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Corresponding Author:	Richard Lamar Bio Huma Netics, Inc. Gilbert, Arizona UNITED STATES
Corresponding Author's Institution:	Bio Huma Netics, Inc.
Corresponding Author E-Mail:	richlamar22@gmail.com
Order of Authors:	Richard Lamar
	Hiarhi Monda
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TITLE:

Quantification of Humic and Fulvic Acids in Humate Ores, DOC, Humified Materials and Humic Substance-Containing Commercial Products

AUTHORS AND AFFILIATIONS:

Richard T. Lamar, Hiarhi Monda

Bio Huma Netics, Inc., Gilbert, AZ. USA

rich@bhn.us

hiarhi@bhn.us

CORRESPONDING AUTHOR:

Richard T Lamar (rich@bhn.us)

KEYWORDS:

humic substances, humic acid, fulvic acid, quantification method, ash-free basis, gravimetric method

SUMMARY:

This method provides a gravimetric quantification of humic substances (e.g., humic and fulvic acids) on an ash-free basis, in dry and liquid materials from soft coals (i.e., oxidized and non-oxidized lignite and sub-bituminous coal), humate ores and shales, peats, composts and commercial fertilizers and soil amendments.

ABSTRACT:

The purpose of this method is to provide an accurate and precise concentration of humic (HA) and/or fulvic acids (FA) in soft coals, humic ores and shales, peats, composts and humic substance-containing commercial products. The method is based on the alkaline extraction of test materials, using 0.1 M NaOH as an extractant, and separation of the alkaline soluble humic substances (HS) from nonsoluble products by centrifugation. The pH of the centrifuged alkaline extract is then adjusted to pH 1 with conc. HCl, which results in precipitation of the HA. The precipitated HA are separated from the fulvic fraction (FF) (the fraction of HS that remains in solution,) by centrifugation. The HA is then oven or freeze dried and the ash content of the dried HA determined. The weight of the pure (i.e., ash-free) HA is then divided by the weight of the sample and the resulting fraction multiplied by 100 to determine the % HA in the sample. To determine the FA content, the FF is loaded onto a hydrophobic DAX-8 resin, which adsorbs the FA fraction also referred to as the hydrophobic fulvic acid (HFA). The remaining non-fulvic acid fraction, also called the hydrophilic fulvic fraction (HyFF) is then removed by washing the resin with deionized H_2O until all nonabsorbed material is completely removed. The FA is then desorbed with 0.1 N NaOH. The resulting Na-fulvate is then protonated by passing it over a strong H^+-exchange resin. The resulting FA is oven or freeze dried, the ash content determined and the concentration in the sample calculated as described above for HA.

INTRODUCTION:

Humic substances (HS) are dynamic residues that result from the microbial decomposition and transformation of dead plant tissues[1,2,3] augmented with microbial by-products and biomass[3,4,5] through a process that is termed humification[6]. HS are present in soils, natural waters, lake sediments, peats, soft coals and humic shales and represent an estimated 25% of total organic carbon on the earth[7]. These substances are complex mixtures of thousands of unique molecules that are fractionated into three main fractions based on their different solubilities in strongly basic and acid aqueous solutions. These fractions are humic acids (HAs), which comprise the alkali-soluble but acid-insoluble fraction; fulvic acids (FAs), the fraction soluble in both alkali and acid; and the humin fraction, which is insoluble at all pH values[6,8]. The fulvic fraction (FF) is further subdivided into the hydrophobic FA (HFA) and hydrophilic (HyFA) fractions. These fractions are defined as the part of the FF that binds to a hydrophobic DAX-8 resin (HFA) and the part that does not bind to the resin (HyFA).

HS are increasingly being used in agriculture, where they are widely used as crop biostimulants, in animal husbandry, in particular as a livestock feed additive, in mining in drilling muds, and environmental remediation as electron shuttles. Research in the use of HS in human medical applications is also increasing.

Many methods for the quantitation of HA and FA exists. However, most of these methods are neither accurate nor precise. For example, the two most widely used methods for the determination of HA in the USA are the colorimetric method[9] and the California Department of Food and Agriculture (CDFA) method, both of which were shown to overestimate the amount of HA in a range of ore and extract sources from the western US and Canada[10].The colorimetric or spectrophotometric method is inaccurate because it relies on the absorbance of alkaline extracts that include, in addition to HA, FA and other chromophores that all absorb at the wavelength used and the standard is not representative of the materials being tested[10].The CDFA method is not accurate because it does not provide HA concentrations on an ash-free basis. Because different ores have different amounts of ash, some of which is carried with the extraction and the extraction process itself adds ash, this method does not provide an accurate value for HA concentrations[10]. In response to the need for an accurate and precise method, a standardized gravimetric procedure based on the one detailed by[11] was published in 2014 to address quantitation of both HA and FA on an ash free basis[12]. This method was then adapted, with minor modifications, by the International Organization for Standardization (ISO) in 2018 under Fertilizers and soil conditioners as “Determination of humic and hydrophobic fulvic acids concentrations in fertilizer materials”[13].

This paper outlines the protocol for extraction and quantitation of humic and hydrophobic fulvic acids and gives details on the accuracy and precision of the data produced from the method.

PROTOCOL:

1. Solid sample preparation

1.1. Crush approximately 5 g of the sample to be analyzed, using a mortar and pestle, so that 100% of the crushed sample passes through a U. S. Standard Sieve mesh size No. 200 (i.e., 74 µm) making sure that the powder is well mixed.

1.2. Determine the moisture content of the powder gravimetrically.

1.2.1. Weigh an aluminum weigh boat and record the mass (W_{wb}).

1.2.2. Transfer approximately 2 g of sample powder into the weigh boat and record the mass (W_{ws+wb}).

1.2.3. Place the weigh boat in a drying oven for 24 h at 102 °C (do not exceed 102 °C). After 24 h, remove the weigh boat from drying oven and place in a desiccator to cool for at least 1 h.

1.2.4. Weigh and record the mass of the weigh boat and dried sample powder (W_{ds+wb}).

1.2.5. Determine the moisture content using formula 1.1.

Formula 1.1 Moisture content of solid sample powder

$$\% \text{ moisture} = (((W_{ws+wb} - W_{wb}) - (W_{ds+wb} - W_{wb}))/ (W_{ws+wb} - W_{wb})) * 100$$

2. Extraction procedure

2.1. Solid samples

2.1.1. Weigh approximately 2.5 g of the remaining <200 mesh sample powder (W_{samp}) into a plastic or aluminum weigh boat and record the weight to four decimal places.

2.1.2. Load the sample into a 1 L graduated cylinder and fill the cylinder to 1 L with 0.1 M NaOH (4 g NaOH x L^{-1}).

2.1.3. Add a magnetic stir bar (e.g., 5 - 7 cm in length) and stir rapidly (i.e., 300 – 400 rpm) on a stir plate until the sample is thoroughly mixed.

2.1.4. Transfer the entire contents of the graduated cylinder into a 1 L Erlenmeyer flask, evacuate the headspace of the flask with N_2 gas and cover the flask opening with an airtight cover.

2.1.5. Place the Erlenmeyer flask on a stir plate and mix at 300 - 400 rpm for 16 - 18 h.

2.2. Liquid samples

2.2.1. For liquid materials, thoroughly mix the sample by shaking to ensure that the test liquid is mixed homogeneously. Make sure any residue that may have fallen to the bottom of the container is thoroughly mixed.

2.2.2. Add approximately 5 g of the test liquid, weighed to 4 decimal places (W_{TL}), to a 1 L graduated cylinder.

2.2.3. Fill the graduated cylinder with 0.1 M NaOH to a final volume of 1 L.

2.2.4. Add a magnetic stir bar (e.g., 5 – 7 cm in length) and stir rapidly (e.g., 300 - 400 rpm) on a stir plate until the test sample is completely mixed.

2.2.5. Transfer the mixture into a 1 L Erlenmeyer flask, evacuate the headspace with N_2 gas and cover the flask opening with an airtight cover.

2.2.6. Place the Erlenmeyer flask on a stir plate and mix at 300 - 400 rpm for 1 h.

NOTE: After this point, the handling of solid and liquid samples is the same.

3. Removal of nonsoluble materials from alkaline extracts

3.1. At the completion of stirring, remove the flask from the stir plate, transfer the mixture to suitable centrifuge tubes and centrifuge the entire volume at 4,921 x *g* for 30 min.

3.2. Collect the alkaline supernatant containing the HA and FA in a clean 1 L Erlenmeyer flask containing a magnetic stir bar. Discard the insoluble material. Filtration through glass wool or qualitative 2.5 µm pore size filter paper is recommended if residual particles are not precipitated after centrifugation.

4. Precipitation and separation of HA from FF

4.1. While stirring the alkaline extract at 300 - 400 rpm on a stir plate, insert a pH probe into the middle portion of the solution (vertically) and add conc. HCl dropwise to the alkaline extract until a stable pH of pH 1.0 ± 0.1 is reached.

4.2. Once a pH of pH 1 is reached and remains stable, remove the pH probe from the flask, retrieve the stir bar, cover the flask with an airtight cover and let the flask sit until the precipitated HA has settled to the bottom of the flask.

NOTE: The time it takes a HA to precipitate and drop out of solution will vary depending on the source and amount of HA in the sample. It typically takes 1 -6 h for the HA to fully precipitate and drop out of solution.

4.3. Centrifuge the extract and precipitated HA at 4921 x *g* for 1 h. After centrifugation, pour off the supernatant FF into a clean 1 L Erlenmeyer and cover with an airtight cover.

NOTE: A longer centrifuge time may be necessary in order to pack the HA down firmly enough to allow decanting the FF without inclusion of any of the precipitated HA.

4.4. Place the centrifuge tubes in a drying oven held at 100 °C for 24 h.

4.5. After drying, remove the tubes from the drying oven and place in a desiccator to cool to room temperature. After cooling, quantitatively transfer the residue from the tube by scraping it from the sides and bottom of the tube with a spatula, transfer to a tared weigh boat, and record the mass (W_{EHA}). This residue is the "Extracted HA".

NOTE: If centrifuge tubes greater than 50 mL have been used in the separation of HA and FF, it is convenient to transfer the precipitated HA to temperature resistant 50 mL centrifuge tubes for the drying process. Also, if a freeze dryer is available the precipitated HA can be freeze dried. Collection of the HA in a freeze-dried state is easier because the HA does not stick to the side of the plastic tubes and does not have to be scrapped.

5. Determination of ash content

NOTE: The procedure for determination of ash content of dried HA and FA samples is the same. The procedure using notation for HA is shown.

5.1. Transfer approximately 30 mg of the dried HA (W_{HA}) to a clean, pre-weighed ceramic dish (W_{CD}) that had been previously dried in a drying oven at 100 °C and then cooled in a desiccator to room temperature. After recording the combined mass of the weighted HA and dish (W_{HA+CD}), combust the HA in a muffle oven for 2 h at 600°C.

NOTE: For each HA sample, three replicates should be processed and the average ash content used in the calculation of pure HA.

5.2. After 2 h, remove the dish and contents from the muffle oven and place in a desiccator to cool. Once cool, weigh the dish with ash (W_{ASH+CD}) and calculate the ash ratio (Ash_{rat}) using formula 1.2:

Formula 1.2 $Ash_{rat} = (W_{ASH+CD} - W_{CD}) / (W_{HA+CD} - W_{CD})$

6. Determination of the percentage of purified extracted HA

6.1. Determine the final mass of the pure HA (W_{PHA}) by correcting for ash content using Formula 1.3:

Formula 1.3 $W_{PHA} = W_{EHA} * (1- Ash_{rat})$

7. Determination of the concentration (%) of pure HA in the original source sample

7.1. Determine the concentration of pure HA using Formula 1.4 and 1.5:

Formula 1.4: % pure HA in solid sample = $(W_{PHA}/W_{samp}) * 100$

Formula 1.5: % pure HA in liquid sample = (W_{PHA}/W_{TL}) * 100

8. Column preparation for HFA purification

8.1. Prepare a low-pressure chromatography column packed with polymethylmethacrylate DAX-8 resin. If the resin has not been used previously, soak the resin in methanol for 2 h and then rinse thoroughly with deionized H_2O until all the methanol is removed.

8.1.1. Remove small resin particles that float on the water at this time. If the resin has been used previously, regenerate it as described in section 10.

8.2. Once thoroughly rinsed, pour the resin into a 5 x 25 cm glass chromatography column fitted with an end piece with a 10 µm frit for resin bed support. Leave 2.5 to 5 cm at top of column for resin-free solution to enable mixing of the FF prior to entering the resin bed. Fit the top piece to the column and pump deionized H_2O through the top of the column to pack the DAX-8 resin bed by using a peristaltic pump.

9. Isolation of HFA

9.1. Once the resin bed is packed, load the FF onto the column using a peristaltic pump, under low pressure, via the top of the column. Use a flow rate of 35 – 40 mL/min. It is critical that the top of the resin in the column remains covered with solution throughout the entire loading and rinsing procedure to prevent drying of the resin and channeling of the extract though the resin bed.

9.2. Once the fulvic fraction has been completely loaded onto the resin, wash the resin with deionized water, to remove the non-adsorbed “hydrophilic fulvic fraction” (HyFF) by pumping it through the top of the column using the peristaltic pump under low pressure. Use a flow rate of 35 – 40 mL/min. Discard the HyFF-containing effluent unless it will be used for analysis.

9.3. Wash the column with deionized H_2O until the absorbance at 350 nm of the column effluent is equal (e.g., within 0.015 absorbance units) to that of the deionized H_2O used to wash the column. Use deionized water to zero (i.e., blank) the spectrophotometer.

9.4. Desorb the HFA by back elution by pumping 0.1 M NaOH via the bottom of the column using the peristaltic pump. Use a flow rate of 35 – 40 mL/min. Capture the pump effluent (the Na-fulvate in a clean sufficiently sized container (e.g., 2 L Erlenmeyer).

NOTE: Most of the HFA adsorbs to the very top of the DAX-8 resin bed. Desorption by introducing the 0.1 M NaOH from the bottom of the column minimizes the amount of 0.1 M NaOH needed to fully desorb the FA.

9.5. All the HFA has been desorbed when the absorbance of the column effluent is equal to the absorbance of 0.1 M NaOH influent at 350 nm. Use 0.1 M NaOH as the spectrophotometric

blank. Add the effluent taken to check absorbance of the desorbed FA solution to ensure all FA is captured.

10. HFA de-ashing by protonation

10.1. Pass the Na-fulvate solution, repeatedly, by gravity feed, through strong cation H^+-exchange resin (**Table of Materials**) contained in a 5 × 50 cm column, with glass frit to retain the resin, until the electrical conductivity of the effluent is <120 µS/cm, as measured with an electrical conductivity meter. Prior to each pass, the H^+-exchange resin requires refurbishment as described in Section 11.

10.1.1. To ensure that all the FA is removed from the resin after the final pass, wash the resin with deionized water until the absorbance of effluent at 350 nm is the same (e.g., within 0.015 absorbance units) as the deionized water used to wash the column. Use deionized H_2O as the spectrophotometric blank. Add the wash and any effluent portions taken to check absorbance to the purified FA solution. To help with removal of all FA, the resin can be agitated (e.g., using a long glass or plastic rod) several times.

10.2. Concentrate the FA to a volume of approximately 15 ± 2 mL by using a rotary evaporator at 55 °C.

10.3. Completely transfer the 15 mL FA concentrate to a 50 mL plastic centrifuge tube and dry at 60±3 °C to constant dryness in a drying oven. Freeze-drying is an alternative to oven drying. After drying transfer the tube to a desiccator to cool.

10.3.1. Remove FA from the tube by scraping the tube sides and bottom with a spatula and weigh the collected FA on pre-tared weigh paper. This material is the "Extracted FA" (W_{EFA}).

10.3.2. Determine the ash ratio (ASH_{rat}) of extracted FA as described under Step 6 for HA and calculate the ash ratio using Formula 1.2. Determine the weight of the extracted FA without ash (W_{PFA}) using Formula 1.3, substituting the W_{EFA} for weight of W_{EHA}. Finally determine the % pure FA in the sample using Formula 1.4 substituting W_{PFA} for W_{PHA}.

11. DAX-8 resin regeneration

11.1. Regenerate the DAX-8 resin by pumping 0.1 M HCl (8.33 mL concentrated HCl/1000 mL final volume deionized H_2O) at a flow rate of 35 – 40 mL/min through the bottom of the column until the pH of the effluent is equal to the pH of the influent. Use the peristaltic pump to pump all reagents through the DAX-8 column during regeneration.

11.2. Rinse the column with DI water by pumping it into the top of the column until the pH of the effluent equals the pH of the influent (i.e., DI water).

12. H^+-cation exchange resin regeneration

12.1. Regenerate the H^+ cation exchange resin in a batch process by pouring the resin into a large beaker (e.g., 4 L plastic beaker), rinse several times by covering the resin with DI H_2O, mixing and then pouring off the water.

12.2. Cover the resin with 1 M HCl (83.3 mL concentrated HCl/1000 mL final volume DI water). Let stand for a minimum of 2 h with occasional stirring (e.g., once every 30 min).

12.3. Remove the excess acid from the resin by pouring off the acid and covering the resin with DI water. Stir vigorously with a stirring rod for 15 s, then let the resin drop to the bottom of the flask and then pour off the water. Repeat the process until the electrical conductivity of the rinse water is ≤ 0.7 µS/cm.

12.4. Load the regenerated resin back into the column. Cover with deionized H_2O to make sure the resin remains wet between uses.

REPRESENTATIVE RESULTS

Performance data for the method are provided in **Tables 1 – 5**. The precision of method for extraction of HA and FAH from liquid commercial samples with very different concentrations of HA and FHA are given in **Table 1**.

The RSDs for HA were lower than those for HFA, but the average HFA RSD over the three liquid samples was on 6.83% which indicates a high degree of precision. The Horwitz ratio (HorRat) is a normalized performance parameter that indicates the suitability of a methods of analysis regarding among laboratory precision. Here it was used for intra-laboratory precision. Value < 0.5 may indicate undisclosed averaging or a high level of experience with the method. Values > 2.0 indicate heterogeneity of test samples, a need for method optimization or more extensive training, operating below the limit of detection or an inapplicable method. For analysis of liquid samples, the HorRat was only > 2 for one of the HFA analyses (**Table 1**).

Precision data for the extraction of HA and HFA from three humic ore samples is given in **Table 2**. Again, with the exception of the HFA extracted from Ore 2 and the HA from Ore 3, all of the HorRat's were below 2. This demonstrates a high degree of precision of this method for extraction of HA and HFA for humic ore samples.

Manufacturers of plant biostimulants often formulate products that contain HS in addition to other ingredients like seaweed, inorganic fertilizers, coals or molasses. Table 3 gives the results of an analysis of the inclusion of these types of additives on the precision of the method. None of the additives effected the recovery of HA or HFA significantly (**Table 3**).

Table 4 and **Table 5** report the recoveries of HA and HFA, respectively, from liquid samples that simulated commercial products with very low concentrations. Recoveries were excellent and ranged between 88% and 97% for HA (**Table 4**) and 92% and 104% for HFA (**Table 5**). Mean recoveries for HA and HFA were 93% and 97%, respectively and % RSD for both HS were less than

5%. While precision is excellent, these data indicate the need to perform laboratory replicates. The method detection limit (MDL) and method quantitation limit were 4.62 and 1.47 mg/L for HA and 4.8 and 1.53 mg/L for HFA.

Table 1. Precision of the method in extraction and quantitation of HA and HFA from liquid commercial samples. Extraction conditions were 1 g in 1 L 0.1 M NaOH.

Table 2. Precision of the method in extraction and quantitation of HA and HFA from humic ores. Extraction conditions were 1 g sample in 1 L 0.1 M NaOH. (Data taken from Lamar et al., 2014)

Table 3. Effect of adulterants on quantitation of HA and HFA from a Gascoyne leonardite. (Data taken from Lamar et al., 2015)

Table 4. Recovery of HA from spiked blanks. (Data taken from Lamar et al., 2014)

Table 5. Recovery of HFA from spiked blanks. (Data taken from Lamar et al., 2014)

DISCUSSION:

The initial steps of extraction and isolation of the HA in this method are relatively straightforward. Because the isolation of the HFA involves column chromatography, obtaining repeatable results comes with strict adherence to the details of each step and practice. In particular, correct preparation of the resins is of primary importance. It is extremely important that the polymethylmethacrylate DAX-8 resin is prepared and packed properly. Correct packing of the resin affects both the yield and quality of the HFA. If channeling exists, then neither pretreatment (i.e. acidification) or adsorption of HFA will be complete, and the separation will lead to inaccurate results. If channels or spaces in the resin are observed prior to sample loading the column should be removed and shaken to redistribute the resin beads, by allowing them to settle without channels, and then re-packed by pumping clean DI H_2O through the resin. In addition, as mentioned in the protocol, maintaining a volume of liquid above the resin when loading the FF onto the resin, will allow the FF to mix prior to entering the resin and result in more effective adsorption. For the strong cation H^+-exchange resin (**Table of Materials**), complete regeneration cannot be rushed. The Na^+/H^+ exchange takes time and therefore this is best done in a bulk treatment so that the resin can be mixed while being re-acidified. Mixing the resin while rinsing with DI H2O will help removing the excess HCl. When rising the acidified resin to remove excess acid, mixing the resin help removing the HCl. It is extremely important to remove the acid to the point where an electrical conductivity of ≤ 0.7 µS/cm is reached. If not, the HCl will be carried over with the HFA.

Finally, when desorbing the HFA from the DAX-8 resin, once the absorbance of the influent equals the absorbance of the effluent, it is a good practice to let the column sit for a couple hours to see if any additional HFA will be released. If so, it will be seen as a yellowing of the liquid above the resin. If this occurs, the additional HFA can be removed by continued desorption until influent/effluent absorbances are equal again.

One of the disadvantages of the HFA isolation is that the entire process is time consuming. The complete desorption of HFA from the DAX-8 resin and complete removal from the H^+-exchange resin both result in a significant volume of HFA that has to be reduced by roto vaping. This is definitely a bottleneck in the analysis. In an effort to reduce this time, desorbing the HFA from the DAX-8 resin using acetone rather than 0.1 M NaOH has been suggested[14]. The authors claimed that by using 50% acetone as desorbent in place of NaOH, a similar HFA result was obtained and the DAX-8 was adequately regenerated and thus the H^+-exchange step could be eliminated. This modification resulted in a greatly reduced analysis time as a result of decreased volume produced and quicker roto vaping of acetone compared to water. This modification deservers further study.

This method is limited to the analysis of organic matter that has undergone the process of humification, and for the case of peat and soft coals, the further processes of peatification and both peatification and coalification, respectively. Humification is the process whereby dead, primarily plant material, is decomposed by a sequence of microbes that consume and modify increasingly recalcitrant substrates. Abiotic processes also participate in decomposition and re-synthesis reactions. Humification ultimately results in the production of relatively recalcitrant materials comprising heterogeneous mixtures of thousands of molecules that form a range of molecular weight and carbon, oxygen and hydrogen contents that form HS. HS are further modified by peatification and coalification. Therefore, this method is not appropriate for plants materials that have been modified by chemical processes. For example, lignosulfonate is widely used as an HFA adulterant. Lignosulfonate is a by-product of the sulfite pulping process. Therefore, this material has not been produced by the process of humification. In addition, there are many substances that bind to the DAX-8 resin. For example, DAX-8 resin has been used to adsorb pesticides from solution[15]. Obviously, pesticides are not HS. Thus, binding of a material to DAX-8 resin does not justify a claim that it is an HFA. The prerequisites are both production by humification and binding to DAX-8 resin.

As more is learned about the contribution of the various components of HS in different applications, it may become advantageous to further fractionate HS and thus modify the method accordingly. As it exists, the method does not quantify the HYFA. However, this fraction might also have activity e.g. in plant biostimulation, where the whole FF is generally applied in agricultural treatments rather than purified HFA.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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Table 1. Precision of the method in extraction and quantitation of HA and HFA from liquid commercial samples[a].

Humic substances, %						
Material	L16		L17		L2	
	HFA	HA	HFA	HA	HFA	HA
Rep 1	1.44	17.00	6.59	7.76	0.36	4.46
Rep 2	1.39	16.03	6.25	7.79	0.42	4.93
Rep 3	1.34	16.44	6.02	7.55	0.40	4.46
Rep 4	1.54	16.75	6.20	7.69	0.33	4.53
Mean	1.43	16.56	6.27	7.70	0.38	4.60
SD	0.09	0.42	0.24	0.11	0.04	0.23
RSD, %	6.29	2.53	3.80	1.39	10.4	4.91
Hor Rat(r)	1.58	0.72	1.25	0.47	2.31	1.55

[a]Extraction conditions were 1 g in 1 L 0.1 M NaOH.

Table 1

Humic substances, %						
Material	L16		L17		L2	
	HFA	HA	HFA	HA	HFA	HA
Rep 1	1.44	17	6.59	7.76	0.36	4.46
Rep 2	1.39	16.03	6.25	7.79	0.42	4.93
Rep 3	1.34	16.44	6.02	7.55	0.4	4.46
Rep 4	1.54	16.75	6.2	7.69	0.33	4.53
Mean	1.43	16.56	6.27	7.7	0.38	4.6
SD	0.09	0.42	0 24	0.11	0.04	0.23
RSD, %	6.29	2.53	3.8	1.39	10.4	4.91
Hor Rat(r)	1.58	0.72	1.25	0.47	2.31	1.55

[a]Extraction conditions were 1 g in 1 L 0.1 M NaOH.

Table 2

Humic substances, %						
	Ore 1		Ore 2		Ore 3	
Material	HFA	HA	HFA	HA	HFA	HA
Rep 1	1.75	67.4	1.31	27.01	1.55	8.95
Rep 2	1.69	67.63	1.25	27.48	1.41	7.2
Rep 3	1.63	67.1	1.27	27.34	1.47	8.35
Rep 4	1.77	67.59	1.55	26.89	1.51	7.98
Mean	1.71	67.53	1.35	27.18	1.49	8.12
SD	0.06	0.94	0.14	0.28	0.06	0.73
RSD, %	3.7	1.39	10.33	1.02	4.02	9.02
HorRat(r)	0.99	0.66	2.71	0.42	1.07	3.09

Table 3

Replicate	Adulterant	HA, %	FA, %	Relative Recovery HA, %	Relative Recovery HFA, %
1	None	81.61	12.86		
2	None	80.16	12.78		
1	Seaweed	80.21	12.85		
2	Seaweed	80.72	12.79	99.5	99.6
1	Fertilizer	80.25	12.98		
2	Fertilizer	79.57	123.77	98.8	101.6
1	Coal	78.79	12.92		
2	Coal	81.27	12.84	98.9	101.8
1	Molasses	79.38	12.99		
2	Molasses	81.02	12.72	99.2	100.9
Mean		80.3	12.85		
SD		0.885	0.09		

[a] Final concentration of FA + HA of 2.5 g/L added to 0.1 M NaOH. (data taken from Lamar *et al.* , 2

2015)

Table 4

HA			
Sample ID	Extracted, mg	Recovered, mg	Recovered, %
1	24.6	23.7	96.3
2	22.6	19.9	88.1
3	25.2	23.6	93.7
4	22.5	21.5	95.6
5	23.9	21.8	91.2
6	23.2	20.8	89.7
7	24	23.2	96.7
Mean	23.7	22.1	93
SD	1.01	1.52	3.43
RSD, %	4.35	6.88	3.67

(data taken from Lamar *et al.,* 2014)

Table 5

FA			
Sample ID	Extracted, mg	Recovered, mg	Recovered, %
1	19.9	19	95.48
2	23.1	22.9	99.13
3	20.7	19.4	93.72
4	20.5	19.8	96.39
5	20.8	21.6	103.85
6	21.9	20.1	91.78
7	22.7	22.3	98.24
Mean	21.37	20.73	96.94
SD	1.21	1.53	3.95
RSD, %	5.64	7.36	4.07

(Data taken from Lamar *et al* ., 2014)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Amberlite IR 120 H^+-exchange resin	Sigma-Aldrich	10322	H^+ form
Analytical Balance	Ohaus	PA214	w/ glass draft shield
Centrifuge	Beckman Coulter	Allegra X-14	minimum 4300 rpm
Centrifuge tubes	Beckman Coulter		To fit rotor selected
Ceramic Combustion Crucibles	Sigma	Z247103	
Chromatography column for DAX-8	Diba	Omnifit 006EZ-50-25-FF	
Chromatography column for IR 120	Chemglass	CG-1187-21 2 in. by 24 in.	
Dessicator	Capitol Scientic	Kimax 21200-250	Vacuum type
Drying Oven	Fisher Scientific	Isotemp	Precision±3˚C
Electrical conductivity meter	HM Digital	EC-3	
Erlenmeyer Flasks	Amazon		1L, 2L
HCl concentrated	Sigma-Aldrich	320331	
Magnetic Stir Plate	Barnstead-Thermolyne	Dataplate 721	
Magnetic Stir bars			These can be obtained at many outlets
Muffle Furnace	Fisher scientific	Thermolyne Type 47900	
NaOH	Sigma-Aldrich	795429	
Nitrogen gas	Praxair	UNI1066	99.99% purity
Peristaltic pump	Cole Parmer	Masterflex 7518-00	
Perstaltic tubing	Cole Parmer	Masterflex Pharmed 06508-17	
pH meter	Oakton Instruments	WD-35618--03	
Rotary Evaporator	Buchi	R-210/R-215	

Spectrophotometer	Healthcare SCiences	Ultrospec II	Dual beam 200 to 900 nm with wavelength accuracy of ±1 nm and reproducibility of ±0.5 nm.

Rebuttal letter

Editors comments-all comments addressed

Reviewer #1- Made all suggested changes except we did not change formula symbols and Formula 1.3 is correct as written.

Reviewer #2
L 59/60 we did indicate that medical applications are increasing, not that they are new
Other comments addressed as suggested

Reviewer #3
Tables were edited to be more clear.

Reviewer #4
No suggested changes.