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New methodology to assess the quantity and quality of humic substances in organic materials and commercial products for agriculture

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Abstract
Purpose The traditional method to determine humic content (humic and fulvic acids) in commercial fertilizers, biostimulants, and organic materials is based on the oxidation of the organic carbon contained in the basic-soluble but acid-insoluble fraction (humic acids) and the basic-acid soluble fraction (fulvic acids) of their alkaline water extracts. This methodology, merely operational, makes it impossible to distinguish if the quantified carbon corresponds to substances with "humic" chemical nature or to non-humic organic matter but with similar solubility properties to those of humic matter. The aim of this work is to develop a new methodology that not only quantifies the humic content in commercial products (and raw materials) but also assesses the humic quality of the quantified organic matter.
Materials and methods To this end, humic and fulvic (-like) fractions have been isolated/purified from several humic and non-humic materials and characterized by means of elemental analysis and UV-visible, fluorescence, and infrared spectroscopies, and these data have been used to perform a discriminant analysis (DA).
Results and discussion The model obtained from the DA is able to discriminate humic and fulvic fractions from apparently humic or fulvic ones and provides discriminant classification functions that have proven to successfully predict the "humic quality" of the fractions isolated from commercial products, after their elemental and spectroscopic characterization.
Conclusions Therefore, the combination of the fractionation, characterization, and evaluation by the DA is proposed as an effective methodology for quantifying and assessing the quality of the humic content claimed in the labels of commercial products.

Keywords Agricultural products · Biostimulants · Fulvic acids · Humic acids · Humic quantification · Humic substances · Humification · Organic carbon · Organic matter

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1 Introduction

Humic substances (HSs) are the result of the degradation, oxidation, and transformation of plant and animal organic debris. These modifications of the organic matter are performed by soil enzymes and microorganisms in soil microcosm during indefinite periods of time, yielding HSs with particular chemical and biological properties. The process of the transformation of fresh organic matter into humic matter occurring in soil is called humification. Numerous studies have shown that HSs are beneficial for soil fertility: they act as soil pH buffers (Vaughan and Ord 1985) and as redox agents (Jones and Bryan 1998; Struyk and Sposito 2001); they complex metallic ions, thus enhancing availability of

micronutrients to higher plants (Cabaniss 1992; Donisa et al. 2003; Nardi et al. 2002; Tipping 2002); they adsorb organic solutes, which is especially important when dealing with contaminants or pesticide problems (Hayes et al. 1989; Nardi et al. 2002; Stevenson 1985, 1994); and they are able to stimulate plant growth (Canellas et al. 2015; Mora et al. 2010; Nardi et al. 2002; Stevenson 1994; Vaughan and Ord 1985). Part of the HSs present in soils forms the clay-humate complex, improving the porosity, aeration, moisture retention, and transport of nutrients in soil (Stevenson 1985, 1994; Vaughan and Ord 1985). When HSs are deposited into aquatic ecosystems or soil solutions, they tend to self-assemble, forming new functional and stable colloidal aggregates in the solution, which might be considered as a family of natural supramolecules (Piccolo 2002; Baigorri et al. 2007a, b).

Commercial organic or organo-mineral fertilizers usually contain between 15 and 85 % of humic substances, extracted from lignite, leonardite, or peat (Lamar et al. 2014; de Liñán Carral and de Liñán Vicente 2016, http://www.terralia.com/vademecum_de_productos_fitosanitarios_y_nutricionales/, in Spanish, last accessed: May 2016), which are sources with a very high content of humic substances. On the other hand, composted materials are typically marketed as organic amendments containing composted organic residues. Extraction of the semi-humified fraction from composts for its subsequent addition to other fertilizer formulation is very rare (the industrial requirements, the processes, and the yields make it not worthwhile compared to extracting humic substances from lignite or leonardite). Therefore, in this study, we have not taken into account humic substances from composts, as our goal was to distinguish sedimentary humic substances from other organic components with similar solubility properties that can be added as adulterants to commercial fertilizers.

In Spain, the current official analytical determination of the humic content in commercial products (BOE 1991) is based on the quantification of the total organic carbon soluble at alkaline pH (total humic content) or in the humic content of the acid-insoluble residue remaining in the alkaline extract (content in humic acids) by oxidation with potassium dichromate (Nelson and Sommers 1982). Weaknesses of this method are linked to the need to assume a specific percentage of carbon in humic matter and an oxidation factor, as well as to its low selectivity. Any organic compound with the same solubility properties as those of HSs at these pH conditions will be quantified as humic matter, regardless of its humic chemical nature. Recently, a new method based on the International Humic Substances Society (IHSS) humic fractionation but quantifying humic and fulvic fraction by gravimetric methods has been proposed (Lamar et al. 2014). This new method solved the inaccuracy associated with humic carbon concentration and oxidation factor assumptions but still has potential low selectivity.

Although there does not exist a perfect method for the isolation and purification of humic substances (Hayes and Graham 2000), the fractionation and isolation methodology proposed by the IHSS has gained popularity and it is considered a satisfactory method for the extraction and purification of HSs from most soil types and organic products (Swift 1996; Lamar et al. 2014). The purification of the fulvic acid fraction in this procedure is based on its adsorption onto the DAX-8 resin. Nevertheless, although this methodology is more selective for HS than the mere oxidation of the dissolved organic carbon with potassium dichromate, the use of DAX-8 resin is not exclusive of fulvic acids, as it can eventually adsorb other types of hydrophobic compounds such as protein or lignin derivatives. Likewise, natural non-humic polymers may also precipitate at very acidic pH (for instance, alginates, or some proteinates, etc.). In this context, the development of a new methodology that might improve the capability of distinguishing humic from non-humic materials is of great interest, especially considering that the heterogeneity and variability of the HS as a function of their origin and extraction method makes it difficult to establish universal standards for humic and fulvic acids. Previously, several groups have attempted to evaluate the humic quantity in sedimentary sources (peat, lignite, leonardite), commercial products, or composts (Sequi et al. 1986; Cavani et al. 2003; Francioso et al. 2003; Van Zomeren and Comans 2007; Lamar and Talbot 2009; Quentel and Filella 2011) basing different properties (purification on XAD-8 or PVP columns, organic C oxidation, isoelectrofocusing, 1H-NMR, differential thermal analyses, adsorpting stripping voltammetry, etc.). Nevertheless, we found a main flaw: these methods are either quantitative or qualitative, but not both; besides, these properties require techniques that need a very careful tune-up (i.e., voltammetry) or equipments not so commonly found in modest analytical laboratories (especially NMR spectrometers). In this paper, we propose performing a fractionation and isolation methodology that includes several purification steps based on IHSS methodology (Swift 1996; Lamar et al. 2014), which is combined with a qualitative assessment of the humic nature of the obtained extracts.

Ideally, an analytical methodology should be easy to perform, and the evaluation of the "humic quality" should neither require expensive equipment nor be based on absolute variables that are equipment dependent. In previous studies, we showed the high correlation between different indexes obtained using relatively simple spectroscopic techniques (UV-visible and fluorescence) and the humification degree of several organic materials (Fuentes et al. 2006). In these studies, the humification degree was assumed to be as a result of the time of transformation of fresh organic materials in soils or in microbial digestion systems, such as compost or vermicompost (Fuentes et al. 2006). Further studies showed that, in fact, these simple parameters were as useful as others derived from much more

sophisticated analytical techniques (^{1}H or ^{13}C–NMR and Pyrolysis MS) to characterize humification (Fuentes et al. 2007, 2010). However, these indexes only showed trends and do not allow us to clearly differentiate between humic and non-humic substances when they are considered individually. Hence, in this framework, the use of multivariate statistical analyses of the different indexes arises as a potential useful tool in this assessment of the humic quality of the extracted fractions.

The discriminant analysis (DA) has two main objectives (McGarigal et al. 2000): firstly, to transform a relatively large group of original variables into a smaller group of composite variables (*canonical discriminant functions*) with the minimum loss of information. The DA constructs canonical functions, which are a linear combination of the original independent variables, so that such canonical functions maximize the among-groups variations at the same time that they minimize intra-groups variations. Secondly, the DA also calculates *classification functions*, which can be used to predict to which group an unknown sample belongs. After the characterization of an unknown sample, the scores for each of the classification functions are calculated—the sample is predicted to belong to the group with the higher score.

For the development of this methodology, we have considered a number of humic and fulvic materials, including IHSS standards, and diverse organic materials with no humic nature such as lignosulfonates, protein-derived products, and seaweed extracts. Humic (or humic-like) substances have been extracted and quantified by two different procedures (the carbon oxidation method with dichromate and an isolation method based on that proposed by the IHSS (Swift 1996). The fractions obtained by the second methodology have been characterized by elemental analysis, UV-visible, fluorescence, and infrared (FTIR) spectroscopies. In order to avoid equipment-dependent bias, for UV-visible, FTIR, and fluorescence spectroscopies, indexes based on specific ratios between measurements and molar absorptivity coefficients were considered. These indexes have been introduced as variables in a multivariate statistical analysis (discriminant analysis), in order to test their suitability for discriminating between humic and non-humic materials.

2 Materials and methods

2.1 Organic materials

The group of naturally formed humic substances consists of (i) seven samples purchased from the IHSS: Leonardite Standard Humic Acid (LSHA), Pahokee Peat Reference Humic Acid (PRHA), Suwanne River Reference Fulvic Acid (SRFA), Waskish Peat Reference Fulvic Acid (WRFA), Elliot Soil Standard Fulvic Acid (ESFA), Pahokee Peat Standard Fulvic Acid (PSFA), and Nordic Lake Reference Fulvic Acid (NRFA); (ii) a commercial humic acid obtained from Aldrich Chemicals (AHA); and (iii) humic substances extracted from humified materials: a leonardite from Florida (HS1), a Chinese leonardite (HS2), a Czech leonardite (HS3), a Spanish peat (HS4), and a Russian lignite (HS5).

The so-called apparent humic substances (*apHS*) were extracted from (i) seven lignosulfonates (apHS1–apHS7); (ii) two protein-derived products, consisting of a pool of oligopeptides and amino acids (apHS8 and apHS9); and (iii) an extract from seaweed (apHS10). The elemental composition of all the raw samples is summarized in Table 1.

Humic and fulvic acids extracted from humified materials and reference or standard HS are referred as *HAs* and *FAs* throughout the text, whereas the equivalent fractions obtained from non-humified matter (*apHS*) are referred as *apHAs* and *apFAs*.

The humic (or apparent humic) content of all the samples has been determined by two different methods (Sects 2.2 and 2.3). Three laboratory replicates for each sample (multiple subsamples extracted separately from each sample) were analyzed.

Table 1 Moisture, ash content, and elemental composition (*w*/*w* %) of the raw materials

Sample	Moisture[a]	Ash[b]	C[c]	H[c]	N[c]	O[c]
LSHA	7.2	2.6	63.8	3.7	1.2	31.3
PRHA	11.1	1.1	56.4	3.8	3.7	37.3
SRFA	8.9	1.0	53.0	4.4	0.8	43.9
WRFA	8.3	0.2	53.6	4.2	1.1	41.8
ESFA	9.3	2.64	49.79	4.27	3.25	44.34
PSFA	9.3	0.9	51.31	3.53	2.34	43.32
NRFA	9.2	0.45	52.31	3.98	0.68	45.12
AHA	15.1	24.9	60.4	4.4	0.7	34.5
HS1	14.3	39.0	59.5	3.5	0.6	36.3
HS2	16.9	28.6	74.2	2.8	1.2	21.9
HS3	14.8	31.1	64.7	4.2	1.1	30.0
HS4	36.0	58.6	57.6	7.0	1.3	34.2
HS5	17.1	27.2	65.3	3.7	1.4	29.6
apHS1	12.2	26.1	51.1	5.4	3.8	39.7
apHS2	8.5	3.8	39.2	4.2	0.0	56.7
apHS3	5.8	19.9	48.0	5.5	2.4	44.1
apHS4	10.4	31.9	59.2	5.6	2.1	33.1
apHS5	12.9	37.3	59.6	5.3	0.0	35.2
apHS6	11.9	32.3	62.7	5.5	0.0	32.1
apHS7	10.7	38.1	56.5	4.5	0.6	38.4
apHS8	34.0	25.6	56.7	7.6	16.6	19.0
apHS9	64.1	2.3	44.7	5.1	13.5	36.7
apHS10	8.3	34.6	47.3	4.5	3.5	44.6

[a] Wet matter

[b] Dry matter

[c] Dry and ash-free matter

2.2 Method 1: organic carbon oxidation

Humic content was quantified by organic carbon oxidation, following the Spanish official analysis method for organic fertilizer products (BOE 1991): total humic extracts (THEs) were isolated from the raw materials by adding 100 mL of a 0.1 M $Na_4P_2O_7$ and 0.1 M NaOH solution to 0.5–0.6 g of each sample, shaking for 1 h at room temperature. The supernatant (containing the THEs) was then separated from the solid residue by centrifugation (11,000×*g*, 5 min) and diluted with deionized water until a final volume of 1 L. Two hundred milliliters of this solution was acidified to pH 1–2 with 96 % H_2SO_4, allowing the precipitation of the humic acid (HA) fraction for 8 h. The precipitate was then centrifuged to separate the HAs from the supernatant and redissolved in 50 mL of 0.01 M NaOH (HA solution). The organic carbon content in the THE (C_{THE}) and the HA (C_{HA}) solutions was determined by dichromate oxidation followed by titration with ferrous ammonium sulfate (BOE 1991; Nelson and Sommers 1982) and calculated using Eq. (1):

$$\%\ C = \frac{(V_b - V_s) \times N \times 3 \times 1.3}{w} \times 100 \quad (1)$$

where

$V_b$vol (L) of ferrous ammonium sulfate solution required to titrate the blank

$V_s$vol (L) of ferrous ammonium sulfate solution required to titrate the sample

*N*exact normality of ferrous ammonium sulfate solution (≈0.5 N)

*w*weight (g) of the analyzed sample

3carbon equivalent weight (g)

1.3error factor, as only 76 % of the total C is oxidizable (Walkley and Black 1934)

The carbon content in the fulvic fraction (C_{FA}) was calculated by difference: $\%C_{FA} = \%C_{THE} - \%C_{HA}$ (BOE 1991). The method states that the percentages of THE, HA, and fulvic acid (FA) are calculated assuming that the organic matter has an average of 58 % C (2).

$$\%\ \text{THE, HA, or FA}\ \left(\text{w}/\text{w}\right) = \%\ \text{C} \times 1.724 \quad (2)$$

2.3 Method 2: isolation/purification and characterization of humic and fulvic fractions

This method is based on the IHSS (Swift 1996) method. Humic (or apparent humic) substances were extracted from the original samples with 0.1 M NaOH under N_2 atmosphere (24 h of mechanical shaking in darkness), with a sample/extractant ratio of 1:6. The suspension was then centrifuged at 11,100×*g* for 15 min, discarding the solid residues. Supernatants were acidified to pH 2 with 5 M HCl, allowing the precipitated HAs to settle overnight. HAs were separated from the supernatant (the fulvic extract) by centrifugation at 11,100×*g* for 15 min and washed with distilled water until negative Cl^- test with $AgNO_3$ before freeze-drying. FAs were purified from the fulvic extract by sorption onto a column of Supelite™ DAX-8 resin as follows: the acidic fulvic extracts were passed through a column of DAX-8, discarding the effluent. The column containing adsorbed FAs was rinsed with 0.65 column volume of deionized water. Finally, FAs were desorbed by adding 1 column volume of 0.1 M NaOH. The pH of the solution containing the purified FAs was lowered to 2–3 with an acidic cation-exchange resin (Amberlite IR-120 Ion Plus, Sigma). Immediately afterward, the resin was separated from the solution by centrifugation, before freeze-drying the FAs. Humic and fulvic content were determined by direct weighting of the recovered fractions.

The fractions were subsequently characterized by elemental analysis and UV-visible, fluorescence and FTIR spectroscopies.

2.3.1 Elemental analysis

The content in carbon, hydrogen, and nitrogen of the lyophilized samples was analyzed in triplicates by a LECO CHN 900 analyzer. The oxygen content was determined by difference (ash-free basis).

2.3.2 UV-visible spectroscopy

UV-visible studies were performed on an HP8543 spectrophotometer (Agilent), using a 1-cm path-length quartz cuvette. The following parameters were obtained:

- E_{ET}/E_{Bz} *ratio*: solutions of 15 mg of organic carbon (OC)·L^{-1} were prepared in 10 mM sodium acetate (pH 7 fixed with acetic acid). Ratios of absorbances at 253 and 220 nm (ET and Bz absorption bands of benzene, respectively) were calculated (Fuentes et al. 2006; Korshin et al. 1997).
- E_4/E_6 *ratio*: solutions of different concentrations were prepared in 0.05 M $NaHCO_3$, with absorbance values within the linearity range of the Lambert-Beer law. E_4/E_6 ratios were calculated from the quotient between absorbances at 465 and 665 nm (Chen et al. 1977).
- ε_{600}: molar absorptivity at 600 nm ($L{\cdot}cm^{-1}{\cdot}mol\ OC^{-1}$), measured in 0.1 M NaOH. This parameter is equivalent to the RF parameter defined by Kumada (1987).
- ε_{280}: molar absorptivity at 280 nm ($L{\cdot}cm^{-1}{\cdot}mol\ OC^{-1}$), measured in 10 mM sodium acetate (pH adjusted to 7

with acetic acid), was calculated in order to estimate the relative aromaticity of the samples (Chin et al. 1994; Peuravuori and Pihlaja 1997).

2.3.3 Fluorescence spectroscopy

Fluorescence spectra were recorded on a Perkin Elmer LS50B fluorescence spectrophotometer, with a scan speed of 300 nm min^{-1}, using excitation and emission slit bandwidths of 7 nm. Solutions of 10 mg of $OC{\cdot}L^{-1}$ of each isolated fraction were prepared in 0.05 M $NaHCO_3$. The following spectroscopic indexes were obtained:

- A_4/A_1: emission spectra were measured between 350 and 650 nm, with an excitation wavelength of 240 nm, using the method proposed by Zsolnay et al. (1999). These spectra were divided into four regions, and ratios between the areas under the last quarter (570–641 nm, A_4) and the first quarter (356–432 nm, A_1) were calculated.
- I_{360}/I_{400}: synchronous-scan excitation spectra were measured over a range of 300 to 520 nm, by measuring fluorescence intensity while scanning simultaneously both the excitation and emission wavelengths, keeping a constant difference, $\Delta\lambda = \lambda_{em} - \lambda_{exc} = 55$ nm. The ratios between fluorescence intensities at 360 and 400 nm were calculated (Fuentes et al. 2006; Kalbitz et al. 1999; Milori et al. 2002).

2.3.4 FTIR spectroscopy

FTIR spectra were recorded with a Nicolet Magna-IR550 spectrometer over the 4000–400-cm^{-1} range. Pellets were prepared by mixing 1 mg of each freeze-dried sample fraction with 100 mg of KBr so that the mixture would become homogeneous. Differences in the FTIR spectra were analyzed by calculating ratios of intensities at the mains peaks: 2940, 1715, 1620, 1515, 1400, and 1040 cm^{-1}. The 1715/1620 ratio was calculated because these peaks represent the main differences between sedimentary HAs and FAs; the 1620/2940 ratio, as a measure of the aromatic C/aliphatic C content; and the 1515/1715 and 1040/1400 ratios, as these peaks experimented the greatest variations depending on the considered samples. The peak at 2940 cm^{-1} is accused by symmetric stretching vibrations of the C–H in CH_2 and CH_3 groups. The band at 1715 cm^{-1} is attributed to the C=O stretching vibration of COOH, ketones, aldehydes, and esters. The band centered at 1640 cm^{-1} may be related to aromatic C=C stretching and C=O stretching of quinone and/or conjugated ketone and amide groups (amide I band). The peak at 1400 cm^{-1} is attributed to O–H deformation, C–O stretching of phenolic OH, and C–H deformation of CH_2 and CH_3 groups. The peak centered at around 1040 cm^{-1} is related to the C–O stretching of polysaccharide or polysaccharide-like structures or the aromatic C–H in-plane deformation of syringyl and guaiacyl alcohols, two structural components of lignin.

2.3.5 Statistical analyses

Multivariate statistical analyses (discriminant analysis) were performed using the SPSS software version 12.0 (SPSS Inc., Chicago).

2.4 Testing the methods in mixtures and commercial formulations

Methods 1 and 2 were applied to two different commercial formulations: *CP1*, which consists of a mixture of biostimulants from vegetal origin containing free amino acids and no trace of humic matter, and *CP2*, which is a liquid fertilizer with 15 % of humic extract.

These methods were also applied to two artificial mixtures of humic and non-humic substances: *solution A*, containing 18 g of HS3 and 3 g of apHS5; *solution B*, with 11.5 g of HS3 and 8 g of apHS5; and *solution C*, with 4.5 g HS3 and 12 g apHS5. These solutions were prepared by dissolving the products in water plus 3 g of KOH and brought to a final volume of 100 mL.

Table 2 Humic and fulvic acid contents (*w*/*w* %) in the studied samples, as determined by organic carbon oxidation (method 1) and the isolation/purification method (method 2)

Sample	Method 1		Method 2	
	% HA	% FA	% HA	% FA
AHA	56.7	12.1	72.8	5.7
HS1	40.1	19.7	89.0	4.9
HS2	53.8	2.8	62.7	0.0
HS3	61.4	1.2	77.5	0.6
HS4	12.0	6.4	5.4	3.6
HS5	63.8	11.8	87.1	4.3
apHS1	0.0	58.0	0.0	16.8
apHS2	0.0	73.7	0.0	27.7
apHS3	0.0	59.2	0.0	20.5
apHS4	0.0	79.7	0.0	23.7
apHS5	0.0	70.2	0.0	22.9
apHS6	0.0	82.0	0.0	31.8
apHS7	29.0	37.8	64.0	17.7
apHS8	0.0	49.0	0.0	37.9
apHS9	0.0	41.0	0.0	15.8
apHS10	16.5	35.0	65.4	6.3

3 Results and discussion

3.1 Comparison between methods 1 and 2 when applied to HS and apHS systems

Values of percentages of humic and fulvic content in the different organic materials studied are reported in Table 2. In all the cases, the fulvic content is overestimated by the oxidation procedure, compared to the results from method 2. These data imply that in method 1, all the organic molecules that are soluble at both acidic and alkaline pH are taken into account as a part of the fulvic fractions. Method 2 is more selective, especially for fulvic fractions. The DAX-8 resin binds the protonated form of the fulvic molecules, whereas inorganic salts and other organic macromolecules or small organic compounds pass through (Stevenson 1994). For this reason, the fulvic contents obtained while performing method 2 are considerably lower than the corresponding values obtained with method 1. Nevertheless, in the case of the apHS samples, both methods yield high amounts of apparent humic or fulvic fractions, making necessary a more detailed characterization of these fractions in order to ascertain if their properties are similar or not to those of naturally formed HS (only the fractions that represented more than 5 % of the total sample were analyzed and characterized).

3.2 Characterization of the fractions isolated by method 2

Tables 3 and 4 gather the results obtained in the studies by elemental analysis and UV-visible, fluorescence, and FTIR spectroscopies (further information derived from these techniques—spectra, interpretation—is available as Electronic Supplementary Material). These indexes tend to reflect the humification degree of non-composted, composted, and sedimentary humic substances (Fuentes et al. 2006, 2007); however, the ranges of the values for each considered group (HA, FA, apHA, and apFA) overlap in most cases (Tables 3 and 4). For this reason, it is necessary to resort to multivariate

Table 3 Elemental composition, atomic ratios, and ratios calculated from the FTIR spectra of the humic and fulvic fractions extracted by method 2

Sample	Elemental analysis				Atomic ratios			Ratios from FTIR spectra				
	% C	% H	% N	% O	O/C	C/H	N/C	1715/1620[a]	1620/2940[a]	1620/2850[a]	1515/1715[a]	1040/1400[a]
LSHA	62.2	3.6	1.2	30.5	0.370	1.440	0.0193	0.627	1.913	2.355	0.321	0.175
PRHA	55.7	3.8	3.6	36.9	0.500	1.220	0.0646	0.984	1.410	1.641	0.515	0.389
SRFA	52.5	4.3	0.7	43.5	0.620	1.020	0.0133	1.684	1.142	1.504	0.164	0.596
WRFA	53.5	4.2	1.1	41.7	0.580	1.060	0.0206	1.183	1.344	1.726	0.378	0.46
ESFA	48.5	4.2	3.2	43.2	0.668	0.972	0.0559	1.616	1.338	1.781	0.266	0.551
PSFA	50.8	3.5	2.3	42.9	0.633	1.211	0.0391	1.387	1.589	1.935	0.239	0.396
NRFA	52.1	4.0	0.7	44.9	0.647	1.095	0.0111	1.342	1.616	2.147	0.180	0.489
AHA	53.4	3.7	0.7	42.2	0.590	1.200	0.0131	0.735	1.409	1.858	0.670	0.362
HA1	55.6	1.9	1.1	41.5	0.559	2.460	0.0191	0.778	1.156	1.282	0.878	0.429
HA2	59.6	1.6	1.4	37.4	0.471	3.042	0.0227	0.501	1.469	1.602	0.672	0.297
HA3	59.4	1.7	0.8	38.0	0.479	2.901	0.0143	0.791	1.361	1.518	1.137	0.331
HA4	57.9	3.1	2.7	36.3	0.469	1.555	0.0469	0.855	1.061	1.235	0.378	0.623
HA5	54.0	2.5	1.5	42.0	0.584	1.793	0.0278	0.985	1.409	1.645	0.581	0.267
apHA7	48.1	3.0	0.6	48.3	0.753	1.357	0.0115	0.497	1.385	1.969	0.732	1.109
apHA10	54.0	5.0	2.0	39.0	0.541	0.898	0.0371	0.246	3.942	6.852	0.370	0.367
apFA1	50.2	4.9	2.0	43.0	0.642	0.862	0.0399	0.668	1.224	1.847	1.470	1.503
apFA2	58.3	4.4	0.0	37.4	0.480	1.099	0.0000	0.493	1.280	1.933	1.342	1.582
apFA3	62.0	4.2	2.5	31.3	0.378	1.230	0.0400	1.676	0.983	1.336	1.384	0.811
apFA4	62.0	4.2	1.9	31.9	0.386	1.217	0.0311	0.756	0.889	1.280	1.991	1.957
apFA5	52.5	4.8	0.0	42.8	0.611	0.914	0.0000	0.945	0.880	1.286	1.924	2.143
apFA6	52.4	5.1	0.0	42.5	0.608	0.862	0.0000	0.856	0.859	1.167	1.612	1.621
apFA7	57.5	4.2	0.7	37.6	0.491	1.145	0.0128	1.304	1.023	1.494	1.231	1.616
apFA8	51.2	5.2	7.4	36.2	0.530	0.822	0.1454	1.161	1.277	1.747	0.397	0.501
apFA9	56.0	6.2	11.2	26.6	0.357	0.759	0.1997	0.570	2.283	2.943	0.295	0.217
apFA10	45.2	2.5	2.4	49.8	0.827	1.479	0.0534	1.296	1.164	1.594	0.722	0.615

[a] Ratios of the absorbance values at the frequencies indicated (cm^{-1}) in the infrared spectra

Table 4 UV-visible and fluorescence parameters of the humic and fulvic fractions extracted by method 2

Samples	UV-visible				Fluorescence	
	ε_{600} [a]	ε_{280} [b]	E_{ET}/E_{Bz} [c]	E_4/E_6 [d]	A_4/A_1 [e]	I_{360}/I_{400} [f]
LSHA	80	1402	0.74	4.9	0.145	0.85
PRHA	58	834	0.69	5.1	0.374	0.74
SRFA	5	449	0.52	17.7	0.059	0.97
WRFA	16	500	0.54	9.9	0.062	0.72
ESFA	9	479	0.67	13.1	0.043	1.47
PSFA	17	610	0.74	10.6	0.060	1.02
NRFA	10	633	0.67	15.5	0.001	0.69
AHA	51	777	0.77	5.9	0.475	0.94
HA1	72	979	0.83	4.8	0.317	0.70
HA2	92	869	0.86	4.2	0.118	0.84
HA3	52	947	0.86	7.1	0.173	0.87
HA4	34	626	0.72	5.6	0.085	0.83
HA5	68	1008	0.82	6.1	0.158	0.87
apHA7	21	524	0.49	5.4	0.001	2.60
apHA10	31	454	0.70	4.6	0.296	1.54
apFA1	11	357	0.41	7.1	0.011	2.73
apFA2	7	300	0.26	6.0	0.015	4.54
apFA3	7	336	0.46	11.1	0.035	1.27
apFA4	3	338	0.33	13.4	0.010	3.44
apFA5	5	329	0.30	6.4	0.006	5.23
apFA6	9	410	0.36	8.1	0.008	2.50
apFA7	8	416	0.54	8.7	0.087	2.21
apFA8	8	320	0.53	10.2	0.029	1.50
apFA9	4	78	0.28	8.9	0.014	1.66
apFA10	12	300	0.70	6.0	0.005	2.47

[a] Molar absorptivity at 600 nm ($L \cdot cm^{-1} \cdot mol$ of organic $carbon^{-1}$)

[b] Molar absorptivity at 280 nm ($L \cdot cm^{-1} \cdot mol$ of organic $carbon^{-1}$)

[c] Ratio of absorbances at 253 and 220 nm in the UV spectrum

[d] Ratio of absorbances at 465 and 665 nm in the visible spectrum

[e] Ratio of areas of the last (570–641 nm) and first quarters (356–432 nm) of fluorescence emission spectra with excitation at 240 nm

[f] Ratio of intensities at 350 and 480 nm in fluorescence synchronous-scan excitation spectra

statistical analysis in an attempt to extract further information that could be useful to discriminate among groups.

3.3 Discriminant analysis

Before performing the DA, it is necessary to identify those analytical indexes (variables) that are strongly correlated, inasmuch as those variables distort the DA. To this end, those variables with significant correlations and/or correlated with a high number of other variables ($p < 0.01$) have been discarded for their use in the DA.

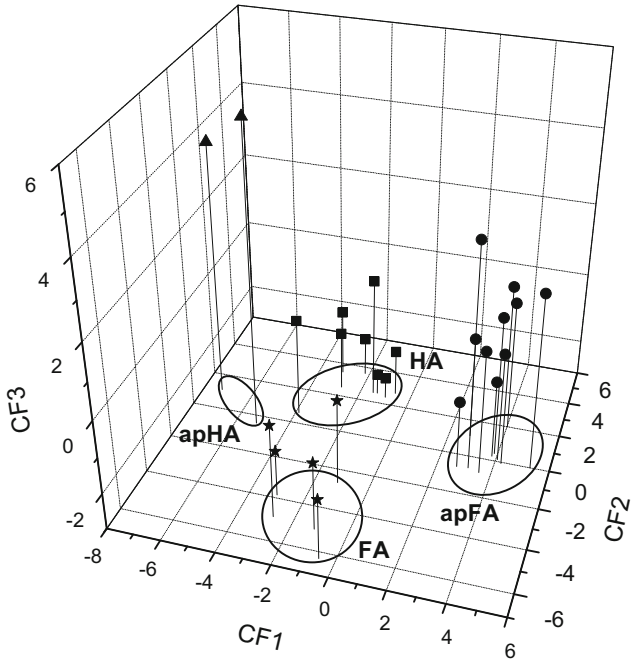

Fig. 1 3D Scatter plot of the canonical discriminant functions for all the samples included in the discriminant analysis

After such correlation analysis, the variables that have been chosen to conduct the DA are: %C, %H, %N, O/C, C/H, N/C (from elemental analysis); A_4/A_1, I_{360}/I_{400} (from fluorescence spectra); E_{ET}/E_{Bz} and E_4/E_6 (obtained by UV-visible spectroscopy); and 1720/1620 and 1040/1400 (from FTIR spectra). Each isolated and characterized fractions (HAs, FAs, apHAs, and apFAs) have been introduced as cases for the DA, taking as the grouping variable the group to which each sample belongs.

Three canonical discriminant functions resulted from the DA (Fig. 1 and Table S1, Electronic Supplementary Material), explaining 100 % of the total variance. Such model is able to discriminate the different groups of samples, what was not feasible in the attempt to evaluate each of the variables (elemental analysis data and spectroscopic indexes) separately. The DA also provides a set of four classification functions (Table S2, Electronic Supplementary Material), with a 96 % of cross-validated grouped cases correctly classified (in cross validation, each case is classified by the functions derived from all cases other than the case).

Table 5 Humic and fulvic acid contents (*w*/*w* %) in the solutions *A*, *B*, and *C* and samples CP1 and CP2, as determined by organic carbon oxidation (method 1) and the isolation/purification method (method 2)

Samples		Sol. *A*	Sol. *B*	Sol. *C*	CP1	CP2
Method 1	% HA	12.1	8.2	3.7	0.0	9.4
	% FA	1.2	4.5	7.6	21	6.1
Method 2	% HA	12	8.3	3.2	1.0	13.2
	% FA	0.5	0.9	3.3	1.3	5.5

3.4 Testing the model

In the wake of these results, it seems advisable to combine method 2 (based on that of the IHSS and Swift 1996) and the subsequent characterization of the fractions and the above-constructed DA, as a tool for evaluating the humic quality of the samples that we are examining. To test the suitability of this methodology, first, we quantified and assessed the quality of several simple and artificial mixtures of humic and non-humic materials following method 1 and method 2 + DA. Solutions *A*, *B*, and *C* were prepared to theoretically contain 12, 8, and 3 % of HA and 3, 8, and 12 % of apFA, respectively. The reason for preparing three different compositions was to take into account the possible interaction between humic and non-humic fractions (with a possible transference of molecules from one fraction to another) and to assess the potential of each method. The quantified content in humic acids was similar in both methods (Table 5), and method 2 was more selective for the fulvic acid fraction. We characterized the "humic" and "fulvic" fractions from solutions *A*, *B*, and *C* obtained by method 2 by elemental analysis and FTIR, UV-visible, and fluorescence spectroscopies, calculating the indexes previously selected to build the DA (Table 6). Afterward, we calculated the scores for each of the six fractions using the classification functions (Table S3, Electronic Supplementary Material). These scores correctly classify the three humic fractions into the group of sedimentary humic acids (HAs), and the isolated "fulvic" fractions are classified into the apparent fulvic acids (apFAs) group (Table S3, Electronic Supplementary Material).

Table 6 Elemental composition, atomic ratios, and ratios calculated from FTIR spectra and UV-visible and fluorescence parameters of the fractions extracted from solutions *A*, *B*, and *C* and CP2

Fraction	Solution *A*		Solution *B*		Solution *C*		CP2	
	Humic	Fulvic	Humic	Fulvic	Humic	Fulvic	Humic	Fulvic
Elemental analysis								
% C	60.6	50.4	60.2	53.0	60.5	52.2	45.6	50.4
% H	4.09	5.83	4.45	5.97	4.29	5.42	5.74	5.83
% N	1.33	1.35	1.01	1.04	1.02	0.23	13.95	1.35
% O	34.0	42.4	34.3	40.0	34.2	42.1	34.7	42.4
O/C	0.420	0.631	0.428	0.565	0.424	0.604	0.571	0.631
C/H	1.236	0.720	1.127	0.740	1.174	0.803	0.662	0.720
N/C	0.019	0.023	0.014	0.017	0.014	0.004	0.262	0.023
FTIR ratios								
1715/1620[a]	0.999	1.344	0.974	1.609	0.996	0.524	0.447	1.128
1620/2940[a]	1.175	1.423	1.190	0.893	1.123	1.746	2.784	1.635
1620/2850[a]	1.365	2.037	1.429	1.286	1.346	2.812	3.545	2.191
1515/1715[a]	0.452	0.379	0.472	0.540	0.519	1.471	0.725	0.462
1040/1400[a]	0.542	1.013	0.725	2.045	0.961	1.598	0.448	0.477
UV-visible parameters								
ε_{600}[b]	43	2	40	4	38	5	32	3
ε_{280}[c]	746	360	670	356	648	346	532	164
E_{ET}/E_{Bz}[d]	0.81	0.48	0.75	0.41	0.7	0.36	0.74	0.52
E_4/E_6[e]	6.5	10.7	6.8	7.3	6.5	9.5	5.5	8.6
Fluorescence parameters								
A_4/A_1[f]	0.010	0.014	0.023	0.028	0.025	0.037	0.039	0.054
I_{360}/I_{400}[g]	0.97	1.85	1.02	2.09	1.06	2.28	1.07	0.82

[a] Ratios of the absorbance values at the indicated frequencies (cm^{-1}) in the infrared spectra

[b] Molar absorptivity at 600 nm ($L \cdot cm^{-1} \cdot mol$ of organic $carbon^{-1}$)

[c] Molar absorptivity at 280 nm (L·cm-1·mol of organic carbon-1)

[d] Ratio of absorbances at 253 and 220 nm in the UV spectrum

[e] Ratio of absorbances at 465 and 665 nm in the visible spectrum

[f] Ratio of areas of last quarter (570-641 nm) and first quarter (356-432 nm) of fluorescence emission spectra with excitation at 240 nm

[g] Ratio of intensities at 360 and 480 nm in fluorescence synchronous-scan excitation spectra

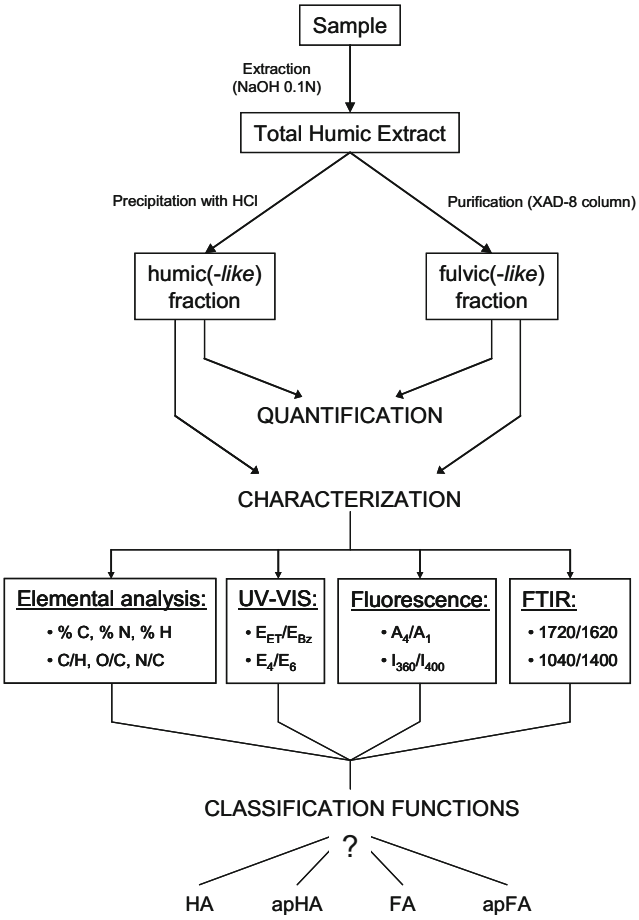

Fig. 2 Workflow of the proposed methodology

Regarding the analysis of commercial products by method 1 and method 2 + DA, we have considered CP1, containing 50 % of organic matter (amino acids and other resides from vegetal origin, but not humic substances), and CP2, containing 10 % of humic acids and 5 % of fulvic acids, as stated in the labels. These formulations have been selected to prove the robustness of the methodology taking into account the possible interferences caused by the matrices of the commercial products, which often consist of complex mixtures of mineral components as well as organic substances of different kinds (including humic substances and other organic compounds).

Theoretically, as the label of CP1 does not claim to contain humic substances, the application of these methodologies would not be necessary. Nevertheless, we wanted to test both methodologies with an organic non-humic commercial product. Method 1 quantifies 21 % of the organic matter contained in CP1 as fulvic acids, whereas method 2 yields negligible percentages (Table 5). On the other hand, the results of the analysis of CP2 by the two methods were comparable (Table 5). In order to assess the humic quality of the fractions extracted from CP1 and CP2 by method 2, they were characterized by elemental analysis and by the three spectroscopic techniques (Table 6); the values of the different variables included in the DA were calculated; and they were introduced in the classification functions to obtain the corresponding scores. According to these scores, the extracted humic fraction is classified into the true humic acids group (HA), whereas the fulvic-like extracted fraction is classified into the apFA group (Table S3, Electronic Supplementary Material).

4 Conclusions

In view of the results presented in this paper, we propose as an effective method for the quantification of the content in humic substances in commercial products, very similar to that proposed by Lamar et al. (2014), and for the assessment of the humic or fulvic quality of those quantified fractions, the combination of the procedure described in method 2—or the procedure described in Lamar et al. (2014)—with the characterization of the extracted fractions (by means of elemental analysis and UV-visible, fluorescence, and infrared spectroscopies), subsequently determining the predicted group membership using the classification functions provided by the DA. The workflow of this new methodology is represented in Fig. 2. This methodology has proven to successfully assess the nature (humic or humic-like) of the extracted fractions, and therefore, it implies an advance in the procedures used to evaluate the quality of the humic fraction labeled in commercial fertilizer products.

During the course of these studies, Lamar et al. (2014) developed a standardized gravimetric analysis of the humic content in commercial products. The principle is very similar to the methodology described here as method 2 (both are based on that described by Swift 1996), although their study was more exhaustive in order to provide a standardized methodology of quantification. Therefore, for the quantification of humic substances, we recommend following their methodology. Nevertheless, in this paper, we have demonstrated the need and profits of an additional characterization of the quantified fractions, which allows us to truly distinguish humic substances from fraudulent materials potentially added to commercial products (seaweed extracts, proteinaceous derivatives, lignosulfonates, etc.).

Notwithstanding the fact that industrial and analytical laboratories would tend to reject a multi-method that implies the use of different techniques, we strongly recommend the implementation of these procedures, as thus far, this has been the only methodology developed not only to quantify but also to identify the humic character of the organic matter contained in commercial products (and, therefore, to identify potential frauds). Besides, the fact that the variables used to build the DA are not based on absolute equipment-dependent magnitudes favors interlaboratory analyses. These measurements may take up more time, but they are quite "cheap," in the sense that

they do not require expensive chemicals or lab ware. It is advisable for labs applying this methodology to construct their own DA with standards and reference materials and target non-humic samples of their interest. These data could be exchangeable for gradually strengthening the DA, as long as the extraction (and quantification) of the humic and fulvic fraction procedures applied are the same.

References

Baigorri R, Fuentes M, Gonzalez-Gaitano G, García-Mina JM (2007a) Analysis of molecular aggregation in humic substances in solution. Colloids Surf A Physicochem Eng Asp 302:301–306

Baigorri R, Fuentes M, Gonzalez-Gaitano G, García-Mina JM (2007b) Simultaneous presence of diverse molecular patterns in humic substances in solution. J Phys Chem B 111:10577–10582

Boletin Oficial del Estado (Spanish State Official Bulletin) (1991) Real Decreto 1110/1991, de 12 de julio, por el que se aprueban los métodos oficiales de análisis de productos orgánicos fertilizantes, (http://www.boe.es/aeboe/consultas/bases_datos/doc.php?id=BOE-A-1991-18408, last accessed: May 2016) pp 23725–23730

Cabaniss SE (1992) Synchronous fluorescence spectra of metal-fulvic acid complexes. Environ Sci Technol 26:1133–1139

Canellas LP, Olivares FL, Aguiar NO, Davey L, Jones DL, Nebbioso A, Mazzei P, Piccolo A (2015) Humic and fulvic acids as biostimulants in horticulture. Sci Hortic 196:15–27

Cavani L, Ciavatta C, Gessa C (2003) Identification of organic matter from peat, leonardite and lignite fertilisers using humification parameters and electrofocusing. Bioresour Technol 86: 45–52

Chen Y, Senesi N, Schnitzer M (1977) Information provided on humic substances by E4/E6 ratios. Soil Sci Soc Am J 41:352–358

Chin YP, Aiken G, O'Loughlin E (1994) Molecular weight, polydispersity, and spectroscopic properties of aquatic humic substances. Environ Sci Technol 28:1853–1858

de Liñán Carral C, de Liñán Vicente C (2016) Vademecum de productos fitosanitarios y nutricionales. Ediciones Agrotécnicas, Madrid

Donisa C, Mocanu R, Steinnes E (2003) Distribution of some major and minor elements between fulvic and humic acid fractions in natural soils. Geoderma 111:75–84

Francioso O, Ciavatta C, Montecchio D, Tugnoli V, Sanchez-Cortes S, Gessa C (2003) Quantitative estimation of peat, brown coal and lignite humic acids humic chemical parameters, ^{1}H-NMR and DTA analyses. Bioresour Technol 88:189–195

Fuentes M, González-Gaitano G, García-Mina JM (2006) The usefulness of UV-visible and fluorescence spectroscopies to study the chemical nature of humic substances from soils and composts. Org Geochem 37:1949–1959

Fuentes M, Baigorri R, González-Gaitano G, García-Mina JM (2007) The complementary use of 1H NMR, 13C NMR, FTIR and size exclusion chromatography to investigate the principal structural changes associated with composting of organic materials with diverse origin. Org Geochem 38:2012–2023

Fuentes M, Baigorri R, Gonzalez-Vila F, González-Gaitano G, García-Mina JM (2010) Pyrolysis–gas chromatography/mass spectrometry identification of distinctive structures providing humic character to organic materials. J Environ Qual 39:1–12

Hayes MHB, Graham CL (2000) Procedures for the isolation and fractionation of humic substances. In: Ghabbour EA, Davies G (eds) Humic substances: versatile components of plants, soil and water. The Royal Society of Chemistry, Cambridge, pp. 91–109

Hayes MHB, MacCarthy P, Swift RS (1989) The search for structure: setting the scene. In: Hayes MHB, MacCarthy P, Swift RS (eds) Humic substances II: in search of structure. Wiley, Chichester, pp. 3–31

Jones MN, Bryan ND (1998) Colloidal properties of humic substances. Adv Colloid Interf Sci 78:1–48

Kalbitz K, Geyer W, Geyer S (1999) Spectroscopic properties of dissolved humic substances - a reflection of land use history in a fen area. Biogeochemistry 47:219–238

Korshin GV, Li CW, Benjamin MM (1997) Monitoring the properties of natural organic matter through UV spectroscopy: a consistent theory. Water Res 31:1787–1795

Kumada K (1987) Chemistry of soil organic matter. Elsevier, Tokyo

Lamar RT, Talbot KH (2009) Critical comparison of humic acid test methods. Comm Soil Sci Plan 40:2309–2322

Lamar RT, Olk DC, Mayhew L, Bloom PR (2014) A new standardized method for quantification of humic and fulvic acids in humic ores and commercial products. J AOAC Int 97: 721–730

McGarigal K, Cushman S, Stafford S (2000) Multivariate statistics for wildlife and ecology research. Springer, New York

Milori DMBP, Martin-Neto L, Bayer C, Mielniczuk J, Bagnato VS (2002) Humification degree of soil humic acids determined by fluorescence spectroscopy. Soil Sci 167:739–749

Mora V, Bacaicoa E, Zamarreño AM, Aguirre E, Garnica M, Fuentes M, García-Mina JM (2010) Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. J Plant Physiol 167: 633–642

Nardi S, Pizzeghello D, Muscolo A, Vianello A (2002) Physiological effects of humic substances on higher plants. Soil Biol Biochem 34:1527–1536

Nelson DW, Sommers LE (1982) Total carbon, organic carbon, and organic matter. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis part 2—chemical and microbiological properties, Soil Science Society of America Book Series. Soil Science Society of America, Madison, pp. 539–579

Peuravuori J, Pihlaja K (1997) Molecular size distribution and spectroscopic properties of aquatic humic substances. Anal Chim Acta 337: 133–149

Piccolo A (2002) The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. Adv Agron 75:57–134

Quentel F, Filella M (2011) A simple method for quantifying the humic content of commercial products. Anal Bioanal Chem 401:3235–3238

Sequi P, De Nobili M, Cercignani G (1986) A new index of humification. Agrochimica 30:175–179

Stevenson FJ (1985) Geochemistry of soil humic acids. In: Aiken GR, McKnight DM, Wershaw RL, MacCarthy P (eds) Humic substances in soil, sediments and water. Wiley-Interscience, New York, pp. 13–52

Stevenson FJ (1994) Humus chemistry: genesis, composition, reactions. Wiley, New York

Struyk Z, Sposito G (2001) Redox properties of standard humic acids. Geoderma 102:329–346

Swift RS (1996) Organic matter characterization. In: Sparks DL (ed) Methods of soil analysis. Part 3—chemical methods, Soil Science Society of America book series. Soil Science Society of America, Madison, pp. 1011–1070

Tipping E (2002) Cation binding by humic substances. Cambridge University Press, Cambridge

Vaughan D, Ord BG (1985) Soil organic matter—a perspective on its nature, extraction, turnover and role in soil fertility. In: Vaughan D, Malcom RE (eds) Developments in plant and soil sciences. Martinus Nijhoff/Dr W. Junk, Dordrecht, pp. 1–35

Van Zomeren A, Comans RNJ (2007) Measurement of humic and fulvic acid concentrations and dissolution properties by a rapid batch procedure. Environ Sci Technol 41:6755–67610

Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci 37:29–37

Zsolnay A, Baigar E, Jimenez M, Steinweg B, Saccomandi F (1999) Differentiating with fluorescence spectroscopy the sources of dissolved organic matter in soils subjected to drying. Chemosphere 38: 45–50