central part of the graph, corresponding to the adjacent areas assigned to lipid-derived and protein-derived formulas (**Figure 2**). Similarities between samples from different tillages, and between HAs and FAs were evident, due to the identical crop rotation, edaphic and climatic conditions. The subtraction graphs highlight the differences between the effects of tillage practices on the structure of HAs and FAs. These graphs are especially helpful to readily identify the most diagnostic compounds because they only display the areas of the van Krevelen diagram where changes occur, while in areas where the subtraction values were close to zero (no changes) the corresponding region appears flat and colourless. The zones marked in green (positive values) indicate selective preservation of organic matter inputs, or the concentration of specific structures as a result of RT management. On the other hand, red colour (negative values), indicates constituents subjected to selective degradation, or that are exported from the structure of humic substances, or decrease in their relative proportion as a consequence of TT. Under RT, the HAs presented a prevalence of unsaturated lipidic and hydroaromatic formulas, whereas under TT a shift toward more oxidized protein-derived formulas was observed. In the case of FAs, proteins and hydrocarbons followed the same trend. Samples under TT also presented higher intensities in the aromatic and condensed aromatic regions, and a higher unsaturation level for the region assigned to lipids.

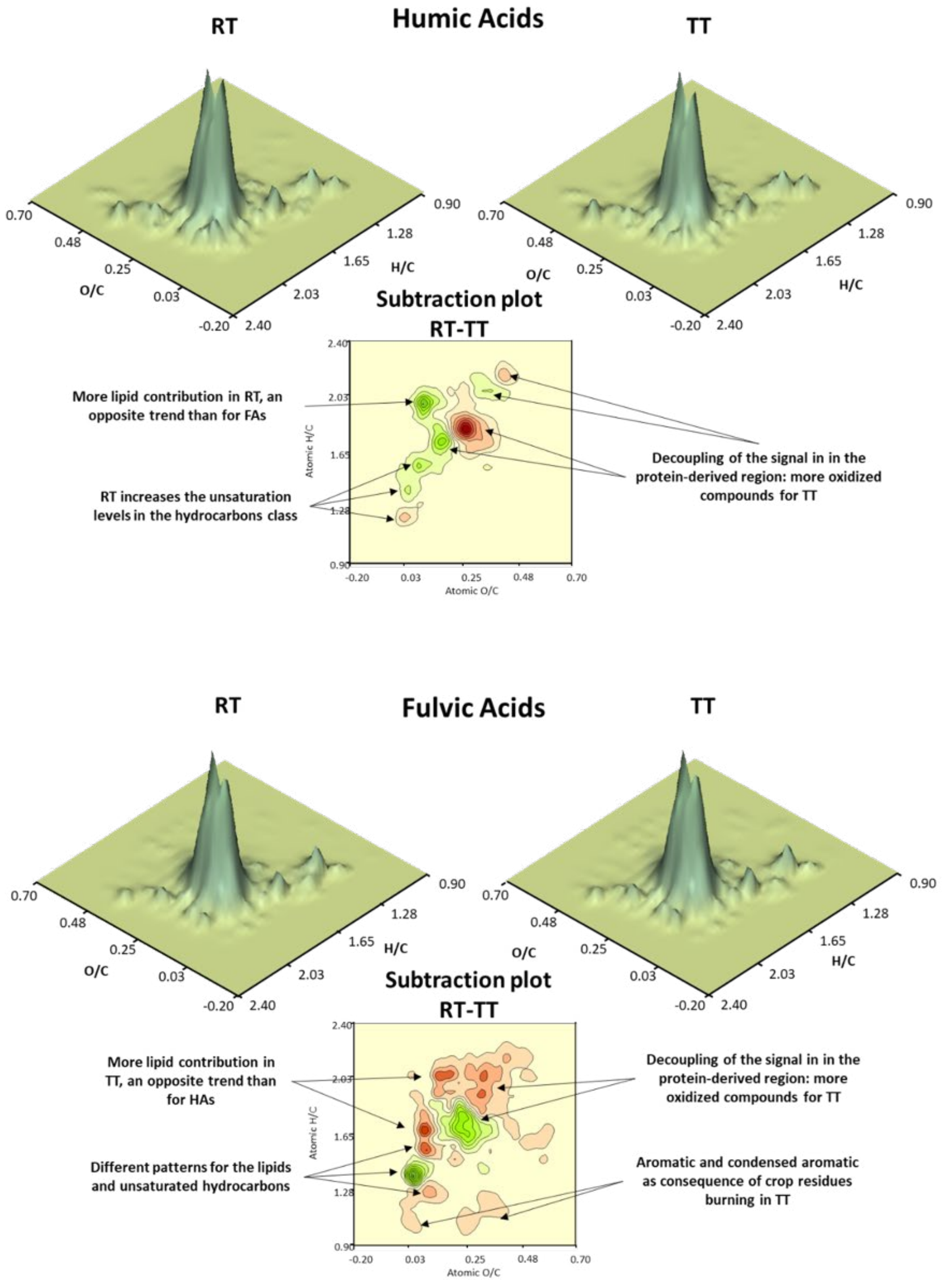
4. Discussion

4.1. Reduced tillage (RT) as a way to improve soil quality

The long-term experiment presented in this study offered a valuable opportunity for assessing the effect of RT under Mediterranean conditions at a molecular level.

Two different conceptual models could be used to interpret the ultrahigh resolution mass spectrometry data: the compound specific approach and the “omic” approach. The most common one requires the identification of individual formulas or compound classes to discuss patterns of SOC degradation between different treatments (Ikeya et al. 2015). A simplified operative approach is the use of “omic” concepts, in which the identified formulas are treated as “species”, and compound richness (expressed as number of identified formulas), distribution (expressed as evenness of the formulas) and relative contribution (expressed using diversity indexes commonly used in ecology) are used to define the overall SOC composition (Zhong et al. 2011; Jiménez-González et al. 2020).

The influence of tillage systems on SOC was



More lipid contribution in RT, an opposite trend than for FAs

RT increases the unsaturation levels in the hydrocarbons class

Decoupling of the signal in in the protein-derived region: more oxidized compounds for TT

More lipid contribution in TT, an opposite trend than for HAs

Different patterns for the lipids and unsaturated hydrocarbons

Decoupling of the signal in in the protein-derived region: more oxidized compounds for TT

Aromatic and condensed aromatic as consequence of crop residues burning in TT

Figure 2. Three-dimensional van Krevelen diagrams showing the distribution and the intensities of identified compounds of humic (above) and fulvic (below) acids extracted from soil samples under reduced tillage (RT, on the left) and traditional tillage (TT, on the right). Two-dimensional van Krevelen diagram showing the subtraction values between reduced tillage and traditional tillage samples. Positive values are shown in green, negative values are shown in red.

reflected by the different chemical richness and diversity indexes found for HAs and FAs extracted from soils under both tillage systems.

Sample diversity indexes revealed that the higher chemical richness is a result of less frequent formulas with a minor contribution to total signal intensity. As a result, the Simpson D index remained unchanged for both tillage systems and only minor changes were detected in Pielou J evenness and the three-dimensional representation of the van Krevelen diagrams. The high Simpson D index showed a highly diverse molecular population with few or no dominant formulas and a considerably high level of evenness (a completely even population would correspond to a Pielou J of 1). On the other hand, HAs and FAs from RT showed higher values for the Shannon *H’* index compared to samples from soils under TT. The Shannon H’ index represents the uncertainty or entropy related to the prediction of formula identity, with samples from RT soils having a higher level of entropy, i.e. higher diversity, than samples from TT soils.

As reflected in the results, RT showed a general trend to a greater chemical diversity for both HAs and FAs, which can be attributed to the potential of this tillage system for higher complexity of the biogeochemical mechanisms/processes involved in soil C storage, higher microbial biomass and activity as previous studies have endorsed (Panettieri et al. 2014). Particularly, long-term implementation of RT increased the relative contribution of protein-derived material to ^{13}C NMR signal (Panettieri et al. 2014), a compound class representing 36-40% of the total number of molecules and signal intensity identified with FTICR-MS within this study.

The higher chemical diversity found for RT soils seems to indicate that different biogeochemical pathways are involved in crop residue degradation and SOC storage in RT compared to TT systems. We can deduce that, starting from the same input from crop residues, tillage-specific compounds are accumulated as new intermediary degradation products or molecular proxies of tillage-adapted microbial communities. Investigating those aspects is crucial to predict SOM persistence at the long-term and to refine prediction models for C storage.

4.2. Chemical diversity as a tool to assess soil functionality

Soil perturbations induced by tillage or land-use changes produce a different shaping of microbial communities (Le Guillou et al. 2018), resulting in different dynamics of SOC mineralization (Panettieri et al. 2020). Those changes in soil functionality may result in different chemical compositions of the SOC due to different pathways of crop input degradation and secondary synthesis of specific microbial compounds mediated by the shifts in microbial community composition (Louis et al. 2016). The higher chemical diversity indexes found within the present study are in good agreement with the increased size of the microbial biomass and enzymatic activities reported for soils under RT in the same experimental farm (Panettieri et al. 2014).

Soil microbial diversity responds to land-use with a non-linear pattern, in which diversity indexes are following a humped-back curve, with a maximum level corresponding to “*optimum*” soil perturbation conditions (Acosta-Martínez et al. 2008). Those conditions leading to the maximum possible diversity are site-specific; increases or decreases of soil perturbation intensity will produce similar decrease in soil microbial diversity in favour of species more adapted to a lower or higher level of perturbation (Acosta-Martínez et al. 2008; Le Guillou et al. 2018). Those shifts have been associated with different levels of soil microbial functions related to SOC degradation (Louis et al. 2016; Panettieri et al. 2020). Comparing the historical performance of TT and RT for the experimental area, the latter improved the overall soil quality in terms of chemical diversity, biological activity, microbial proliferation and agronomical performance (López-Garrido et al. 2014).

The high level of functional redundancy found for soil microbial communities (Rousk et al. 2009) implies a high level of similarity between identified formulas from FA and HA samples. However, there is a considerable level of uncertainty on rare phyla of soil microbes and their functionality (Delgado-Baquerizo 2019) that can contribute to diverging metabolites production together with different agronomical conditions. Increase in soil microbial diversity has been associated with higher C storage potential (Banerjee et al. 2016; Trivedi et al. 2013; Wagg et al. 2019) and with lower susceptibility to priming effect of ancient SOM when fresh OM inputs are added to soil (Panettieri et al. 2020).

In view of the results obtained in this study, the use of omic techniques applied to high throughput techniques of compound specific analysis, such as FTICR-MS (Jiménez-González et al. 2020; Zhong et al. 2011) or pyrolysis coupled to gas chromatography and mass spectrometry (Jiménez-González et al. 2018) are portrayed as two possible ways to assess soil chemical diversity. This proxy should be seen as a comprehensive picture of soil inputs (crop residues, manure), organic matter degradation processes and metabolites from soil microbial communities. This could be considered as a useful tool to compare soil quality under different land-uses and to extrapolate not only the quantity of SOC stored by each land-use, but also its persistence at long-term in a climate change scenario.

4.3. Compound specific patterns of SOC formation under different tillage systems

Van Krevelen plots have proven to be useful to facilitate the interpretation of the complex assemblages of individual compounds released by different analytical methods (Kim et al. 2003; Ikeya et al. 2015). This method lets us, in a very perceptual way, detect the different amounts of compounds which tend to accumulate in the SOC formed under the influence of the different factors (Almendros et al. 2018).

In general, changes were not very pronounced, but much evident in the case of FAs, as corresponds to its presumably active dynamics attributable to its greater chemical reactivity, which can be assumed to be associated with greater biodegradability (Wander 2004).

The lipid and protein components were predominant in HA with respect to FA, as well as compounds corresponding to transformation products from lignocellulosic biomass, suggesting the incorporation of such constituents as originally macromolecular domains in the humic structure. This is the case with formulas corresponding to OH-substituted aromatic structures such as phenols and methoxyphenols, but also of condensed aromatic compounds or alkylbenzenes with O/C ratio close to 1.5.

In the case of HAs, TT showed a relative depletion of structures with typical protein stoichiometry, which could be tentatively assigned to the stabilized N-components of HA, of ill-known structure. Derenne et al. (1991) proposed that they correspond to non-hydrolyzable amides, strongly encapsulated in the complex structure of the HAs.

However, there was a portion of the protein-like domain that does increase with RT. That could be due to the constituents contributed by the new vegetation or soil microbial population subjected to RT with mulching. From the structural point of view, their presence is compatible with that of peptides or oligopeptides of highly aliphatic structure that have not yet been stably incorporated into the HA structure. In particular, and due to its H/C ratio > 2, its stoichiometry is compatible with that of lipoproteins, with a possible origin from microbial metabolism. Moreover, the content of polycyclic aromatic compounds decreased to some extent for both FA and HA as a consequence of the implementation of reduced tillage. Those condensed aromatic structures are typical of high-maturity humic substances, but also of the pyrogenic C from residue burning (González-Pérez et al. 2004) that occurred for several years in TT.

In the case of FAs, the evolution is somewhat opposite to that of HAs, indicating the previously suggested translocation of constituents between both humic compartments in the soil.

The comparison with the subtracted van Krevelen diagram from the HAs clearly suggests that the "aliphatic enhancement" produced by the effect of reduced tillage is only marked in the HA fraction, whereas FAs do not offer a structure or composition adequate for the preservation or accumulation of these nonpolar constituents lacking reactive functional groups.

The same can be said about the differential impact of tillage on the peptide-like domain. The region is clearly differentiated between i) a low H/C and low O/C N-domain, recalcitrant as regards the effect of the change in use, and ii) a peptide-like domain comparatively dynamic as regards the change in use, which is characterized by its more aliphatic nature and higher degree of oxidation favoured by the improved aerated soil structure under TT.

Both HA and FA include a large nitrogen domain in which at least two moieties of different dynamism can be clearly distinguished: i) compounds with comparatively high $H/C > 1.84$, that would correspond to newly incorporated protein and peptide chains, and ii) compounds with lower $H/C < 1.84$, that would correspond to N-compounds of heterocyclic structure and/or associated to aromatic structures. Traditionally, the latter has been considered to be composed of comparatively more resistant, non-hydrolyzable N-forms that increase in extent with the progress of humification.

5. Concluding remarks and future directions

The use of FTICR-MS on HAs and FAs provides detailed information on the chemical composition of those complex SOC pools that can be interpreted using an “omic” approach. This study showed how the reduced tillage favours richness in the chemical composition of SOC, as a consequence of the improvements on soil quality and physical properties induced by this tillage type. The implementation of RT increased the contribution of formulas assigned to lipids and hydroaromatics and reduced the oxidization level of the protein-derived formulas of humic acids compared with TT. A similar pattern was observed for fulvic acids, with the exception of the unsaturated lipids that were more abundant in TT than in RT.

However, despite the fact that HAs and FAs represent the most characterized pool of soil organic matter (Olk et al. 2019; De Nobili et al. 2020; Jiménez-González et al. 2020) their importance has been questioned because they only represent a part of total SOC (Kleber and Lehmann 2019). The complexity of soil matrix implies that there is no silver bullet for compound specific analysis and a multianalytical approach is necessary, assuming an increase of analysis costs and extended/time-consuming data analysis procedures. However, similar problems of costs, difficult extractions, identification, data analysis pipelines and formation of artefacts did not affect the use of metagenomics techniques to study soil microbial diversity (Lombard et al. 2011).

The assessment of chemical diversity can also be applied to analytical pyrolysis coupled to gas chromatography and mass spectrometry. This technique is relatively economic, does not require previous soil extraction (even if the signal to noise ratio is not satisfactory for soils with low C contents) and is very versatile for chemometric data treatments (Almendros et al. 2003; Jiménez-González et al. 2018). Compared to ultrahigh resolution mass spectrometry, exact formulas are more difficult to identify in pyrograms, leading to a lower number of identified compounds (usually hundreds in a complete soil). Data analysis can be supported by automated pipelines (Chen et al. 2018) and the formation of artefacts due to non-specific pyrolytic cleavage is negligible when similar soils are compared. Therefore, we propose the use of chemical diversity as a tool to assess soil quality and persistence at long-term of newly stored SOC using FTICR-MS or the alternative Py-GC-MS to assess chemical diversity in soils.

6. Acknowledgements

Marco Panettieri acknowledges the Spanish Ministry of Science, Innovation and Universities for his post-doctoral fellowship number IJCI-2016-27693. Marco Panettieri thanks Prof. Patrick G. Hatcher of the Department of Chemistry and Biochemistry of the Old Dominion University (Norfolk, VA, USA) for providing access to the FTICR-MS of the College of Science Major Instrumentation Cluster (COSMIC). Marco A. Jiménez-González thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for funding his pre-doctoral FPI fellowship

(BES-2014-069238). The authors thank the collaboration of IRNAS-CSIC members: Dr José Manuel Murillo for the management of the experimental plots, Dr Rosa López-Garrido and Dr Ignacio Girón for the field tasks and Patricia Puente for their help in laboratory analysis. This work was framed in the net of experiments of the “Unidad Asociada Uso Sostenible del Suelo y el Agua en Agricultura” of the IRNAS-CSIC.

REFERENCES

- Acosta-Martínez V, Dowd S, Sun Y, Allen V. 2008. Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biol Biochem. 40:2762-2770. https://doi.org/10.1016/J.SOILBIO.2008.07.022.

- Almendros G, Hernández Z, Sanz J, Rodríguez-Sánchez S, Jiménez-González MA, González-Pérez JA. 2018. Graphical statistical approach to soil organic matter resilience using analytical pyrolysis data. J Chromatogr A. 1533:64-173. https://doi.org/10.1016/j.chroma.2017.12.015.

- Almendros G, Kgathi D, Sekhwela M, Zancada MC, Tinoco P, Pardo MT. 2003. Biogeochemical assessment of resilient humus formations from virgin and cultivated northern Botswana soils. J Agric Food Chem. 51:4321-4330. https://doi.org/10.1021/jf034006u.

- Banerjee S, Kirkby CA, Schmutter D, Bissett A, Kirkegaard JA, Richardson AE. 2016. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. Soil Biol Biochem. 97:188-198. https://doi.org/10.1016/j.soilbio.2016.03.017.

- Baveye PC, Wander M. 2019. The (Bio)chemistry of Soil Humus and Humic Substances: Why Is the “New View” Still Considered Novel After More Than 80 Years? Front Environ Sci. 7:27. https://doi.org/10.3389/fenvs.2019.00027.

- Chen H, Blosser GD, Majidzadeh H, Liu X, Conner WH, Chow AT. 2018. Integration of an automated identification-quantification pipeline and statistical techniques for pyrolysis GC/MS tracking of the molecular fingerprints of natural organic matter. J Anal Appl Pyrolysis 134:371-380. https://doi.org/10.1016/j.jaap.2018.07.002.

- Chenu C, Angers DA, Barré P, Derrien D, Arrouays D, Balesdent J. 2019. Increasing organic stocks in agricultural soils: Knowledge gaps and potential innovations. Soil Tillage Res. 188:41-52. https://doi.org/10.1016/j.still.2018.04.011.

- Dang YP, Moody PW, Bell MJ, Seymour NP, Dalal RC, Freebairn DM, Walker SR. 2015. Strategic tillage in no-till farming systems in Australia’s northern grains-growing regions: II. Implications for agronomy, soil and environment. Soil Tillage Res. 152:115-123. https://doi.org/10.1016/j.still.2014.12.013.

- De Nobili M, Bravo C, Chen Y. 2020. The spontaneous secondary synthesis of soil organic matter components: A critical examination of the soil continuum model theory. Appl Soil Ecol. 154:103655. https://doi.org/10.1016/j.apsoil.2020.103655.

- Delgado-Baquerizo M. 2019. Obscure soil microbes and where to find them. ISME J. 13:2120-2124. https://doi.org/10.1038/s41396-019-0405-0.

- Derenne S, Largeau C, Casadevall E. 1991. Occurrence of tightly bound isoprenoid acids in an algal, resistant biomacromolecule: possible geochemical implications. Org Geochem. 17:597-602. https://doi.org/10.1016/0146-6380(91)90003-3.

- DiDonato N, Chen H, Waggoner D, Hatcher PG. 2016. Potential origin and formation for molecular components of humic acids in soils. Geochim Cosmochim Acta 178:210-222. https://doi.org/10.1016/j.gca.2016.01.013.

- Dignac MF, Derrien D, Barré P, Barot S, Cécillon L, Chenu C, Chevallier T, Freschet GT, Garnier P, Guenet B, Hedde M, Klumpp K, Lashermes G, Maron PA, Nunan N, Roumet C, Basile-Doelsch I. 2017. Increasing soil carbon storage: mechanisms, effects of agricultural practices and proxies. A review. Agron Sustain Dev. 37:14. https://doi.org/10.1007/s13593-017-0421-2.

- Gajri PR, Arora VK, Prihar SS. 2002. Tillage for sustainable cropping. Lucknow, India: Int B Distrib co. ISBN: 9781560229032

- González-Pérez JA, González-Vila FJ, Almendros G, Knicker H. 2004. The effect of fire on soil organic matter - A review. Environ Int. 30:855-870. https://doi.org/10.1016/j.envint.2004.02.003

- Hayes MHB, Swift RS. 2020. Vindication of humic substances as a key component of organic matter in soil and water. Advances in Agronomy 163:1-37. https://doi.org/10.1016/bs.agron.2020.05.001.

- Ikeya K, Sleighter RL, Hatcher PG, Watanabe A. 2015. Characterization of the chemical composition of soil humic acids using Fourier transform ion cyclotron resonance mass spectrometry. Geochim Cosmochim Acta 153:169-182. https://doi.org/10.1016/j.gca.2015.01.002.

- Jiménez-González MA, Almendros G, Waggoner DC, Álvarez AM, Hatcher PG. 2020. Assessment of the molecular composition of humic acid as an indicator of soil carbon levels by ultra-high-resolution mass spectrometric analysis. Org Geochem. 143:104012. https://doi.org/10.1016/j.orggeochem.2020.104012.

- Jiménez-González MA, Álvarez AM, Hernández Z, Almendros G. 2018. Soil carbon storage predicted from the diversity of pyrolytic alkanes. Biol Fertil Soils 54:617-629. https://doi.org/10.1007/s00374-018-1285-6.

- Kim S, Kramer RW, Hatcher PG. 2003. Graphical Method for Analysis of Ultrahigh-Resolution Broadband Mass Spectra of Natural Organic Matter, the Van Krevelen Diagram. Anal Chem. 75:5336-5344. https://doi.org/10.1021/ac034415p.

- Kleber M, Lehmann J. 2019. Humic Substances Extracted by Alkali Are Invalid Proxies for the Dynamics and Functions of Organic Matter in Terrestrial and Aquatic Ecosystems. J Environ Qual. 48:207-216. https://doi.org/10.2134/jeq2019.01.0036.

- Koch BP, Dittmar T. 2006. From mass to structure: An aromaticity index for high-resolution mass data of natural organic matter. Rapid Commun. Mass Spectrom. 20:926-932. https://doi.org/10.1002/rcm.2386.

- Lal R. 2008. Sequestration of atmospheric CO_2 in global carbon pools. Energy Environ Sci. 1:86-100. https://doi.org/10.1039/b809492f.

- Lal R. 2003. Soil erosion and the global carbon budget. Environ Int. 29:437-450. https://doi.org/10.1016/S0160-4120(02)00192-7.

- Lal R, Logan TJ, Fausey NR. 1989. Long-term tillage and wheel traffic effects on a poorly drained mollic ochraqualf in northwest Ohio. 2. Infiltrability, surface runoff, sub-surface flow and sediment transport. Soil Tillage Res. 14:359-373. https://doi.org/10.1016/0167-1987(89)90054-8.

- Le Guillou C, Chemidlin Prévost-Bouré N, Karimi B, Akkal-Corfini N, Dequiedt S, Nowak V, Terrat S, Menasseri-Aubry S, Viaud V, Maron PA, Ranjard L. 2018. Tillage intensity and pasture in rotation effectively shape soil microbial communities at a landscape scale. Microbiologyopen 8:e00676. https://doi.org/10.1002/mbo3.676.

- Lehmann J, Kleber M. 2015. The contentious nature of soil organic matter. Nature 528:60. https://doi.org/10.1038/nature16069.

- Leifeld J, Kögel-Knabner I. 2005. Soil organic matter fractions as early indicators for carbon stock changes under different land-use? Geoderma 124:143-155. https://doi.org/http://dx.doi.org/10.1016/j.geoderma.2004.04.009.

- Lombard N, Prestat E, van Elsas JD, Simonet P. 2011. Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. FEMS Microbiol Ecol. 78(1):31-49. https://doi.org/10.1111/j.1574-6941.2011.01140.x.

- López-Garrido R, Madejón E, Moreno F, Murillo JM. 2014. Conservation Tillage Influence on Carbon Dynamics Under Mediterranean Conditions. Pedosphere 24:65-75. https://doi.org/10.1016/S1002-0160(13)60081-8.

- Louis BP, Maron PA, Menasseri-Aubry S, Sarr A, Lévêque J, Mathieu O, Jolivet C, Leterme P, Viaud V. 2016. Microbial Diversity Indexes Can Explain Soil Carbon Dynamics as a Function of Carbon Source. PLoS One 11:e0161251. https://doi.org/10.1371/journal.pone.0161251.

- Melero S, Panettieri M, Madejón E, Macpherson HG, Moreno F, Murillo JM. 2011. Implementation of chiselling and mouldboard ploughing in soil after 8 years of no-till management in SW, Spain: Effect on soil quality. Soil Tillage Res. 112:107-113. https://doi.org/10.1016/j.still.2010.12.001.

- Minasny B, Malone BP, McBratney AB, Angers DA, Arrouays D, Chambers A, Chaplot V, Chen ZS, Cheng K, Das BS, Field DJ, Gimona A, Hedley CB, Hong SY, Mandal B, Marchant BP, Martin M, McConkey BG, Mulder VL, O'Rourke S, Richer-de-Forges AC, Odeh I, Padarian J, Paustian K, Pan G, Poggio L, Savin I, Stolbovoy V, Stockmann U, Sulaeman Y, Tsui CC, Vågen TG, van Wesemael B, Winowiecki L. 2017. Soil carbon 4 per mille. Geoderma 292:59-86. https://doi.org/10.1016/J.GEODERMA.2017.01.002.

- Moreno F, Pelegrín F, Fernández JE, Murillo JM. 1997. Soil physical properties, water depletion and crop development under traditional and conservation tillage in southern Spain. Soil Tillage Res. 41:25-42. https://doi.org/10.1016/S0167-1987(96)01083-5.

- Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2018. Vegan: Community Ecology Package. R Package Version 2.2-0.

- Olk DC, Bloom PR, Perdue EM, McKnight DM, Chen Y, Farenhorst A, Senesi N, Chin YP, Schmitt-Kopplin P, Hertkorn N, Harir M. 2019. Environmental and Agricultural Relevance of Humic Fractions Extracted by Alkali from Soils and Natural Waters. J Environ Qual. 48:217-232. https://doi.org/10.2134/jeq2019.02.0041.

- Panettieri M, Berns AE, Knicker H, Murillo JM, Madejón E. 2015. Evaluation of seasonal variability of soil biogeochemical properties in aggregate-size fractioned soil under different tillages. Soil Tillage Res. 151:39-49. https://doi.org/10.1016/j.still.2015.02.008.

- Panettieri M, Guigue J, Chemidlin Prevost-Bouré N, Thévenot M, Lévêque J, Le Guillou C, Maron PA, Santoni AL, Ranjard L, Mounier S, Menasseri S, Viaud V, Mathieu O. 2020. Grassland-cropland rotation cycles in crop-livestock farming systems regulate priming effect potential in soils through modulation of microbial communities, composition of soil organic matter and abiotic soil properties. Agric Ecosyst Environ. 299:106973. https://doi.org/10.1016/j.agee.2020.106973.

- Panettieri M, Knicker H, Berns AEE, Murillo JMM, Madejón E. 2013. Moldboard plowing effects on soil aggregation and soil organic matter quality assessed by ^{13}C CPMAS NMR and biochemical analyses. Agric Ecosyst Environ. 177:48-57. https://doi.org/10.1016/j.agee.2013.05.025.

- Panettieri M, Knicker H, Murillo JM, Madejón E, Hatcher PG. 2014. Soil organic matter degradation in an agricultural chronosequence under different tillage regimes evaluated by organic matter pools, enzymatic activities and CPMAS ^{13}C NMR. Soil Biol Biochem. 78:170-181. https://doi.org/10.1016/j.soilbio.2014.07.021.

- Pielou EC. 1966. The measurement of diversity in different types of biological collections. J Theor Biol. 13:131-144. https://doi.org/10.1016/0022-5193(66)90013-0.

- Plaster EJ. 1992. Soil Science and Management. New York: Delmar Publ, Inc. 514 p.

- Plaza C, Fernández JM, Pereira EIP, Polo A. 2012. A comprehensive method for fractionating soil organic matter not protected and protected from decomposition by physical and chemical mechanisms. Clean - Soil, Air, Water. 40:134-139. https://doi.org/10.1002/clen.201100338.

- Rousk J, Brookes PC, Bååth E. 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl Environ Microbiol. 75:1589-1596. https://doi.org/10.1128/AEM.02775-08.

- Simpson EH. 1949. Measurement of diversity. Nature. 163:688. https://doi.org/10.1038/163688a0.

- Sleighter RL, Hatcher PG. 2007. The application of electrospray ionization coupled to ultrahigh resolution mass spectrometry for the molecular characterization of natural organic matter. J Mass Spectrom. 42:559-574. https://doi.org/10.1002/jms.1221.

- Soil Survey Staff. 2014. Keys to Soil Taxonomy, 12th ed. Washington, DC: USDA-Natural Resources Conservation Service.

- Trivedi P, Anderson IC, Singh BK. 2013. Microbial modulators of soil carbon storage: Integrating genomic and metabolic knowledge for global prediction. Trends Microbiol. 21:641-651. https://doi.org/10.1016/j.tim.2013.09.005.

- van Krevelen DW. 1950. Graphical-statistical method for the study of structure and reaction processes of coal. Fuel 29:269-284.

- Wagg C, Schlaeppi K, Banerjee S, Kuramae EE, van der Heijden MGA. 2019. Fungal-bacterial diversity and microbiome complexity predict ecosystem functioning. Nat Commun. 10:1-10. https://doi.org/10.1038/s41467-019-12798-y.

- Wander MM. 2004. Soil Organic Matter Fractions and Their Relevance to Soil Quality. In: Weil RR, Magdoff F, editors. Soil Organic Matter in Sustainable Agriculture. Boca Raton (FL): CRC Press. p. 86-130.

- Zhong J, Sleighter RL, Salmon E, McKee GA, Hatcher PG. 2011. Combining advanced NMR techniques with ultrahigh resolution mass spectrometry: A new strategy for molecular scale characterization of macromolecular components of soil and sedimentary organic matter. Org Geochem. 42:903-916. https://doi.org/10.1016/j.orggeochem.2011.04.007.