SPECIAL GUEST EDITOR SECTION

A New Standardized Method for Quantification of Humic and Fulvic Acids in Humic Ores and Commercial Products

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Increased use of humic substances in agriculture has generated intense interest among producers, consumers, and regulators for an accurate and reliable method to quantify humic acid (HA) and fulvic acid (FA) in raw ores and products. Here we present a thoroughly validated method, the new standardized method for determination of HA and FA contents in raw humate ores and in solid and liquid products produced from them. The methods used for preparation of HA and FA were adapted according to the guidelines of the International Humic Substances Society involving alkaline extraction followed by acidification to separate HA from the fulvic fraction. This is followed by separation of FA from the fulvic fraction by adsorption on a nonionic macroporous acrylic ester resin at acid pH. It differs from previous methods in that it determines HA and FA concentrations gravimetrically on an ash-free basis. Critical steps in the method, e.g., initial test portion mass, test portion to extract volume ratio, extraction time, and acidification of alkaline extract, were optimized for maximum and consistent recovery of HA and FA. The method detection limits for HA and FA were 4.62 and 4.8 mg/L, respectively. The method quantitation limits for HA and FA were 14.7 and 15.3 mg/L, respectively.

The objective of the study was to validate analytical protocols that are based on the preparative method for humic acid (HA) and fulvic acid (FA) used by the International Humic Substances Society (IHSS) for preparing their Standard and Reference samples (1, 2) that was subsequently modified and adopted by the Humic Products Trade Association (HPTA). This modified Swift protocol (2) is referred to as the new standardized method (NSM) for quantification of humic and fulvic acids in humic ores and commercial products. This single-laboratory validation (SLV) study was conducted under the guidance of the Association of American Plant Food Control Officials (AAPFCO) to validate a quantitative analytical method for analysis of HA and FA in commercial humic products. Until this work, there has been no validated analytical method for determining the quantity of HA and FA in any material.

The proposed NSM is intended to quantify HA and FA in solid and liquid commercial humic products, peat, soil, and humate-containing geological deposits. This method is based on a procedure for extracting HA and FA from natural materials. Like the method of Swift (2), the proposed method is a modified form of the "classical" technique described in detail by Stevenson (3). The classical method of extracting HA and FA from soil humus utilizes a strong base to extract the alkaline-soluble materials, and then, after removal of nonsoluble components, the alkaline solution is acidified to precipitate the HA. Waksman (4) credits Oden, a German scientist who worked to determine the chemical nature and structure of humic substances (5), with naming the remaining substances in solution after alkaline and acid treatment "FA". The method described by Swift adds a column adsorption step to separate FA as the more hydrophobic component, as distinct from the more hydrophilic biological molecules in the FA-containing extract (2, 3) of the classical method. Both the method of Swift and the classical method were developed as preparative methods for the fractionation of soil organic matter, and they were not intended to be used as quantitative analytical methods for commercial purposes.

Similar to Swift, the proposed NSM defines FA as the material that binds to a nonionic macroporous acrylic ester resin of moderate polarity at low pH, i.e., DAX-8 (6, 7). This definition displaces the classical definition of FA, which is defined as all organic material extracted with strong base that is soluble in both acid and base (4), with a more rigorous definition. This stricter definition of FA facilitates the distinction of hydrophobic FA from the more hydrophilic components in the classical fulvic fraction, i.e., polysaccharides, amino sugars, amino acids, proteins, fatty acids, carbohydrates, lipids, etc., that may be extracted by strong base along with humic substances (3, 8).

The products currently sold as FA in agricultural amendments often contain the fulvic fraction, complete with hydrophilic

Guest edited as a special report from the AOAC Agricultural Community on "Collaborations in New and Improved Methods of Analysis for Plant Food Materials" by Nancy Thiex.
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Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI:10.5740/jaoacint.13-393

components. However, in this study we follow the practice of the IHSS (1) and determine FA through separation from nonhumic substances in the fulvic fraction by selective adsorption onto DAX-8 resin. The FA separated with DAX-8 and similar resins is sometimes called the hydrophobic strong acid fraction of soluble organic matter (7). The agriculture amendment industry has proposed calling this fraction hydrophobic FA to distinguish it from the fulvic fraction, which contains hydrophilic components that might also provide benefits for plant growth (Mayhew, L., Humic Products Trade Association, personal communication, 2014).

The NSM utilizes several techniques from Swift (2) that differentiate it from the classical method including: initial alkaline extraction is performed under a N_2 atmosphere, determination of the quantity of humic substances on a dry ash-free basis, and nonionic resin separation of FA. In addition, it establishes protocols to differentiate FA from certain nonhumic materials that are purportedly marketed as genuine humic products.

The NSM determines ash-free quantities of HA and FA gravimetrically after separation from their matrix using procedures similar to the method published by Lamar and Talbot (9). They compared their gravimetric method to two other methods that are in general use: the California Department of Food and Agriculture (CDFA) method (10) and a colorimetric method proposed by Mehlich (11).The authors concluded that relative to their modified Swift gravimetric method, the CDFA and Mehlich methods overestimated the HA content of eight different humic ores.

In the current study, the ash-free mass of HA is determined after precipitation and drying and then ashing the dried HA. The material remaining in solution after acidification, the fulvic fraction, is passed through a DAX-8 resin. The FA, which adsorbs to the resin, is separated from other nonadsorbed components by rinsing the column with deionized water. FA is then eluted with NaOH and subsequently protonated on a strong acid exchange resin. The FA extract volume is reduced through use of a rotary evaporator and brought to dryness in an oven at 90°C. The ash content of the dried FA is determined to provide the ash-free mass.

For analysis of liquid agricultural amendment products, HA is flocculated with HCl after dilution with 0.1 M NaOH to about 500 mg/L of total FA plus HA. This is done in order to establish a consistent ionic environment for the flocculation of HA by HCI. This wide dilution is also used when extracting solid materials with 0.1 M NaOH. It is important to have approximately the same HA plus FA concentration for all analytical samples and standards because the partitioning between HA and FA is dependent on the concentration during the addition of HCl. At higher concentrations generally more HA is recovered (12).

In order to analyze a broad range of humic and fulvic products, HPTA members submitted three liquid and four solid commercial sources of HA and FA. These commercial sources were compared to an IHSS reference Leonardite, a source of HA and FA, a high content FA material from China, a Leonardite HA IHSS standard, and a Pahokee peat FA IHSS standard.

As this procedure was developed according to AAPFCO requirements, the SLV study had to demonstrate that the method was able to distinguish HA and FA from adulterants. Any materials containing sugars and other carbohydrates or amino acids and most proteinaceous materials will not adsorb to the resin (6, 7). However, in the course of this study, the authors observed that lignosulfonates do adsorb to DAX-8 resin and thus cannot be separated from FA. Therefore, additional protocols were established to determine the presence of lignosulfonates.

Description of the Proposed NSM

Chemicals

All chemicals were ACS reagent grade.

(**a**) *NaOH and HCl.*—Sigma-Aldrich (St. Louis, MO).

(**b**) *Nitrogen gas.*—(UN1066) 99.99% purity (Praxair, Danbury, CT).

(**c**) *Supelco Supelite DAX-8 resin 21567-U.*—Sigma-Aldrich.

(**d**) *Amberlite IR120 strong cation exchange resin.*—Hydrogen form 10322 (Sigma-Aldrich).

Equipment

(**a**) *Analytical balance with glass draft guard.*—Capacity 210 g with readability to 0.0001 g (Ohaus PA214, Parsippany, NJ).

(**b**) *Drying oven.*—Precision ± 3°C (Isotemp, Fisher Scientific, Fairlawn, NJ).

(**c**) *Centrifuge.*—Minimum relative centrifugal force 1500 × *g* (International Equipment Co., Chattanooga, TN).

(**d**) *Polyethylene centrifuge tubes (50 mL).*—VWR Scientific (Batavia, IL).

(**e**) *Rotary evaporator.*—Rotavapor R-210/R-215 with Heating Bath B-491 (Buchi, New Castle, DE).

(**f**) *Magnetic stir plates and stir bars.*—Dataplate 721 (Barnstead-Thermolyne, Dubuque, IA).

(**g**) *pH Meter and electrode.*—WD-35618-03 (Oakton Instruments, Vernon Hills, IL).

(**h**) *Electrical conductivity meter.*—HM Digital EC-3 (Amazon.com).

(**i**) *Spectrophotometer.*—Dual beam 200 to 900 nm, with wavelength accuracy of ±1 nm and reproducibility of ±0.5 nm (Ultrospec II, G.E. Healthcare Sciences, Pittsburgh, PA).

(**j**) *Peristaltic pump and tubing.*—Masterflex 7518-00, Pharmed 06508-17 (Cole Parmer, Vernon Hills, IL).

(**k**) *Combustion oven, i.e., muffle furnace.*—Thermolyne Type 47900 Furnace (Fisher Scientific).

(**l**) *Rotating shaking mixer.*—GlasCol 099A RD 4512, (A. Daigger & Co., Vernon Hills, IL).

(**m**) *Desiccator.*—Vacuum type, KIMAX 21200-250, 250 mm (Capitol Scientific, Austin, TX).

Glassware

(**a**) *Erlenmeyer flasks.*—1000 and 2000 mL.

(**b**) *Graduated cylinder.*—1000 mL.

(**c**) *Glass chromatography columns.*—4 × 25 cm for DAX-8 resin; 5 × 60 cm for IR120 cation exchange resin.

(**d**) *Ceramic combustion crucibles*.—Sigma-Aldrich.

Analytical Procedure

Step 1. Alkaline Extraction

If the material is a dry solid, prepare analytical samples by crushing them to a fine powder using a mortar and pestle so that 100% of the crushed analytical sample passes through a U.S.

Standard Sieve mesh size No. 60 making sure that the powder is well mixed.

Determine the moisture content gravimetrically as follows: Weigh an aluminum weigh boat and record mass (W_{t1}); transfer 2 ± 0.5 g test portion of the analytical sample into the weigh boat, weigh, and record mass (W_1); place in drying oven for 24 ± 0.2 h at 90°C; after 24 h, remove from drying oven and place in desiccator to cool for 1 h; weigh and record mass of weigh boat and dry test portion (W_2). Determine the moisture ratio using Equation 1 (Step 7).

Next, weigh a second test portion from the analytical sample estimated to contain approximately 2.5 g HA and record the test portion mass to three decimal places (W_3) into a precalibrated/premarked 1 L Erlenmeyer flask. Add 0.1 M NaOH (i.e., 4 g NaOH/L distilled H_2O) with stirring, and make to a final volume of 1 L. Determine the dry weight of the test portion using Equation 2 (Step 7).

For liquid materials, thoroughly mix the analytical sample by stirring with a glass rod for 1 min, ensuring that any residue that may have fallen to the bottom of the container is thoroughly mixed. Then add an aliquot of a test portion of the analytical sample, noting the volume (V_1), into a precalibrated/premarked 1 L Erlenmeyer flask, and bring to a final volume of 1 L with 0.1 M NaOH. The test portion should result in a concentration of 200 to 600 mg/L HA plus FA after dilution with 0.1 M NaOH. The aliquot volume will be based on the HA and FA product concentrations that were claimed by the manufacturer. Determine the density (g/mL) of the liquid material by weighing 10 mL of well-mixed analytical sample in a pretared graduated cylinder (D_1). Determine the weight of the liquid test portion using Equation 3 (Step 7).

Add a 0.8 to 1.2 cm long magnetic stir bar, replace the air in the headspace with N_2, and cover with Parafilm. Mix vigorously on a stir plate (e.g., 200–300 rpm). Stir solid materials for 6 h to extract humic substances and the liquid materials for 1 h to ensure dissolution of all HA and FA.

From this point on, the method is the same for both solid and liquid materials.

After stirring, remove the flask from the stir plate, transfer the contents to centrifuge tubes, and centrifuge the entire volume to separate any insoluble material from the dissolved HA and FA. Centrifuge at 3900 × *g* for 10 min (use 50 mL centrifuge tubes, or larger, as available). Discard the insoluble precipitates and collect the alkaline supernatant containing the HA and FA in a clean 1 L Erlenmeyer flask.

While the extract solution is being mixed with a stir bar, carefully insert a pH electrode into the middle portion of the solution. To flocculate the HA, add concentrated HCl (1:1) dropwise to the alkaline extract until pH 1.0 ± 0.05 is reached.

Cover the flask with Parafilm and mix for 1 h. Check pH and readjust to pH 1.0 with additional concentrated HCl if necessary. If the pH should fall below 0.95, adjust pH back to 1.0 ± 0.05 with the 0.1 M NaOH solution. Continue to let the acidified extract mix and check pH occasionally until it is stable at pH = 1 ± 0.05 for 5 min.

Once the pH is stable, remove the flask from mixer and cover with Parafilm. Let the mixture sit unstirred until precipitated HA has fallen to the bottom of the flask. The time for HA to precipitate from solution varies greatly among products, but a typical time range is 1 to 6 h.

Step 2. Separation of HA

(**a**) Once the HA has completely precipitated, decant the FA-containing extract (fulvic fraction) into a clean 1 L Erlenmeyer flask, being careful not to include any of the HA precipitate. Typically >900 mL can be decanted while excluding any HA precipitate. With some products, the precipitated HA will remain in suspension as colloidal particles. If so, centrifuge the entire volume to separate the flocculated HA from the acidified fulvic fraction.

(**b**) Pour the remaining mixture into centrifuge tubes and centrifuge at 3900 × *g* for 0.5 h to separate the HA precipitate. If necessary a higher g force or longer centrifugation time can be used to obtain a clean separation of the fulvic extract supernatant from the HA precipitate. Add the supernatant to the FA-containing acidified extract.

Step 3. Determination of HA Concentration

(**a**) Place the centrifuge tubes containing the precipitated HA in a drying oven set at 90°C, and dry the HA to constant weight (typically 24 h). Constant weight is achieved when the tube and HA (or FA) weigh the same after an additional 2 h drying time.

(**b**) 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_{4HA}). This residue is the "Extracted HA".

Step 4. Determination of Ash Content

Transfer the extracted HA to a preweighed (W_{t2}) ceramic dish that had been previously dried in a drying oven set at 90°C and then cooled in a desiccator to room temperature. After recording the combined mass of the extracted HA and dish (W_5), combust in a muffle oven for 4 h at 500°C. While still warm, remove the dish and contents from the muffle oven and place in a desiccator to cool. Once cool, weigh the dish with ash (W_6) and calculate the ash ratio (Equation 4, Step 7). Determine the final mass of the extracted HA by correcting for ash content using Equation 5 (Step 7).

Step 5. Separation of Fulvic Acid

Separate FA from the other acid-soluble compounds in the fulvic fraction by using a 40 × 250 mm glass column prepared with a nonionic macroporous acrylic ester resin (i.e., Supelite DAX-8). Through selective adsorption, hydrophilic acid-soluble components do not bind to the resin and are removed. Pass the fulvic fraction through the column using a peristaltic pump, under low pressure, via the top of the column. It is critical that the top of the resin in the column remains covered with solution until all the extract has been added to prevent drying of the resin.

Once the fulvic fraction has been completely loaded onto the resin, wash the resin with deionized water by pumping it through the top of the column using the peristaltic pump under

low pressure. Discard the effluent. Wash the column until the absorbance at 350 nm of the column effluent is equal (e.g., within 0.015 absorbance units) to that of the deionized water used to wash the column, using deionized water to zero (i.e., blank) the spectrophotometer. A wavelength of 350 nm gives strong absorbance by FA and allows the use of a spectrophotometer that only measures visual wavelengths.

Desorb the FA by back elution (i.e., influent introduced into the bottom of column) by pumping 0.1 M NaOH using the peristaltic pump. Most of the FA is adsorbed to the very top of the DAX-8 resin. Desorption from the column bottom uses a minimal amount of 0.1 M NaOH to fully desorb the FA. All the FA has been desorbed when the absorbance of the column effluent is equal to the absorbance of 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.

Protonate and de-ash the FA by passing repeatedly (by gravity feed) through Amberlite IR120 hydrogen form ion exchange resin contained in a 5 × 50 cm column until the electrical conductivity of the effluent is <120 uS/m as measured with a conductivity meter. To ensure that all the FA is removed from the resin after the final pass, wash the column 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.

Concentrate the FA to a volume of approximately 15 ± 2 mL by using a rotary evaporator at 55°C. Completely transfer the 15 mL fulvic acid concentrate to a 50 mL plastic centrifuge tube and dry at 90°C to constant dryness in a drying oven. Freeze-drying is an alternative to oven drying. After drying, as described for the HA above under Step 4, place the tube in a desiccator to cool. Remove FA from the tube by complete scraping of the tube sides and bottom with a spatula, and weigh it on pretared weigh paper (W_8). This material is the "Extracted FA". Determine the residual ash content of extracted FA as described under Step 4 for HA and calculate the ash ratio (Equation 4, Step 7). Finally, determine the weight of the extracted FA without ash using Equation 6 in Step 7.

Step 6. Column Regeneration

Regenerate the DAX-8 resin by pumping 0.1 M HCl [8.33 mL concentrated HCl/1000 mL final volume deionized (DI) water] 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. Next 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).

Regenerate the H^+form cation exchange resin in a batch process by pouring the resin into a large beaker (e.g., 4 L plastic beaker), pour off the water, and cover the resin with 1 M HCl (83.3 mL concentrated HCl/1000 mL final volume DI water). Let stand for a minimum of 30 min with occasional stirring (e.g., once every 5 min). 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 and rinse water sit for 5 min. Repeat the process until the pH of the rinse water equals the pH of the DI water.

Load the regenerated resin back into the column. Once loaded, rinse the resin with DI water and check the pH of the effluent. If it is still lower than the pH of the DI water before it is passed through the column, continue to rinse the column with DI water until the pH of the effluent is within 0.1 pH units of the pH of the influent.

Step 7. Calculations

$$\text{Moisture ratio} = [(W_1 - W_2)/(W_1 - W_{t1})] \quad (1)$$

$$\text{Dry test portion dry weight} = (W_3)\,(1 - \text{moisture ratio}) \quad (2)$$

$$\text{Liquid test portion weight (g)} = (V1)\,(D1) \quad (3)$$

$$\text{Ash ratio} = [(W_5 - W_6)/(W_5 - W_{t2})] \quad (4)$$

$$\text{Weight of extracted HA without ash (g)} = (W_{4HA})(1 - \text{Ash Ratio}) \quad (5)$$

$$\text{Weight of extracted FA without ash (g)} = (W_{4FA})(1\text{-Ash Ratio}) \quad (6)$$

where W_1 = weight of test portion taken for moisture plus ceramic dish before drying, g; W_2 = weight of test portion taken for moisture plus ceramic dish after drying, g; W_{t1} = weight of ceramic dish, g; W_3 = test portion weight, g; V_1 = volume of liquid test portion, mL; D_1 = weight/unit volume of liquid test portion, g/mL; W_{4HA} = weight of extracted HA, g; W_{4FA} = weight of extracted FA, g; W_{t2} = weight of ceramic dish used for ashing, g; W_5 = weight of extracted HA or FA taken for ash plus ceramic dish before combusting, g; and W_6 = weight of ash plus ceramic dish after combusting, g.

(**a**) For solid materials, determine the percentages of FA and HA (dry weight basis) as follows:

$$\text{FA, \%} = [\text{Ashless FA (g)/test portion dry weight)}] \times 100$$

$$\text{HA, \%} = [\text{Ashless HA (g)/test portion dry weight)}] \times 100$$

(**b**) For liquid materials:

$$\text{FA, \%} = [\text{Ashless FA (g)/liquid test portion (g)}] \times 100$$

$$\text{HA, \%[Ashless HA (g)/liquid test portion (g)}] \times 100$$

Materials and Methods

Validation Materials

Validation materials for this study included three commercial liquid humic products supplied by humic product vendors and four solid humate ores supplied by humate mining companies. The products were typical of commercial humic products that are distributed worldwide and raw materials for preparation of

HA- and FA-containing products. Therefore, they reflected the types of products that are routinely tested for HA and FA.

Liquid Test Materials

Liquid test materials consisted of commercial products referred to as L16, L17, and L2. These liquid materials were analyzed in triplicate (data not presented) at an early stage of the NSM development, and their HA and FA concentrations were reported as percentage of initial sample mass. They were selected for their reported relative differences in concentrations of both HA and FA for the purpose of validating the analysis of liquid humic materials across a range of concentrations using the proposed method. The concentrations of HA and FA, respectively, in materials from preliminary analyses were: 16.5 and 1.7% (L16), 7.7 and 6.3%, (L17), and 3.9 and 9.8% (L2). L16 represented a product with relatively high concentration of HA with relatively low concentration of FA. L17 represented a product with medium concentrations of HA and FA, and L2 represented a product with relatively low concentration of HA with a relatively high concentration of FA.

Solid Test Materials

Test materials referred to as D1, D2, D3, and D4 were supplied by North American mining operations and consisted of humate-bearing ores. The mined materials that were chosen for this study were found, in preliminary triplicate analyses (data not shown), to have relatively low, medium, and high concentrations of HA and FA within a range of numerous solid materials that had been gathered for the purpose of validating the analysis of solid materials across a range of concentrations using the proposed method. With the exception of D1, the ore materials contained concentrations of FA between 1 and 2%, which is typical for many mined humic materials that are humate-bearing ores used by the humic products industry. The concentrations of HA and FA, respectively, for materials found in preliminary analyses were: 60.3 and 1.3% (D2), 26.3 and 1.2% (D3), and 5.2 and 1.1% (D4), representing mined ore materials with relatively high, medium, and low concentrations of HA and typical levels of FA.

Reference Standards

IHSS Gascoyne Leonardite, which is rich in HA, was from a mine near Gascoyne, ND (IHSS Cat. No. 1BS104L). It was used as the HA standard and was supplied by the IHSS. The IHSS Gascoyne Leonardite is a mined ore that was ground, sifted, and dried. The FA standard was a commercial FA manufactured through a proprietary process by Hangzhou Dayangchem Co. Ltd, Hangzhou Zhejiang, China. The composition of the FA material was verified by ^{1}H-NMR and ^{13}C-NMR spectrometry analysis (data not shown). In addition, IHSS Pahokee Peat standard FA (2S103F) and IHSS Leonardite standard HA (1S104H) were provided by the IHSS. They were prepared by the IHSS according to protocols described in http://www.humicsubstances.org/sources.html and verified by FTIR and ^{13}C-NMR spectral analysis, http://humicsubstances.org/spectra.html (data not shown).

Preparation of Test Samples

Test samples were stored in opaque white plastic bottles and prepared as per the NSM protocol described above prior to analysis.

SLV Parameters

Test Material Precision (Repeatability SD)

The repeatability design included the analysis of liquid products, ore products, and the Gascoyne Leonardite and Hangzhou FA. Four replicate extractions of each test sample were performed. Extractions were done using 1 g ore in a final volume of 1 L 0.1 M NaOH. Liquid products were extracted as follows: L2, 11 g; L16, 3 g; and L17, 3 g in a final volume of 0.1 M NaOH. The different extraction amounts for the liquid products approximated and estimated final recovery of 500 mg HA plus FA. To maximize variability, each replicate of the test samples was analyzed on four separate days by different analysts. Repeatability or RSD and SD, predicted RSD (PRSD) and HorRat(r) were calculated for each test sample. The HorRat(r), which is determined by dividing the RSD by the PRSD (i.e., RSD/PRSD), is used to support the data generated on repeatability and precision.

Accuracy

Method accuracy was evaluated by extracting in triplicate a mixture containing 0.5 g IHSS Gascoyne Leonardite and 0.2 mg Hangzhou FA in a final volume of 200 mL 0.1 M NaOH.

Ruggedness

The ruggedness parameters reported below were evaluated by varying major factors with potential to contribute to method variability.

(**a**) *Initial sample quantity*.—One liquid (L16) and two dry (D1 ore and the IHSS Gascoyne Leonardite) test materials were used to test the effect of the ratio of initial test portion weight to extract volume on extraction efficiency of HA and FA. Initial weights of 1, 2.5, and 5 g solid were extracted in a final volume of 1 L 0.1 M NaOH. Aliquots of L16 were added to attain 0.200 and 0.600 g/L (HA + FA). These treatments were not replicated.

(**b**) *Extraction time*.—Initially, a 24 h extraction time for solid materials was proposed based on the procedure of Swift (2). However, in preliminary testing we found a 6 h extraction time to be sufficient to give optimum extraction efficiency. To confirm that the 6 h extraction time was as efficient as extracting for 24 h, extraction for 6 and 24 h were compared using the D1 ore material and the Gascoyne Leonardite with 1 g test portion in a final volume of 1 L 0.1 M NaOH. These treatments were not replicated.

(**c**) *Effect of pH*.—Currently, the generally accepted pH values for acidification of the alkaline extract to flocculate HA are either pH 2 or 1. Both pH values were evaluated for their

Table 1. Method precision in recovery of HA and FA from liquid commercial materials[a]

	Humic substances, %					
	L16		L17		L2	
Material	FA	HA	FA	HA	FA	HA
Rep 1	1.44	17.00	6.59	7.76	0.36	4.46
Rep2	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
PRSD, %	3.78	2.62	3.03	2.94	4.61	3.17
HorRat(r)	1.58	0.72	1.25	0.47	2.31	1.55

[a] Extraction conditions were 1 g which contained approximately 500 mg HA + FA/L 0.1 M NaOH.

Table 2. Method precision in determination of HA and FA contents of IHSS Gascoyne Leonardite standard and Hangzhou FA standard[a]

	Humic substances, %			
	IHSS Gascoyne Leonardite		Hangzhou FA	
Standard	FA	HA	FA	HA
Rep 1	7.25	75.48	63.18	0.00
Rep 2	7.14	74.95	62.58	0.00
Rep 3	8.10	74.57	63.45	0.00
Rep 4	7.69	75.86	62.89	0.00
Mean	7.55	75.22	63.03	0.00
SD	0.44	0.57	0.37	0.00
RSD, %	5.83	0.76	0.59	0.00
PRSD, %	3.75	2.12	3.81	0.00
HorRat(r)	1.55	0.36	0.16	0.00

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

effect on recovery of HA and FA from the D1 ore, the Gascoyne Leonardite, and the L16 liquid product. Extraction conditions were 1 g test portion in a final volume of 1 L 0.1 M NaOH for the dry samples and 0.600 g/L for the L16 liquid product.

(**d**) *Effect of time and temperature on ash content*.—In order not to overestimate the HA or FA content of any product that contains inorganic substances, ash content must be determined (8). The effects of temperature (400 and 500°C) and time (4, 8, 12, and 24 h) of ashing on the ash content determination were evaluated for HA generated from the experiment on pH variation. Humic acid extracted from the D1 ore, Gascoyne Leonardite, and L16 liquid were used.

Selectivity for HA and FA

Dark colored nonhumic materials are reportedly sold in the marketplace as humic products, and it is important that a method of analysis be specific enough to discriminate HA and FA from adulterants in commercial products. Potentially fraudulent materials tested were liquid seaweed (kelp) extract, NPK fertilizer, coal, molasses, and lignosulfonates.

(**a**) *Seaweed, NPK fertilizer, coal, and molasses*.—The effects of these adulterants on the determination of HA and FA were studied using the Gascoyne Leonardite solid reference standard. The Gascoyne Leonardite reference standard was added to the matrix blank, i.e., 0.1 M NaOH, to produce a mixture with a final concentration of 2.5 g/L. Adulterants were tested individually and prepared by adding to the Gascoyne Leonardite/NaOH mixture in a final volume of 1 L 0.1 M NaOH. Adulterants added were seaweed (Neptune's Harvest Organic Seaweed Plant Food), approximately 8 g/L; NPK fertilizer (Miracle Gro All Purpose Plant Food), approximately 2 g/L; coal (bituminous), approximately 2 g/L, and molasses, at approximately 2 g/L. These mixtures with and without adulterant were analyzed in duplicate.

(**b**) *Lignosulfonate*.—The effect of adding lignosulfonate to liquid humic products was evaluated by preparing a liquid solution containing 1.8 g IHSS Gascoyne Leonardite HA and 0.25 g IHSS Pahokee Peat FA in 1 L 0.1 M NaOH with about 5 g lignosulfonate added. The resulting mixture was analyzed for HA and FA contents using the NSM protocol for liquid materials.

Method Detection and Quantification Limits

Guidance for determining the method detection limit (MDL) for HA and FA was based on communications with the humic products industry and the fact that we had consistently extracted levels as low as 0.025 g/L HA and 0.020 g/L FA from several liquid products prior to this study.

The MDL is the lowest concentration of HA and FA that can be distinguished from a blank sample but cannot necessarily be quantified. It was calculated by multiplying the sample SD by the Student's *t*-value. The MDL for HA was experimentally determined by preparing seven replicates of about 0.025 g/L of IHSS Leonardite standard HA (1S104H) in 0.1 M NaOH and subjecting them to the NSM. This freeze-dried HA standard had been extracted and purified from the IHSS Gascoyne Leonardite ore by the IHSS. The extractions were conducted by the same analyst on various days over a 1 month period. These

Table 3. Method precision in recovery of HA and FA from humic ore materials[a]

	Humic substances, %					
	D2		D3		D4	
Material	FA	HA	FA	HA	FA	HA
Rep 1	1.75	67.40	1.31	27.01	1.55	8.95
Rep 2	1.69	67.63	1.25	27.48	1.41	7.20
Rep 3	1.63	67.10	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, %	3.70	1.39	10.33	1.02	4.02	9.02
PRSD, %	3.75	2.12	3.81	2.43	3.76	2.91
HorRat(r)	0.99	0.66	2.71	0.42	1.07	3.09

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

Table 4. Analysis of blanks for known quantities of HA and FA reference materials

	1% Hangzhou FA 2.5% IHSS Gascoyne Leonardite, g		Expected recovery, g[a]		Actual recovery, g		Recovery, %	
	IHSS Gascoyne Leonardite[b] HA plus FA	Hangzhou[b] HA	FA	HA	FA	HA	FA	HA
Rep 1	4.367	1.753	1.432	3.262	1.397	3.342	97.6	102.5
Rep 2	4.427	1.758	1.439	3.330	1.432	3.471	99.5	104.2
Rep 3	4.378	1.724	1.414	3.293	1.392	3.340	98.5	101.4
Mean	NA[c]	NA	NA	NA	1.407	3.384	98.5	102.7
SD	NA	NA	NA	NA	0.022	0.075	1.0	1.4

[a] Expected recovery based on previous analysis of IHSS Gascoyne HA = 75.22% and FA = 7.5%, Hangzhou FA = 63%.

[b] Dry mass (g) added to 200 mL 0.1 M NaOH (final volume).

[c] NA = Not applicable.

HA-amended blanks (in 0.1 M NaOH) were used to determine the recovery of standard additions of HA at low concentrations.

Similarly, seven additional 1 L 0.1 M NaOH solutions were spiked with about 0.020 g/L IHSS Standard Pahokee Peat FA (2S103F), and FA was determined by the same analyst on various days over a 1 month period. These FA-amended blanks in 0.1 M NaOH were used to determine the recovery of FA at low concentrations.

The method quantitation limit (MQL) values were calculated according to guidelines published by the Wisconsin Department of Natural Resources (13). The MQL is the lowest level of analyte above which quantitative results may be obtained. The MQL was calculated as 10 times the SD at the concentrations analyzed.

Results and Discussion

Test Material Precision

The RSD for HA content of the commercial liquid humic products concentration ranged from 1.39% for L17 to 4.91% for L2 (Table 1).The RSD for FA ranged from 3.8% for L17 to 10.4% for L2. As expected, the relative error was higher for FA overall, and the highest was for sample L2, the material with the lowest concentration. Using L2 as an example, at 0.38% FA, the mass of FA measured was only 4 mg. For the dry materials, FA and HA concentrations for the IHSS Gascoyne Leonardite were 7.6 and 75.2%, respectively (Table 2). The mean FA concentration for the Hangzhou FA was 63.0%, and no HA was detected as expected for this purified FA (Table 2). The RSD of the IHSS Gascoyne Leonardite FA and HA were 5.8 and 0.8%, respectively (Table 2). The RSD of the Hangzhou FA analyses was 0.6%.The RSD for FA in the mined ores ranged from 3.7 to 10.3%, and the RSD for HA ranged from 1.4 to 9.0% (Table 3). HorRat(r) values within the range of 0.3 to 1.3 are considered acceptable without further explanation. In eight of 15 cases, HorRat(r) values fell within this range (Tables 1–3). Therefore, the method is both precise and reliable.

Accuracy

The results for the accuracy study are reported in terms of mass recovered from 200 mL of 0.1 M NaOH (Table 4). The solutions used to measure method accuracy were prepared without considering the moisture content of the two materials, i.e., the IHSS Gascoyne Leonardite and the Hangzhou FA, both of which contained about 12% moisture. The expected recoveries were calculated based on 12% moisture content and the contents of HA and FA in Gascoyne Leonardite and Hangzhou FA that are reported in Table 2. Based on those numbers the actual recoveries of both HA and FA were excellent for all three replicates, ranging only from 97.6 to 99.5% for FA and 101.4 to 104.2% for HA (Table 4).

Ruggedness

Based on prior experience, four major factors potentially contributing to method variability were tested individually. Results and interpretation are provided below.

(**a**) *Initial quantity of sample.*—Initial mass of dry sample appeared to have an effect on the amount of HA recovered from the solid ore samples, with more HA being recovered at 2.5 and 5 g/L of ore in 0.1 M NaOH than at 1 g/L 0.1 M NaOH for both D1 and IHSS Gascoyne Leonardite (Table 5). Initial sample mass also affected recovery of FA from the IHSS Gascoyne Leonardite, with more being recovered at initial concentrations of 2.5 and 5 g/L 0.1 M NaOH than at 1 g/L 0.1 M NaOH. However,

Table 5. Ruggedness testing variable—initial sample mass[a]

Material	D1		IHSS Gascoyne Leonardite		L16-Liquid		
	Humic substance in solid sample, %					Humic substance in liquid sample, %	
Initial dry mass, g	FA	HA	FA	HA	Initial mass of HA + FA, mg	FA	HA
1	4.59	55.18	7.39	70.02	200	2.37	15.42
2.5	4.6	62.28	12.66	78.19	600	3.62	15.98
5	4.48	62.77	12.87	76.66			

[a] All extractions done in 1 L 0.1 M NaOH.

Table 6. Ruggedness testing variable—extraction time[a]

	Humic substances in solid sample, %			
Material	D1		IHSS Gascoyne Leonardite	
Extraction time, h	FA	HA	FA	HA
6	4.26	55.76	4.28	68.89
24	4.50	55.18	7.39	70.02

[a] Extraction conditions were 1 g sample/L of 0.1 M NaOH.

no effect was observed for FA recovery from D1. The results obtained from the addition of 2.5 or 5 g of these ores suggests varying quantities of HA + FA added according to the quantity in these samples will not affect the results. The least HA + FA added was 1.7 g for 2.5 g of the D1 ore sample and the largest addition of HA + FA was 4.5 g for the Gascoyne Leonardite. At the concentrations tested for the liquid L16 material, initial sample masses of 0.2 g/L 0.1 M NaOH and 0.6 g/L 0.1 M NaOH combined concentrations of HA and FA did not appear to affect recovery of HA or FA (Table 5). Therefore, the initial solids concentration for dry samples should be changed to a range of 1.7 to 4.5 g of HA + FA/L 0.1 M NaOH to determine HA content in dry samples. No change in the initial sample content for liquid samples is necessary, and it can remain in the range of 500 mg/L 0.1 M NaOH HA and FA combined. Liquid products have already been subjected to extensive extraction, most likely in KOH. Therefore, HA in liquid products is already in solution.

(**b**) *Extraction time*.—There was little difference in the HA and FA concentrations observed after 6 or 24 h of extraction for the dry mined material D1 sample, suggesting that extraction times greater than 6 h had little or no further benefit for extraction of the dry mined material (Table 6).

The effect of extraction time was greater for the IHSS Gascoyne Leonardite with somewhat more HA and FA extracted after 24 h than after 6 h , but somewhat less than that reported for determination of extraction precision using a 6 h extraction time using an ore/NaOH ratio of 2.5 g/L 0.1 M NaOH (Table 5). These data support the recommendation of extracting solid materials for a minimum of 6 h.

(**c**) *Effect of pH on the acidified alkaline extract.*—Of all the steps in the procedure, the final pH to which the alkaline extract was acidified had the greatest effect on HA and FA concentrations (Table 7). With a decrease in final pH from 2.0 to 1.0, the amounts of extracted HA increased while the amounts of extracted FA decreased for all three samples. Hence, lowering the pH of the alkaline extract from 2.0 to 1.0 resulted in more humic substances precipitating out of the alkaline solution as HA, which is consistent with the findings of Kipton et al. (14).

Table 7. Ruggedness testing variable—pH at which HA is flocculated[a]

	Humic substances, %					
Material	D1		IHSS Gascoyne Leonardite		L16	
pH	FA	HA	FA	HA	FA	HA
1.0 ± 0.05	4.59	55.18	7.39	70.02	1.44	17.00
2.0 ± 0.05	6.66	53.76	16.46	57.73	4.51	14.92

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

It is also consistent with the fact that FA has more total acidity relative to HA, having pKa < 2 (15) because of a higher concentration of carboxyl [-COOH] groups.

Under conditions of high pH in the presence of strongly hydrated monovalent cations such as sodium, nonprotonated [R-COO–] binding sites on HA provide sufficient surface charge repulsion to resist precipitation. But at low pH, protonation of phenolic hydroxyl and COOH groups on the HA causes conformational changes in its molecular structure as protons replace the Na^+ counter ions causing reduction in surface charge density and reduction in volume. With increased hydrophobic interactions, water is excluded from the shrinking matrix (16) and an aggregated hydrophobic material (HA) precipitates out of solution leaving the FA in solution (15).

The choice of pH 1.0 as the standard endpoint for aggregating HA dissolved in alkaline solution is further supported by Prado et al. (17), who demonstrated a continued decrease in surface charge density as pH was adjusted from 2 to 1 for a commercial HA product. In addition, use of the pH 1.0 endpoint is very common in the literature, and the IHSS uses this pH end point in preparation of their HA and FA standard and reference samples (2).

(**d**) *Effect of time and temperature on ash content*.—The data in Table 8 suggest that there is no benefit for ashing at durations greater than 8 h either at 400 or 500°C and that ashing for 4 h at 500°C works well.

Selectivity Against Adulterants

(**a**) *Seaweed, NPK fertilizer, coal, and molasses.*—The additions of seaweed, NPK fertilizer, coal, or molasses had no effect on concentrations of the HA and FA that were determined in the solid Gascoyne Leonardite (Table 9).

(**b**) *Lignosulfonates*.—The addition of lignosulfonate increased the quantities of both HA and FA measured in the artificial liquid sample (Table 10). The effect was greater for the FA.

As a result of this selectivity study, protocols for prescreening and detection of the presence of lignosulfonates were investigated. Phloroglucinol (Appendix A on the *J. AOAC Int.* website), barium chloride, and ^{13}C-NMR spectrometry protocols for detecting lignosulfonates were tested. Both wet chemistry methods failed to detect the presence of lignosulfonates, and ^{13}C-NMR spectrometry analysis was inconclusive (data not shown). The investigation proceeded to establish prescreening

Table 8. Ruggedness testing variable—effect of ashing time and temperature on ash content in HA

	Ash, %					
	D1		L16		IHSS Gascoyne Leonardite	
	Temperature, °C					
Time, h	400	500	400	500	400	500
4	6.00	4.97	7.54	6.22	8.10	6.42
8	4.86	4.91	6.73	6.25	5.93	6.62
12	4.75	4.71	6.49	6.13	5.26	6.23
24	4.75	4.93	6.04	6.15	5.28	6.37

Table 9. Effect of adulterants on the determination of HA and FA in IHSS Gascoyne Leonardite[a]

	Adulterant	HA, %	FA, %	Relative recovery HA, %	Relative recovery FA, %
1	None	81.61	12.86		
2	None	80.16	12.78		
3	Seaweed	80.21	12.85		
4	Seaweed	80.72	12.79	99.5	99.6
5	Fertilizer	80.25	12.98		
6	Fertilizer	79.57	12.77	98.8	101.6
7	Coal	78.79	12.92		
8	Coal	81.27	12.84	98.9	101.8
9	Molasses	79.38	12.99		
10	Molasses	81.02	12.72	99.2	100.9
Mean		80.30	12.85		
SD		0.885	0.09		

[a] Final concentration of FA plus HA of 2.5 g/L added to 1 L 0.1 M NaOH.

protocols for the presence of lignosulfonates based on physical and chemical properties of lignosulfonates.

Prescreening for the presence of lignosulfonates is accomplished by organoleptic inspection and testing for elemental sulfur. Solid and liquid humic acids are dark brown to black in color, insoluble in acid aqueous media, and either odorless or having a slight petroleum-like smell. Fulvic acids are light yellow in color, soluble in both acid and alkaline aqueous media, and odorless.

Lignosulfonates have a distinctive sulfurous odor because they are derived from wood lignins treated using sulfites to sulfonate the lignins (18) and to separate lignin from cellulose in processing wood for paper or cellulose production. Total sulfur concentration in lignosulfonates is typically approximately 5% (19). As the concentration of sulfur in humic substances is typically <1%, with an average of about 0.6% (1), we propose that for regulatory purposes any product that exceeds 0.75% total elemental sulfur will have to be tested by FTIR analysis to exclude the presence of lignosulfonates.

In IR spectra, the sulfur-oxygen bonds in lignosulfonates demonstrate characteristic symmetric stretching of S=O bands at about 1041 cm–1 and asymmetric stretching of S=O bands at about 1182 cm–1 (Piccolo, A., Università di Napoli Federico II, personal communication, 2012), which differ from the band for sulfate anions in fertilizers. Therefore, the presence of lignosulfonates can be detected by FTIR spectrometry in both simple and complex mixtures. *See* Appendix B on the *J. AOAC Int*. website for FTIR spectra of lignosulfonates.

Table 10. Effect of lignosulfonate on recovery of HA and FA from Gascoyne Leonardite in 0.1 M NaOH[a]

Recovery	HA	FA
Expected, g	1.8	0.25
Observed, g	3	2.6
%	166	1,040

[a] Extraction conditions: 1.8 g IHSS Gascoyne Leonardite HA, 0.25 g IHSS Pahokee Peat FA, and 5 g lignosulfonate in 1 L 0.1 M NaOH.

HA and FA have IR spectral bands that are similar to each other, with characteristic peaks at 1620 and 1720 cm^{-1} (3). The 1720 cm^{-1} peak is much stronger in spectra of FA than HA because of the occurrence of more COOH groups. These bands are either very weak or absent in spectra for lignosulfonates. *See* Appendix C on the *J. AOAC Int.* website for FTIR spectra of FA.

MDL and MQL Values for HA and FA

Tables 11 and 12 report the recoveries of HA and FA, respectively, from liquid samples that simulated commercial products with very low concentrations. Recoveries for such low concentrations were excellent, ranging between 88 and 97% for HA and between 92 and 104% for FA. No HA or FA were recovered from the 0.1 M NaOH solutions that received no HA or FA (data not shown). Mean recoveries for HA and FA were 93 and 97%, respectively, and RSD values were less than 2%. Nevertheless, these results indicate the need to perform laboratory replicates. The MDL and MQL for HA were 0.00462 and 0.0147 g/L, respectively. The MDL and MQL for FA were 0.0048 and 0.0153 g/L, respectively.

Table 11. Recovery of HA from spiked blanks

	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	24.0	23.2	96.7
Mean	23.7	22.1	93.0
SD	1.01	1.52	3.43
RSD, %	4.35	6.88	3.67

Table 12. Recovery of FA from spiked blanks

	FA		
Sample ID	Extracted, mg	Recovered, mg	Recovered, %
1	19.90	19.0	95.48
2	23.10	22.90	99.13
3	20.70	19.40	93.72
4	20.50	19.80	96.39
5	20.80	21.60	103.85
6	21.90	20.10	91.78
7	22.70	22.30	98.24
Mean	21.37	20.73	96.94
SD	1.21	1.53	3.95
RSD, %	5.64	7.36	4.07

Conclusions

A standardized method, the NSM, for measuring the concentrations of HA and FA in commercial humic products and their source ores was evaluated for both liquid and solid materials. It features gravimetric determination of the HA and FA on a dry, ash-free basis; moderated concentrations of HA, FA, and salts during extraction to control the partitioning between HA and FA; and a definition of FA based on adsorption to DAX-8 resin. This definition, which corresponds to that used by the IHSS, is stricter than the classical definition of solubility in both acid and base, and it allows the distinction of FA from hydrophilic components present in the fulvic extract as well as potential adulterants. Through testing of reference standards and humic test materials provided by HPTA members, this procedure was found to be precise, reliable, and accurate. It was relatively rugged, as only one of four selected steps that were systematically varied clearly altered the amounts of HA and FA determined, namely the pH at which the HA was flocculated. The procedure proved to be highly selective with quantitative recovery of amended amounts of added humic products when added to blank solutions and complete distinction of humic products from four of five amended adulterants. The evaluation of MDL and MQL demonstrated that the method is sensitive enough for determination of low concentrations of FA and HA. This method is intended for routine regulatory and industrial use to establish and verify contents of commercial products. To compensate for its inability to distinguish FA from lignosulfonates, an alternative approach for detecting lignosulfonates based on elemental S concentration and FTIR spectrometry was proposed.

Acknowledgments

This study was entirely funded by the Humic Products Trade Association. We gratefully appreciate the assistance and reference materials donated by the IHSS, and gratefully acknowledge the valuable assistance provided by James Bartos, Office of Indiana State Chemist, the AAPFCO, and the SLV study monitors: Elaine Wong (CDFA), John Peterson (Wilber Ellis), Nancy Thiex (South Dakota State University), and Debra Wong (Oregon Department of Agriculture).

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