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EVALUATION OF OPTICAL TECHNIQUES FOR CHARACTERISING SOIL ORGANIC MATTER QUALITY IN AGRICULTURAL SOILS

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ABSTRACT

Soil organic matter (SOM) is one of the main global carbon pools. It is a measure of soil quality as its presence increases carbon sequestration and improves physical and chemical soil properties. The determination and characterisation of humic substances gives essential information of the maturity and stresses of soils as well as of their health. However, the determination of the exact nature and molecular structure of these substances has been proven difficult. Several complex techniques exist to characterise SOM and mineralisation and humification processes. One of the more widely accepted for its accuracy is Nuclear Magnetic Resonance (NMR) spectroscopy. Despite its efficacy, NMR needs significant economic resources, equipment, material and time. Proxy measures like the fluorescence index (FI), cold and hot-water extractable carbon (CWC and HWC) and SUVA-$_{254}$ have the potential to characterise SOM and, in combination, provide qualitative and quantitative data of SOM and its processes. Spanish and British agricultural cambisols were used to measure SOM quality and determine whether similarities were found between optical techniques and ^{1}H-NMR results in these two regions with contrasting climatic conditions. High correlations ($p<0.001$) were found between the specific aromatic fraction measured with ^{1}H-NMR and SUVA-$_{254}$ (Rs=0.95) and HWC (Rs=0.90), which could be described using a linear model. A high correlation between FI and the aromatics fraction measured with ^{1}H-

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Abbreviations: (SOM) Soil organic matter; (NMR) Nuclear magnetic resonance; (FI) Fluorescence index; (CWC) Cold-water extractable carbon; (HWC) Hot-water extractable carbon; (LOI) Loss of ignition; (DOM) Dissolved organic matter; (DOC) Dissolved organic carbon; (EEM) Excitation-emission matrix; (GS-UK) Soil with a grass cover, United Kingdom; (CC-UK) Cereal crops from United Kingdom; (CC-ES) Cereal crops from Spain; (OG-ES) Olive grove from Spain; (HIX) Humification index; (BIX) Biological/autochthonous index; (RU) Raman units; (SUVA-254) Specific absorbance at 254 nm

NMR (Rs=-0.976) was also observed. In view of our results, optical measures have a potential, in combination, to predict the aromatic fraction of SOM without the need of expensive and time consuming techniques.

KEYWORDS: SOM quality; hot-water extractable carbon; cold-water extractable carbon; fluorescence index; EEM; SUVA-$_{254}$; ^{1}H-NMR; aromatic fraction.

1. Introduction:

1.1. The importance of organic matter

Soil organic matter (SOM) is composed of organic residues that are originated from plant and animal remains and microbial products at different stages of decomposition or humification (Hur *et al.*, 2013). Additionally, it is one of the main global carbon pools, storing three times more carbon than living organisms or the atmosphere (Fischlin *et al.*, 2007; Brevik, 2012). Aside from carbon sequestration, SOM is also a measure of soil quality because of the beneficial function it has on a variety of soil processes. For instance, it reduces erosion and, therefore, increases crop production by increasing the elasticity and resistance to deformation and compactability as well as porosity and water retention (Sellami *et al.*, 2008; Paradelo and Barral, 2013). Increased water retention decreases potential runoffs by improving water infiltration in to soils and provides a store of water for plant uptake, buffering against moisture and rainfall fluctuations (Lal, 2004). This is of importance considering that the lack of water retention leads to a change in the hydrological patterns of agricultural areas and promotes the quantity and severity of floods and water-led erosion. Also, SOM leads to an increased vegetative cover, which ultimately reduces soil erosion (Cerdà, 1998, 2000; Novara *et al.*, 2011; Zhao *et al.*, 2013). Carbon mineralisation is crucial in SOM dynamics and along with carbon input, determines how much carbon accumulates in soil and releases nutrients that are essential for plant growth. Factors that affect mineralisation are the size of labile carbon, environmental conditions and the local microbial community (Zhao *et al.*, 2008; Li *et al.*, 2013). SOM and soil assemblage; SOM decomposition and transport by organisms contribute to soil stabilisation and the improvement of soil structure (Brevik *et al.*, 2015). Moreover, SOM quantity has been directly related to the preservation of soil aggregates, which in turn reduces soil erodibility (Novara *et al.*, 2011). Also, the direct processing of SOM along with its decomposition contribute to the improvement of soil chemical properties and stability (Brevik *et al.*, 2015). Therefore, optimal quantities of SOM improve structure, water retention, and nutrient holding capacity of soils, which has an effect in multiple aspects of the soil system. These are essential considering the wider context of Earth System, as SOM conservation techniques have been proven to improve the fertility of degraded soils of a wide variety of ecosystems that are the main resource of large communities of developing areas of our planet, as well as have an influence on biogeochemical cycles and climate change mitigation (Batjes, 2014; Saha *et al.*, 2014; Srinivasarao *et al.*, 2014).

1.2. SOM carbon fractions and their importance

SOM has been conceptualised as containing three pools, with different residence/turnover times (Trumbore, 2000). These pools are the active SOM (living biomass of microorganisms and partially decomposed residues; associated with 1 year turnover); the slow SOM (resistant plant material; associated to a turnover from years to centuries); and passive SOM (humic substances and inert organic matter), which has been traditionally associated with longer residence time (thousands of years) and more stability. Some authors consider that the inert organic matter should not be considered as part of the passive pool, but as a fourth pool (Trumbore, 1997; Ohno, 2002; Agren and Bosatta, 2002; Sparks, 2003; Bell and Lawrence, 2009; Dungait *et al.*, 2012). Although the traditional view has been that decomposition led to complex molecules that were very stable as a result of their structure; it has recently been known that environmental conditions, organo-mineral associations and other processes influence more in SOM stability than structure, which only plays a secondary role. As a result of this new view, recent research has found that humic substances, which have always been considered high molecular mass polymers, could be simpler than originally thought (Kleber and Johnson, 2010; Schmidt *et al.*, 2011). Still, their structure is on discussion and the separation of SOM into fractions with different turnovers remains a major challenge (Kleber, 2010; Schmidt *et al.*, 2011; Schrumpf and Kaiser, 2015).

Non-humic substances are composed by microbial biomass, decomposable plant material (active SOM); and resistant plant material, mainly waxes, lignified tissues and polyphenols (slow SOM) (Dungait *et al.*, 2012). Microbial biomass has been used for comparing natural and degraded ecosystems and as an early indicator of soil processes, fertility and health (García-Gil *et al.*, 2000; Brevik, 2009; Chen *et al.*, 2013).

1.3. Current SOM quality measurements. Challenges

Measures such as % Organic Matter measured by Loss of Ignition (LOI) are useful, popular and inexpensive methods to determine bulk SOM (Luke *et al.*, 2009; Salehi *et al.*, 2011). However, some studies have concluded that bulk SOM measurements cannot be used, on their own, as a representative indicator of carbon in soil due to their limitations (Koarashi *et al.*, 2005; Salehi *et al.*, 2011).

Humic substances have also been measured to determine soil quality, as their presence has been associated with a higher quality of soils as stated in section 1.2. Their study is relevant in agricultural soils, as they increase crop yield and root dry weight, although this response is not fully understood (Rose *et al.*, 2014). Therefore, the determination and characterisation of humic substances gives essential information of the maturity and stresses of soils as well as of their health.

Traditionally, alkali and acid abstraction methods have been used, to later interpret the chemistry of the extracted functional groups (Olk and Gregorich, 2006). Afterwards, these were combined with other complex techniques that enabled scientists to obtain new information on the structure and dynamic associations of humic substances (Sutton and Sposito, 2005; Schmidt *et al.*, 2011). Despite these advances, SOM dynamics and cycling still have many questions to answer, with models differing in SOM fluxes results for the future, due to their sensitivity to SOM turnover time assumptions (Schmidt *et al.*, 2011). There are a number of powerful but complex and expensive techniques that have been used for the study of soil fluxes (Helal *et al.*, 2011). The economic resources needed, along with the time required to prepare the samples and conduct the analyses, make its use with a large number of samples difficult and delays experiments, while more work is still needed to accurately determine and define the molecular structures and linkages between the SOM components (Weishar *et al.* 2003; Helal *et al.*, 2011).

Nuclear Magnetic Resonance (NMR) spectroscopy is a non-destructive technique that is valuable for the characterisation of SOM and humification processes, providing information on static and dynamic properties of molecules. This is due to its high performance to assess intermolecular interactions. The relationship between SOM, contaminants and metals can also be studied with NMR (Cardoza *et al.*, 2004). Of the various variants that exist, ^{1}H-NMR spectroscopy was used in this study. This technique analyses humic and fulvic acids dissolved in neutral or alkaline solutions to characterise the components of the substance, and gives a semi-quantitative notion of aromatic, aliphatic and carboxylic groups (Hemminga and Buurman, 1997). One of the main drawbacks of this technique is the quantity of economic resources that are necessary for its regular application in research laboratories/centres. This is due to the expensive deuterated solvents and NMR tubes, as well as the expensive equipment and significant sample preparation that are required (Weishar *et al.*, 2003; Cardoza *et al.*, 2004; MIT, 2008). Also, the technique is time consuming not only when measuring, but when interpreting 2-D or 3-D data resulting from it (Cardoza *et al.*, 2004). Simpler methods for the characterisation of SOM are required.

1.4. Proxy measures. Opportunities to improve the ability to characterise SOM quality

Water extractable carbon is the most active component in the carbon cycle. Its quantity and biological nature is affected by the extraction temperature (Bu *et al.*, 2010). Hot-water extractable carbon (HWC) contains simple compounds such as microorganisms, soluble carbohydrates and other compounds that account for the labile fraction of SOM (Ghani *et al.*, 2003). HWC responds to land use changes in the short term and has been used to detect the effects of different land management practices and for determining the effects of soil amendments such as biochar or agricultural residues (Leifeld and Kögel-Knabner, 2005; Uchida *et al.*, 2012; Alburquerque *et al.*, 2014; Fernández-Romero *et al.*, 2014). For these reasons, it has been proven useful to obtain information about soil quality (Ghani *et al.*, 2003; Xue *et al.*, 2013).

Fluorescence has become popular because of its potential to characterise SOM and study humic substances, as it is non-destructive, simple, non-separative and accurate. As a result, it has been used for determining the compositional and structural properties of SOM (Chen *et al.*, 2003; Senesi and D'Orazio, 2005; Sun *et al.*, 2007; Kwiatkowska *et al.*, 2008; Henderson *et al.*, 2009; Hur and Kim, 2009; Tang *et al.*, 2011). The intensity and position of the peaks detected in the spectra are unique to each substance structural and functional characteristics. For instance, higher fluorescence intensities are related to a higher humification (Martins *et al.*, 2011).

The Fluorescence Index (FI) was developed to assess different properties of dissolved organic matter (DOM). It was defined by McKnight *et al.* (2001) as the ratio of emission intensities at 450-500 nm excited at 370 nm. The 450 nm point was chosen for specific characteristics of the experiment. Later, Cory *et al.* (2010) modified the ratio to 470-520 nm to reflect corrections specific to the instruments used. This index has been correlated to the aromaticity of DOM (Korak *et al.*, 2014).

Fluorescence spectroscopy can be used in combination with UV-Visible spectroscopy to characterise humic substances, as absorbance measures transitions from the ground state to the excited state, as opposed to fluorescence spectroscopy (Skoog *et al.*, 2007). Its spectra are usually uniform and provide with qualitative data when a specific wavelength is selected (Hassouna, *et al.*, 2012). Also the specific absorbance at 254 nm ($SUVA\text{-}_{254}$) has been recognised as a method to determine SOM aromaticity (Fuentes *et al.*, 2006; Chow 2006). This parameter is very useful for assessing the nature of the general composition of dissolved organic carbon (DOC), due to its high correlation with it (Weishar *et al.*, 2003).

Considering what has been described in sections 1.3 and 1.4 above, both the fluorescence and NMR techniques can be used in combination to determine the humic substances properties and the degree of aromaticity; while HWC could contribute further to the understanding of soil quality, given its usefulness to detect the biodegradation of soil biochemical properties (Ghani *et al.*, 2003; Saab and Martin-Neto, 2007; González-Pérez *et al.*, 2007).

As an illustrative and additional way to characterise and represent some of the analyses conducted and the results obtained, Excitation-Emission Matrix (EEM) spectra have been plotted. These provide information on the relative intensity of fluorescence at different excitation and emission wavelengths regions in a fast manner that is also easy to interpret (Coble, 1996). Several peaks have been identified that are used to describe EEM fluorescence spectra. Peak A and C refer to humic peaks. Peak A is referred to as UVC-excited and is located at an excitation wavelength (λ_{Ex}) between 240-260 nm and an emission wavelength (λ_{Em}) between 400-460 nm. Peak C, also referred to as UVA-excited; is located at a λ_{Ex} between 320-360 nm and a λ_{Em} between 420-460 nm. There are also peaks that indicate biological activity material (peaks B and T, which are defined as tyrosine-like and tryptophan-like peaks respectively). B has λ_{Ex} of 270-280 nm and λ_{Em} of 300-315 nm whereas T has λ_{Ex} between 270-280 nm and λ_{Em} of 345-360 nm (Birdwell and Engel, 2010).

1.5. Aim/objective of this study

The aim of this study was to evaluate the use of fluorescence spectroscopy to measure SOM quality (specifically the grade of humification). The specific objectives were: (1) characterise water extractable SOM quality using liquid state ^{1}H-NMR; (2) characterise the quality of water extractable organic matter using fluorescence spectroscopy and UV-VIS; (3) compare measures of quantity and quality of water extractable organic matter with the specific organic matter fractions measured by ^{1}H-NMR like aromaticity. If robust relationships and similarities between optical measures and ^{1}H-NMR are found, there may be potential for fluorescence spectroscopy as a fast and more cost-effective method of organic matter characterisation.

2. Materials and methods

2.1. Field sites description

Cambisols were sampled in two regions with contrasting climatic conditions; Andalusia (South Spain) and Berkshire (South East England) (Table 1). Berkshire has a temperate climate, characterised generally by relatively mild winters and summers and rainfall throughout the year. The annual mean temperature ranges from 6.7 ºC and

14.5 ºC (30 years, annual mean temperature: 10.5 ºC) and the average annual rainfall is 635.4 mm (UK Met Office, 2014).

Andalusia has a Mediterranean climate, which characteristics are hot and dry summers contrasted with cool and wet winters. In the province of Cordoba (Hinojosa del Duque and Pozoblanco), the annual mean temperature is 17.6 ºC and the average annual rainfall is 536 mm (Aemet, 2014). As for the province of Jaen (Torredelcampo), its annual mean temperature is 16.2 ºC and the average annual rainfall is 646.3 mm (REDIAM, 2007).

In Berkshire, samples were collected from University of Reading Farms at Sonning, Arborfield and Shinfield. The site sampled at Sonning (GS-UK-1) has been covered with grass for over 15 years. Arborfield (CC-UK-1) was permanent pasture until autumn 2012, and has been subsequently drilled with wheat or winter barley. Shinfield (CC-UK-2) has been in an arable rotation for over 20 years and sown with either winter wheat, maize or spring barley.

In Andalusia, soil samples were collected between the provinces of Jaen (Torredelcampo) and Cordoba (Hinojosa del Duque, Pozoblanco) that were managed with conventional tillage of cereals and olive crops. CC-ES-3 was managed with a wheat-barley-fallow cycle, whereas the other sites (CC-ES-1 and CC-ES-2) were covered by wheat crops throughout the year. OG-ES-1, OG-ES-2, and OG-ES-3 were covered by olive grove.

2.2. Sample collection and preparation

Soil samples were collected from each horizon. Total soil depths are included in Table 2. Only the first horizon from each soil (specified in section 2.3) was used for the analyses. Samples were air dried and sieved with a 2 mm sieve.

2.3. Analytical methods

Cold and hot water extractable carbon were determined following Ghani *et al.* (2003). This consists in a cold and a hot extraction of the supernatant of the samples (room temperature and 80 ºC respectively). To do this, 30 ml of ultrapure water was added to 3 g of soil. Then, there was a 30-minute extraction in a shaker at 20ºC. After this, the sample was centrifuged during 20 minutes at 3500 rpm. Once this was done, the supernatant was extracted (cold extraction) and analysed (DOC). The resulting pellet was used for the rest of the steps. 30 ml of ultrapure water were added to the pellet, which was then shaken on a Vortex to mix the ultrapure water with the pellet. The sample was then left in a bath at 80 ºC during 16 hours. The sample was centrifuged at 3500 rpm during 20 minutes and was filtered with a 0.45 µm cellulose nitrate filter (hot extraction). The filtered supernatant was analysed for DOC, fluorescence, absorbance and NMR.

The techniques that were compared in this study were applied to the first horizon of each soil sample, as this was considered sufficient for the purposes of comparing these techniques. This horizon was at different depths depending on the soil sample (at 12 cm in GS-UK-1; 10.5 cm in CC-UK-1; 5.7 cm in CC-UK-2; 15 cm in CC-ES-1; 20 cm in CC-ES-2; 30 cm in CC-ES-3; 10 cm in OG-ES-1; 10 cm in OG-ES-2 and 18 cm in OG-ES-3).

Fluorescence of all the hot water extracts was measured in a Varian Eclipse Fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at an emission wavelength from 300 to 600 nm at 5-nm increments and an excitation of 240-450 nm at 5-nm increments. All samples were run in 1 cm quartz cuvettes and in triplicate.

FI was calculated as the ratio of intensities at 450 over 500 nm with an excitation of 370 nm, as described by Cory *et al.* (2010). McKnight *et al.* (2001) introduced this index approach for the characterization of the fulvic and fraction of DOM.

The Humification Index (HIX) was calculated with the following formula:

$$(\Sigma\ I_{435 \rightarrow 480}) / (\Sigma\ I_{300 \rightarrow 345}) + (\Sigma\ I_{435 \rightarrow 480})$$

where I is the fluorescence intensity at each wavelength (modified of Zsolnay *et al.*, 1999 by Ohno, 2002).

The Biological/Autochthonous Index (BIX) has been calculated as the ratio of intensities at 380 nm over 430 nm with an excitation of 310 nm, as described by Huguet *et al.* (2009). This index assesses the relative contribution of autochthonous DOM in water and soil samples.

A subsample of the HWC extract was frozen and subsequently freeze-dried to remove all the water present. Deuterium oxide was added as a solvent to avoid disruptions in the spectrum, as other solvents have a proton signal that causes disruptions, as demonstrated by Cardoza *et al.* (2004), prior to a second freezing and a second freeze-drying. This second stage of freezing-freeze-drying was used to avoid that H_2O peaks interfered in the spectra. After that, deuterium oxide (100%, density of 1.107 g/ml at 25 °C) was added again as a solvent to conduct the NMR tests. NMR was measured in 1.5-1.7 ml of sample with a Bruker Avance III 700 MHz NMR spectrometer (Bruker Corporation, Billerica, MA, USA). The deuterium oxide peak was used as a calibration reference and placed in 4.75 ppm.

Absorbance of all the hot water extracts was measured with a Varian Cary 300 UV-Visible spectrometer (Agilent Technologies, Santa Clara, CA, USA), from 200-800 nm with 1 nm intervals using a 1 cm cuvette.

SUVA-$_{254}$ has been used for this study. This is the absorbance at 254 nm divided by the DOC concentration (of the hot water extracts). Values are expressed in $l \cdot mg^{-1} \cdot m^{-1}$.

DOC of the cold and hot water extracts was calculated with a Shimadzu TOC 5000 total organic carbon analyser (Shimadzu Corporation, Kyoto, Japan). Standards were calculated using Stock solutions of 1,000 ppm.

% Organic matter measured by LOI was calculated using a modified version of Hieri *et al.* (2001). A soil sample of 10 g was heated at 105 ºC for 24 hours. Then, the sample was weighted again (w_1) and heated in a Muffle furnace at 550 ºC for 16 hours to ignite the organic matter. The sample was weighted after this (w_2) and the % of organic matter was obtained by weight difference between w_1 and w_2.

Soil pH was determined in 1:2.5 soil to water ratio. Texture was determined by laser granulometry, using a Coulter LS 230 (Beckman/Coulter Inc. Brea, CA, USA). This technique uses polarised light at three different wavelengths (450 nm, 600 nm and 900 nm) to analyse the particle size distribution specifically in the 0.04 µm to 0.4 µm range.

EEM fluorescence spectra were obtained by collecting a series of emission scans of λ_{Ex} 240-450 nm at 5 nm intervals and λ_{Em} 300-600 nm, also at 5 nm intervals. EEM spectra were plotted using RStudio v0.98.1091 (Rstudio Inc., Boston, MA, USA). Intensities are reported in Raman units (RU). Raman scattering was mitigated subtracting blanks that had been collected on ultrapure water from each spectrum.

2.4. Statistical methods

Data were tested for normality to verify the model assumptions. As the data failed the normality test, non-parametric tests were used. The effect of the hot water extraction in the extractability of carbon (compared to the cold water extraction) was analysed using a Paired Sample Wilcoxon Signed Rank Test. The correlation between the different analytical methods was tested using the Spearman Rank and assessing the significance of the resulted Spearman Correlation Coefficient (Minitab 16 for Windows. Minitab Inc., State College, PA, USA). Linear regressions of the Spearman Correlations were also plotted (SigmaPlot 12.0 for Windows. Systat Software Inc., San Jose, CA, USA). Differences of $p<0.05$ were considered statistically significant.

3. Results and discussion

3.1. Soil characteristics

The majority of CC-UK soils had sandy texture, with a relatively high proportion of silt (Table 2). The only exception was CC-UK-2, although the sand proportion was quite close to that of silt. This texture was similar to that of CC-ES-1 and CC-ES-2, although CC-ES-3 had a higher proportion of silt and higher proportion of clay than of sand. All the OG soils presented a texture of a majority of silt (56.7-59.5%), followed by clay. It is worth considering that silt is the most erodible fraction (Table 2).

pH in the UK soils was generally acid, although GS-UK-1 and CC-UK-1 had values between 3.58-5.66 and CC-UK-2 had values close to 7. ES soils had generally higher values, ranging from 5.53-6.98 (Table 2). Some authors have related pH and measurement of aromatics (Weishar *et al.*, 2003). GS-UK-1 has the lowest pH (3.58) and is the one with the highest aromatics content and highest HWC.

3.2. Total extractable carbon by cold and hot extractions

CWC and HWC measurement results are in Table 3. HWC extracted significantly more carbon ($P<0.01$) than CWC, thus proved to be a more exhaustive extraction method. The increases in the values ranged from 161-605%. This was equivalent to higher values by a factor between 3 and 7 respectively. The most significant increase was obtained in GS-UK-1. Gregorich *et al.* (2003) obtained a similar trend in maize-cropped soils of Ottawa, Canada, and found HWC exceeding CWC by a factor of two.

Moreover, Landgraf *et al.* (2006) found that HWC had higher carbon concentrations than CWC by a factor that varied from 4 to 6 in surface horizons of forest soils in SE Germany.

A correlation analysis was run to assess whether the C extractability differed between the different soils and locations. The correlation resulted to be high (Rs=0.91, $p<0.01$). Wang and Wang (2007) found a similar correlation (r=0.93, p=<0.01) in forest oxisols of Southern China. Klose and Makeschin (2003) also found that HWC and CWC increased with the same proportion in forest soils of NE Germany. On the other hand, Ghani *et al.* (2003) found a positive but poor correlation.

HWC and CWC data were compared with SOM (measured by LOI) to assess which of the two extraction methods would imply a higher correlation with the organic matter from the samples (Table 4). HWC implied a higher correlation with SOM measured with LOI (Rs= 0.70, $p<0.05$) than CWC (Rs= 0.55). Despite the fact that the correlation between CWC and SOM was lower than that of HWC; other authors have found even poorer correlations. For instance, Van Migroet *et al.* (2005) found a r^2 of 0.2 in forest soils in Utah, USA.

3.3. Quality of cold-water extractable carbon using UV-Vis and Fluorescence

Cold-water extractable carbon (CWC) data was compared with UV-Vis and fluorescence spectroscopy, using $SUVA\text{-}_{254}$ and FI respectively. The Spearman Rank Correlation was calculated to evaluate the quality and reproducibility of CWC using optical techniques (Table 4).

There were high correlation patterns between CWC and $SUVA\text{-}_{254}$ (Rs=0.82, $p<0.05$) although not between CWC and FI (Rs=-0.29), where the slight correlation that could be appreciated was inverse. Chow (2006) obtained a worse correlation between CWC and $SUVA\text{-}_{254}$ (R^2=0.38), whereas that of Van Migroet *et al.* (2005) was even lower

(R^2=0.01). As Weishar *et al.* (2003) point out; some authors have found conflicting conclusions when using SUVA-$_{254}$ to determine the aromaticity of DOC.

3.4. Quality of hot-water extractable carbon using UV-Vis and Fluorescence

HWC data was compared with SUVA-$_{254}$ and FI to assess whether it had higher correlation than CWC. As it can be seen in Table 4, there is a high correlation (Rs=0.88, $p<0.001$) between HWC and SUVA-$_{254}$ whereas that of HWC with FI is not as significant (Rs=-0.53). In both cases, HWC was more significantly correlated to the results obtained with fluorescence and SUVA-$_{254}$ than CWC.

3.5. EEM spectra general characteristics

A set of reference fluorescence spectra has been represented in Figure 1. As it can be seen in Figure 1.a and 1.b, the two soils with the highest % aromatics measured with ^{1}H-NMR as well as high SUVA-$_{254}$ and HWC values (GS-UK-1 and CC-UK-1) showed strong peaks C (10-12 RUs), which indicate UVA-excited humic peaks as described by Coble (1996). They also showed weak peaks A.

On the other hand, Figure 1.c. represents the soil with the highest FI, but lowest % aromatic measured with ^{1}H-NMR as well as comparatively low values of SUVA-$_{254}$ (CC-ES-1). The EEM spectrum of this soil is very different from the ones represented in Figures 1.a and 1.b, with a strong peak A or UVC-excited (8 RUs), and weak peaks C and T. The presence of the latter indicates biological activity.

3.6. Relationship between optical measures and NMR

The aromaticity of isolated fulvic acid samples was calculated as the ratio of the area of aromatic hydrogen region to the total area of the ^{1}H-NMR spectrum (% aromaticity). An analogous method was used to calculate the ratio of the area of aliphatic hydrogen and carbohydrate hydrogen regions.

As it can be seen in Figure 2, the aromatic hydrogen region is situated in a chemical shift of 6-8ppm by frequency. On the other hand, the carbohydrate hydrogen and aliphatic hydrogen regions are situated in a chemical shift of 3-4.2 ppm and 0.5-3 ppm respectively. For Figure 2, the two soils with the highest % aromatics were chosen for representation purposes (GS-UK-1 and CC-UK-1).

Results of these measurements can be found in Table 3, along with the results from the optical techniques. Correlation analyses were conducted comparing the results from all of them (Table 4).

The largest carbon fraction of the UK soils was carbohydrates. The same trend was found in the CC-ES soils, but not in the OG-ES soils, where the largest carbon fraction was aliphatics.

The proportion of aromatics of the UK soils ranged from 3.91%-5.98%, whereas for the CC-ES and OG-ES soils, it ranged from 2.40-3.47% and 3.90%-4.33% respectively. As these were taken from samples in the first horizon of each soil, the low level of aromaticity can be explained by the fact that plant residues may have accumulated in the surface, while microorganisms did not have enough capacity to decompose them. As Cardoza *et al.* (2004) point out, NMR is a powerful tool for investigating humic substances interactions at the molecular level.

Aromatics have positive correlations for $SUVA_{254}$, CWC and HWC but are inversely correlated to FI. Correlations are high for aromatics compared with $SUVA_{254}$ and HWC (Figures 3 and 4. Rs of 0.95 and 0.90 respectively; $p<0.001$), but not as good for FI (Figure 5). The correlation between aromatics and CWC, although significant, is not as strong as the one with HWC (Rs=0.74, $p<0.05$). Weishaar *et al.* (2003) found that $SUVA_{254}$ is a good predictor of the humic fraction (higher $SUVA_{254}$ indicates higher humic acid content and molecular weight in DOM solutions) and the general chemical properties of HWC, although it does not predict the reactivity of HWC from different types of source materials nor information of the individual molecules of samples unless these are humic substances (Chin *et al.*, 1997; Weishar *et al.*, 2003). They obtained a good correlation between SUVA-$_{254}$ and aromaticity determined by ^{13}C-NMR (R=0.97), which was slightly higher than ours. Yeh *et al.* (2014) also found a high correlation between SUVA-$_{254}$ measurements and the aromatic fraction of samples of organic matter of river sediments of Taiwan. Jamieson *et al.* (2014) found that higher SUVA-$_{254}$ implied higher aromaticity in biochar from sugar maple, thus demonstrating that this effect is currently studied when characterising organic material with a high recalcitrance and that is able to retain carbon for thousands of years, due to its stability (Lehmann, 2007).

According to McKnight (2001) and other authors (Rodríguez *et al.*, 2014; Wei *et al.,* 2014), there is a correlation between the FI values and those from methods that indicate the aromaticity of humic substances such as the ratio of the aromatic carbon region area in the total NMR spectra and SUVA-$_{254}$. This pattern was observed in our soils, with negative correlations between FI and these 2 parameters (Rs=-0.63 and Rs=-0.52 respectively), although only the trend with the aromatic carbon region area in the total NMR spectra was statistically significant ($p<0.05$).

Kim *et al.* (2006) and Rodríguez *et al.* (2014) indicated that differences in the FI higher than 0.1 would imply significant differences in the aromaticity of the samples. If this assumption is applied to our case, CC-UK-2 has differences in its aromaticity with GS-UK-1 but not with CC-UK-1. CC-ES-2 has a different aromaticity than CC-ES-1 and CC-ES-3. In the same way, OG-ES-1 has differences with OG-ES-2 and OG-ES-3. When all the types are compared, there are combinations of differences in the aromaticity but a single pattern cannot be obtained.

Given the results above, the regressions between the aromatic carbon region measured with ^{1}H-NMR and SUVA-$_{254}$ and HWC are powerful. The equations we obtained can explain very significantly the relationship between these variables ($p<0.001$ for the results of ^{1}H-NMR predicted with SUVA-$_{254}$ and $p<0.01$ if they are predicted using HWC). These equations (Figure 3 and Figure 4) could therefore be used to predict the aromaticity of water-extractable carbon.

The regression of the aromaticity measured with ^{1}H-NMR with FI, although significant ($p<0.05$), did not have the same level of confidence as the one with HWC and SUVA-$_{254}$. As it can be seen in Figure 5, an outlier was identified in the Regression analysis (FI value of CC-ES-3). Figure 5 showed how the regression model improved when the outlier was removed from the analysis (Rs=-0.98). The linear regression equation that resulted after the elimination of the outlier is able to explain the ^{1}H-NMR values using FI with a very high significance ($p<0.001$).

However, according to McKnight (2001), the FI would not be sufficient to estimate aromaticity and other techniques are necessary (e.g. SUVA-$_{254}$), as geological processes can alter aromaticity without changing the FI.

SUVA-$_{254}$ did not demonstrate a particularly strong correlation with FI (Rs=-0.52, Table 4). The correlation was negative (higher SUVA-$_{254}$ led to lower FI) which coincides with authors like Kothawala *et al.* (2012) with minerals soils collected across Canada although their trend is less clear than in the study of other authors such as Williams *et al.* (2010), where the slope is more pronounced. The correlation of Williams *et al.* (2010) was similar to ours (r=-0.57 in their study and Rs=-0.52 in ours), although the statistical analysis in their case was the Pearson correlation, as their data followed a normal distribution. Hassouna *et al.* (2012) found a negative correlation as well, with slightly higher correlation in Mediterranean calcareous soils (r=-0.69). As in the case described in Figure 5, if the same outlier was removed, the correlation improved significantly (Rs=-0.98) and the equation of the linear regression explained with a very high degree of significance (p<0.001) the relation between SUVA-$_{254}$ and FI. Analogously, the same outlier could be removed in the analysis of HWC vs. FI, obtaining a significant correlation between them (Rs=-0.88). A linear regression model explains significantly (p<0.01) the relationship between HWC and FI and therefore similar conclusions can be stated as in the case of the correlation of SUVA-$_{254}$ to FI. Given the strong intrinsic relationships between the results of SUVA-$_{254}$, HWC and FI; and how they are able to predict the proportion of aromaticity measured with ^{1}H-NMR, we can conclude that UV-Vis absorbance and fluorescence spectroscopy can be used to characterise the aromaticity of carbon and may be a plausible substitute for ^{1}H-NMR, given that they are more cost-effective. Other authors such as Zornoza *et al.* (2008, 2015) demonstrated the effectiveness of spectroscopy (near infrared reflectance spectroscopy in their study) as an accurate, cost- and time-effective method for predicting and/or estimating soil biogeochemical properties and other soil parameters. Correlations between aliphatics and the data from the optical measures and CWC/HWC were poor (Table 4), although slight trends can be observed for SUVA-$_{254}$, FI and CWC. In these cases, the correlation was positive for FI and CWC but negative for SUVA-$_{254}$. Correlations were also poor between carbohydrates and the data from the optical measures and HWC/CWC, with only one trend detected. This was the inverse correlation (Rs=-0.35) between HWC and carbohydrates.

BIX and HIX did not correlate well with any of the other parameters (Table 4), with very few trends detected. Of the trends detected, HIX was positively correlated with FI (Rs=0.52). It is worth noting that BIX was positively correlated with carbohydrates but inversely correlated to aliphatics (Rs=0.31 and -0.31 respectively), whereas the trend was the contrary for HIX (Rs=-0.28 and 0.22). A similar trend than ours was also found by Kalbitz *et al.* (2003) between HIX and carbohydrates in forest soils, arable soils and a fen area; although theirs was stronger (r^2=0.81). Williams *et al.* (2010) studied the correlation between HIX and SUVA-$_{254}$ in watershed from mixed land use in Canada. They obtained a positive correlation (r=0.74), whereas our correlation was negative. On the other hand, Yeh *et al.* (2014) did not find a clear trend between SUVA-$_{254}$ and HIX. The significance of our comparison between SUVA-$_{254}$ and HIX values is extremely low and therefore robust conclusions on this issue cannot be stated. Bu *et al.* (2010) compared HWC and HIX in various soils with different vegetation types in Wuyi Mountain (SE China), obtaining a poor negative correlation, just like ours. However, Kalbitz *et al.* (2003) found a strong correlation between the aromatic fraction of DOM and HIX (r^2=0.80).

Yeh *et al.* (2014) calculated BIX as well, obtaining that higher SUVA-$_{254}$ values implied lower BIX values, which coincides with our comparison in this case, although its significance is not relevant enough to establish a robust conclusion.

Plotting the results from Birdwell and Engel (2010) for FI, HIX, and BIX did not led to significant correlations, which is similar to our results and, along with the authors commented above, demonstrate that a number of studies have found difficulties when correlating HIX and BIX to other parameters.

4. Conclusions

Different techniques to measure the quantity and quality of SOM were tested in cambisols from very different climatic locations and under different cropping systems. HWC extracted a higher amount and carbon than CWC, and correlated better with the %SOM (LOI) than CWC.

SUVA-$_{254}$ and HWC correlated significantly with the proportion of aromatics measured with ^{1}H-NMR, demonstrating their complementary nature. Linear regression models fitted to the data were able to explain the relationship between the specific fraction of aromatics measured with ^{1}H-NMR and SUVA-$_{254}$ and HWC, and therefore allow the aromatic fraction to be estimated at lower cost using SUVA-$_{254}$ and HWC.

A linear regression model was also able to explain the relationship between ^{1}H-NMR measurements and FI, after an outlier was removed.

BIX and HIX were not useful indexes for our purposes, as they did not correlate well to the carbon fractions measured with ^{1}H-NMR or with FI, SUVA-$_{254}$, or HWC.

The EEM spectra showed a difference in the peak types that appeared in the CC-UK soils and in the CC-ES soil. The fact that figures 1.a and 1.b showed a stronger peak C and figure 1.c. showed a stronger peak A indicated subtle qualitative differences in their humic fractions. This could have been caused by the different soil management (more intense agricultural activity in CC-ES-1). More work will be needed to confirm this.

In view of our results, optical measures have a potential, in combination, to predict the aromatic fraction of SOM without the need of expensive and time consuming techniques like ^{1}H-NMR; which could be very useful when the equipment is not available or in instances when a high number of samples need to be analysed simultaneously.

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6. References

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Figure captions

Figure 1. EEM plots from: a) GS-UK-1; b) CC-UK-1; c) CC-ES-1

Figure 2. Liquid state ^{1}H-NMR spectra from: a) GS-UK-1; b) CC-UK-1

Figure 3. Correlation between % Aromatics and SUVA-$_{254.}$ A linear regression model was calculated to explain the relationship between the variables. The equation was y [% Aromatics] = 0.250 + 0.950 x [SUVA-$_{254}$], $p<0.001$

Figure 4. Correlation between % Aromatics and HWC. A linear regression model was calculated to explain the relationship between the variables. The equation was y [% Aromatics] = 0.500 + 0.900 x [HWC], ($p<0.01$)

Figure 5. Correlation between % Aromatics and FI. A linear regression model was calculated to explain the relationship between the variables. The equation was y [% Aromatics] = 8.17 – 0.633 x [FI], ($p<0.05$), represented by the solid line. The removal of an outlier resulted in the equation: y [% Aromatics] = 8.89 – 0.976 x [FI], which explained the relationship with a higher significance ($p<0.001$). The second equation is represented by the dashed line