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Richard T. Lamar^a & Karen H. Talbot^a

^a EarthFax Development Corporation, North Logan, Utah, USA

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Critical Comparison of Humic Acid Test Methods

Richard T. Lamar and Karen H. Talbot

EarthFax Development Corporation, North Logan, Utah, USA

Abstract: The colorimetric method and the California Department of Food and Agriculture (CDFA) method of evaluating the humic acid content of five raw humate ores and three humate products were compared to the classical technique of extraction in a dilute base followed by precipitation of humic acid by extract acidification and ash removal by hydrochloric/hydrofluoric acid (HCl/HF) wash. Compared to the classical procedure, the colorimetric and CDFA methods overestimated the humic acid content of the eight samples by 120% and 52%, respectively. Therefore, these procedures do not produce a reliably accurate value for the humic acid contents of humates and products produced using materials extracted from them.

Keywords: Humic, humate, humic analysis, humate analysis, CDFA method, colorimetric method

INTRODUCTION

Humic substances (HSs) are a series of relatively high-molecular-weight, light-brown- to black-colored, complex and heterogeneous organic polymers formed by secondary synthesis reactions (Stevenson 1982). These substances are partitioned into three main fractions based on their solubilities in alkaline and acidic extraction solutions. These are humic acids (HAs), which comprise the alkali-soluble but acid-insoluble fraction; fulvic acids (FAs), the fraction soluble in both alkali and acid;

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Address correspondence to Rich Lamar, EarthFax Development Corp., 1770 N. Research Park Way, N. Logan, UT 84341. E-mail: rlamar@earthfax.com

and the humin fraction, which cannot be extracted by either dilute base or acid (Stevenson 1982; Schnitzer 1982). Chemically, the three fractions are similar, but they differ in molecular weight, ultimate elemental analyses [i.e., carbon (C), hydrogen (H), oxygen (O), nitrogen (N) contents], and functional group content (Stevenson 1982). Humic acids and FAs from different sources and from the same source can also vary considerably in structure (e.g., degree of aromaticity/aliphaticity) (Wilson 1987). Indeed, HAs produced from sequential extractions from the same source have been shown to have significant chemical and structural differences (Kang and Xing 2005). Humic acids and FAs are extracted in large quantities from humates, which include a variety of naturally occurring organic lithologies with high HS content (Simandl, Simandl, and Ayles 2001). These materials include leonardite (oxidized lignite from a particular geologic deposit in North Dakota), weathered (i.e., oxidized) lignite, subbituminous coal, and a variety of carbonaceous rocks such as mudstones, shales, and claystones (Kohanowski 1957, 1970; Hoffman et al. 1993). Humates as raw ores and their extracts (i.e., HA and FA) are marketed and sold to the agricultural and horticultural communities as soil amendments and as fertilizers.

Because HA does not possess a clearly defined and consistent chemical structure, an analytical technique to accurately quantify it does not exist. However, there are currently three methods offered by commercial laboratories to estimate the HA or HS content of humates and HA derivatives (i.e., extracts of humates). All three methods are based on the solubility of HA in dilute alkaline solutions and two take additional advantage of its precipitation when alkaline extracts are acidified. These procedures include a qualitative colorimetric method based on a procedure developed by Mehlich (1984), the California Department of Food and Agriculture (CDFA) method, a semiquantitative method developed by the CDFCA, and a quantitative method, referred to in this article as the classical method based on a modification of the procedure detailed by Swift (1996).

The colorimetric method involves an alkaline extraction with a solution composed of 0.2 M sodium hydroxide (NaOH), 0.002 diethylenetriamine penta acetic acid (DTPA), and 2% alcohol. This solution is used to extract HSs from humates or humate extracts. The method attempts to estimate the quantity of HS by comparing the intensity of color of the alkaline extract of the sample to the intensity of color produced by the extract of a standard amount of Aldrich humic acid. However, because FA and other water- and base-soluble constituents including amino acids, proteins, sugars, fatty acids, and humic substances (Hayes and Graham 2000) will also be extracted, this method does not estimate HA content alone but the content of HA, FA, and base-soluble constituents that are spectrally active at the analytical wavelength employed.

The colorimetric method is based on the Beer–Lambert law:

$$\log(I_0/I) = kcd$$

where I_0 is the intensity of incident light, I is the intensity of transmitted light, k is the extinction coefficient of the substance (e.g., HA), c is the concentration of the substance in solution, and d is the path length of the cell (i.e., distance the light travels through the substance in solution). The extinction coefficient, k , is equal to the optical density or absorbance ($\log I_0/I$) when the cell length is 1 cm and the concentration of the substance is 1 mol L^{-1} . For equivalent concentrations, the extinction coefficient of humic compounds increases with increase in molecular weight, C percentage, degree of condensation, and the ratio of C in aromatic rings to C in aliphatic structures (Stevenson 1982). Thus, if all humic compounds had identical molecular weights and chemical structure, the colorimetric method could be used to give an accurate estimate of the humic matter concentration. However, HSs from different sources, or even from the same source, can vary greatly in molecular-weight distribution, degree of condensation, C content, and degree of aromaticity to aliphaticity (Stevenson 1982). Additionally, the standard used in the colorimetric procedure is Aldrich HA (Aldrich Chemical Company, Milwaukee, Wisc.). According to Aldrich (personal communication with Aldrich technical department), their HA is obtained from mines in Germany and is composed of a mixture of decomposing plant parts, peat, and soft coal. It is highly probable that Aldrich HA is not consistent from batch to batch in the qualities mentioned (e.g., molecular-weight distribution) and is not representative of HA extracted from different deposits. Therefore, it is a poor standard.

To accurately determine the concentration c of a substance, the Beer–Lambert law relies on the extinction coefficient (i.e., molar absorptivity) of the sample being equal to that of the standard. Thus, the accuracy of the colorimetric technique might be improved somewhat by creating standards for each source of raw humate or HA derivative tested and determining the extinction coefficient of the standards. Once the extinction coefficient of the standard is determined, the concentration of HA could be estimated. The technique might also be made more accurate if the FA and other compounds spectrally active at the wavelength employed were removed prior to analysis so that only HA remained in the extraction solution. This could be easily done by acidifying the extraction solution to $\text{pH} \leq 2$, separating the precipitated HA by centrifugation, and redissolving it in fresh extraction solution prior to colorimetry.

The CDFA method uses 0.5 N NaOH to extract HSs from solid samples. Once extracted, the pH of the extract is adjusted to $\text{pH} \leq 2$ using concentrated hydrochloric acid (HCl). The precipitated HA is then removed from the acidified extraction solution, which contains any FA

that might have been contained in the initial sample. The HA is then washed, dried, and weighed to determine its concentration. Thus, the CDFA method primarily measures the nonpurified (i.e., ash is not removed) HA content. The value is always less than that given by the colorimetric test because the FA and other spectrally active components that are still soluble at pH 2 are removed. However, the ash content of humates from different sources varies considerably (Ozdoba et al. 2001). The ash (i.e., the inorganic fraction complexed by the HA and FA) content of raw humates from six different geographic locations including humalite from Alberta, Canada; leonardite from southeastern Saskatchewan, Canada, and North Dakota; weathered subbituminous coals and carbonaceous shales from Wyoming and New Mexico; and carbonaceous shales from Idaho were evaluated (Ozdoba et al. 2001). The ash contents varied between 11.1% for the Alberta humalite and 84.7% for the Idaho carbonaceous shale. Thus, because the CDFA method does not remove the ash, it will overestimate the HA content of humates and humate derivatives.

The classical method for extraction and purification of HA involves dissolving a solid humate sample in 0.1 N NaOH or adjusting the pH of a liquid sample to pH 11 with concentrated NaOH and shaking overnight under an atmosphere of nitrogen (Swift 1996). The mixture is then centrifuged to remove undissolved inorganic materials. The pH of the supernatant is then adjusted to pH 2 with concentrated HCl and allowed to sit overnight. The mixture is then centrifuged, and the precipitate, which contains the HA with inorganic contaminants, is collected and purified. Purification is achieved by washing the HA several times in an HCl/HF solution (Schnitzer 1982). Because the HA is extracted and purified, this method is the only one of the three that reports the true HA content of a humate or humate extract. Recently, a rapid batch procedure based on this method was developed that allows analysis of multiple samples to be completed in 1.5 to 4 h (Zomeran and Comans 2007).

The objective of the work described in this article was to determine if the colorimetric and CDFA methods provide accurate estimates of the HA contents of humates and humate extracts, based on a comparison of the HA contents determined using the classical method. In addition, modifications to the colorimetric method were investigated to determine if the accuracy of the method could be improved.

MATERIALS AND METHODS

Humate and Humate Extracts

Eight solid humate ore or processed humic extracts, referred to as the test HSs, from a variety of sources were obtained and subjected to testing for

Table 1. Test humic substances used to compare the colorimetric, CDFAs, and classical test procedures for HA/substance content

Humic substance (HS)	Lithology	Geographic origin	Ore/ext.
1. North Dakota ore	Leonardite	North Dakota	Ore
2. New Mexico ore	Subbituminous coal	New Mexico	Ore
3. Humate extract	Subbituminous coal	New Mexico	Extract
4. Senonian compost	Humic shale	Emery County, Utah	Ore
5. Humate ore	Subbituminous coal	Emery County, Utah	Ore
6. Idaho ore	Unknown	Idaho	Ore
7. Humic acid extract	Leonardite extract	Williston, N.D.	Extract
8. Humic acid extract	Humilite extract	Pine River, Canada	Extract

HA/HS concentration using the protocols for the three test methods as detailed in Table 1.

Colorimetric Determination of Humic Matter

Each tested HS was analyzed for HA content in triplicate. An extraction solution composed of 0.2 M NaOH, 0.002 DPTA, and 2% ethyl alcohol was prepared. The standard used in the procedure was Aldrich HA sodium salt (Aldrich Chemical Co., Milwaukee, Wisc.; H1,675-2). A standard curve was prepared in the following manner: 0, 25, 50, 100, 200, 250, 400, and 500 mg of Aldrich HA were weighed and placed in 50-mL polypropylene screw-cap vials. Twenty mL of extraction solution were poured into each tube, and the mixture was vortexed on high for 10 s. After 1 h, another 20 mL of extraction solution was added, and the mixture was vortexed. After allowing the mixture to stand overnight, 5 mL of undisturbed supernatant were added to 30 mL of deionized water in a clean 50-mL polypropylene tube, and the mixture was vortexed. After vortexing the transmittance (%T) at 650 nm, a 1-mL aliquot of each standard solution was measured using an LKB Ultraspec II spectrophotometer (Pharmacia, Irvine, Calif.). Initial weight of Aldrich HA was plotted against the %T at 650 nm to construct a standard curve.

For sample analysis, 1 g of HS 3, 4, and 6; 0.5 g of HS 5, 7, and 8; and 0.25 g of HS 1 and 2 were placed in 50-mL screw-cap polypropylene tubes, in triplicate. The different amounts used for the various HS were determined by trial and error to determine an amount that would allow transmission values in the linear area of the standard curve. Twenty mL of extraction solution were added to each tube, the caps were secured, and the mixtures were vortexed on high for 15 s. After 1 h, an additional 20 mL of extraction solution were added, and the mixture was vortexed as before. After allowing the mixture to stand overnight, 5 mL of

undisturbed supernatant were transferred to a clean 50-ml polypropylene tube with 30 mL of deionized water, and the mixture was vortexed. The %T at 650 nm was determined for each sample, and the milligrams of HA was determined from the standard curve.

CDFA Humic Acid Method

Each tested HS was analyzed by the CDFA method (CDFA 1999) in triplicate as follows: 1 g of HS and 50 mL of 0.1 N NaOH were placed into a 50-mL polypropylene screw-cap centrifuge tube. The tube was capped and shaken on a rotating shaker (Glas-Col) for 1.5 h. The cap was then rinsed with 5 mL of 1% (0.25 N) NaOH, the rinse was added to the tube, and the cap was replaced. The tube was then centrifuged at 1000 g for 20 min. The supernatant was decanted into a second, preweighed, 50-mL polypropylene centrifuge tube. An additional 5 mL of 1% (2.5 M) NaOH was added to the first tube; the tube was vortexed to resuspend the residue and then centrifuged as before. The supernatant was then added to the second tube. The pH of the combined extracts in the second tube was adjusted to $\text{pH} \leq 1$ with 6 N HCl, followed by shaking for 20 min. The tube was then centrifuged at 1000 g for 20 min, and the supernatant was decanted and discarded. The precipitate (i.e., the HA) was then washed by the addition of 25 mL of distilled water previously adjusted to $\text{pH} \leq 1$ with concentrated HCl, vortexed to resuspend the precipitate, and centrifuged at 1000 g for 20 min. The washing process was repeated again. After washing, the HA was dried in the preweighed tube at 100 °C overnight. Prior to weighing, the tubes were allowed to cool in a desiccator.

Classical Extraction and Purification of HA and FA

The classical method is based on a modification of the method described by Schnitzer (1982). One g of test HS was placed in a 1-L graduated cylinder, which was then filled to 1 L with 0.1 N NaOH. After mixing to partially dissolve the test HS, the alkaline mixture was completely transferred to a 1-L Erlenmeyer flask, and the sample was fully dissolved by adding a stir bar to the cylinder and mixing it on a magnetic stir plate. After mixing for 1–2 h, the alkaline extract was centrifuged to remove the undissolved mineral and organic components. Both of these procedures were done after evacuating the headspaces of the cylinder and flask with nitrogen and sealing the openings with parafilm. The pH of the alkaline extract was then acidified to pH 2.0 with concentrated HCl. The headspace of the flask was evacuated with N₂, sealed with parafilm,

and left to stand for 24 h. The HA-containing precipitate was removed from the FA-containing supernatant by centrifugation in 50-mL polyethylene centrifuge tubes. The HA was then purified (i.e., ash content was minimized) by repeated washing of the HA with dilute HCl/HF solution (5 mL of conc. HCl and 5 mL of 52% HF dissolved in 990 mL of deionized water) (Schnitzer 1982). Twenty mL of HCl/HF was added to the HA, mixed by vortexing, and centrifuged. The supernatant was discarded, and the process was repeated two times with the dilute HCl/HF solution and once with deionized water. The HA was then dried in the centrifuge tubes at 100 °C for 24 h. Prior to weighing, the purified HA-containing tubes were placed in a desiccator to cool to room temperature. This procedure results in HA content on a dry, ash-free basis.

The pooled supernatant, which contained the FA, was filtered through a 0.22-mm filter (Whatman 142128, 142 mm) using pressure filtration with N₂. Five hundred mL of filtrate were then slowly passed through a DAX-8 resin (Supelco, Bellefonte, Penn.) column using a peristaltic pump. The FA was adsorbed in the upper part of the column. The DAX-8 was then desalted by passing 4 to 6 L of deionized water from the top with the peristaltic pump. The DAX-8 was then back eluted (i.e., from the bottom) with 0.1 N NaOH to desorb the FA. The FA-containing eluent was then passed through an Amberlite IR-120 (Supelco, Bellefonte, Penn.) hydrogen ion exchange column twice, by gravity, followed by 500 mL of deionized water. The filtrate was collected and concentrated by rotovapping at 60 °C. The concentrated extracts were lyophilized, and the dried, purified FA was collected and weighed.

Determination of Ash Content Test of HS

The ash content of each of the test HS was determined by weighing 1 g of material into a preweighed and tared ceramic crucible. The dry weight of each crucible was first determined after drying for 2 h at 80 °C and cooling to room temperature in a desiccator. The crucibles containing the test HS were then placed in a muffle oven and heated at 650 °C for 24 h. Prior to weighing to determine the weight of residual ash, the crucibles were again placed in the desiccator to cool to room temperature. Moisture contents of the test materials were determined gravimetrically.

Evaluation of Method Changes to Improve the Accuracy of the Colorimetric Method

The effects of using a HA standard generated from the New Mexico ore and of isolating the HA in samples from acid-soluble products (e.g., FA)

on the accuracy of the colorimetric procedure were evaluated. Humic acid from a pooled sample of several New Mexico ore samples was extracted and purified using the classical procedure described previously. The purified HA standard was then compared to Aldrich HA as the standard in the colorimetric procedure to evaluate the HA content of several ore samples that were collected from the same mine in New Mexico as the one used to generate the standard. In addition, the isolation of the HA from acid-soluble products, in the samples, was compared to conducting the procedure using the original protocol. Isolation was accomplished by acidifying the extract with concentrated HCl and separating the precipitated HA from the acid supernatant by centrifugation. The HA was then redissolved in 40 mL of extraction medium by vortexing. The extract was then tested after letting it settle for 10 min and after centrifugation.

Duplicates of three New Mexico ore samples were subjected to the original colorimetric protocol. Another set of duplicates were subjected to the same protocol, which was modified by acidifying the extracts after they were allowed to stand overnight. After acids were extracted and the supernatant was separated, the duplicates were washed with 40 mL pH 1 deionized water and redissolved in 40 mL extraction solution. Colorimetry was conducted before and after centrifugation for 10 min. Initial weight of samples was 0.5 g and 0.3 mL rather than 5 mL of undisturbed sample, and the standard extract was diluted with 30 mL to make up the final extract. Standard curves (i.e., %T vs. mg of standard) were prepared using the following initial weights of New Mexico HA or Aldrich HA standards: 0, 25, 50, 100, 200, 250, 400, and 500 mg. The %T of the diluted extracts was read at 650 nm. The HA concentrations of the ore samples were determined from the standard curves. As a control and for comparison purposes, the HA contents of the three ore samples were also determined using the classical procedure described previously.

The procedure was repeated using only the New Mexico standard and the same samples with the exceptions that initial weight of samples was 0.25 g and colorimetry was conducted on diluted sample extracts only after centrifugation.

Data Analysis

Data on HA content obtained from application of the colorimetric, CDFA, and classical procedures were subjected to analysis of variance (ANOVA) with humate source and HA method as main effects. Significant differences among main effects were determined using Sheffe's test ($\alpha = 0.05$).

Table 2. Effect of HA analytical method on the HA content of eight humates and humate extracts

Procedure	HA content (mg/g) ^a
Colorimetric	539 a
CDFA	310 b
Classical	248 c

^aAverage HA contents that are followed by different letters are significantly different (Scheffe's test $\alpha = 0.05$).

RESULTS

HA Content

There was a significant difference among the methods for HA content (Table 2). The CDFA method gave significantly greater HA contents than the classical method, and the colorimetric method gave significantly greater HA contents than either of the other methods (Table 2).

The colorimetric method gave the greatest values for all samples (Table 3). Values for HA contents of all the HS were significantly greater (average = 50.8%) than the HA values obtained using the CDFA method in five out of the eight tested HS. Two of the three tested HSs that did not have significant differences between these two methods included the Senonian compost and the Idaho ore, both of which had extremely low HS contents. The colorimetric procedure produced significantly greater (average = 120.2%) HA values than those obtained using the classical procedure with the exception again of the Senonian compost and the Idaho ore (Table 3). It was expected that the colorimetric procedure

Table 3. Average HA concentration in humates and humic extracts based on colorimetric, CDFA, and classical methods

Humate/dry extract	Colorimetric [mg g ⁻¹]	CDFA [mg g ⁻¹]	Classical [mg g ⁻¹]
North Dakota	1065a	514b	496b
New Mexico	1080a	600b	423c
Utah Mining-Agri. Magic	175a	128b	113b
Semonian compost	7a	1a	6a
Live earth (raw ore)	797a	555b	537b
Idaho ore	30a	19a	17a
Williston, N.D.	580a	491b	191c
Pine River, Canada	579a	519a	201b

Means within columns followed by a different letter are significantly different (Scheffe's test $\alpha = 0.05$).

would produce greater values than those obtained by the other two methods because it includes FA and other extracted substances that absorb at 650 nm. A general rule of thumb that has been in use is that doubling the CDFA method HA value will equal the colorimetric method HA value. This was only true for the North Dakota leonardite, one HS sample out of eight. Based on the data from these samples, it is obvious that this practice is not reliable.

Values obtained using the CDFA method were, on average (again without including the Senonian compost), 55.6% greater than those produced using the classical procedure (Table 3). The values produced using the CDFA method were significantly greater in four of the eight tested HSs. This was expected because the CDFA method does not remove ash associated with the HA whereas the classical procedure does. However, the CDFA method produces HA values far closer to those produced using the classical method than does the colorimetric procedure. However, because of differences in ash contents in raw ores or extracts (Table 4), use of the CDFA method can far overestimate the HA contents (e.g., HS from Williston, N.D., and Pine River, Canada).

Evaluation of Method Changes to Improve the Accuracy of the Colorimetric Method

Humic acid concentrations in three New Mexico ore samples, determined using the colorimetric method with and without modifications, are given in Table 5. The HA concentrations determined using the classical procedure are also given. We have shown that the colorimetric method consistently overestimates the HA content compared to values obtained using the classical procedure. This was true even when the extracts were acidified to remove FA and other acid-soluble compounds that might absorb at 650 nm. In fact, acidification without centrifugation resulted in

Table 4. Humic acid, fulvic acid, and ash contents of the test humic substances

Test humic substance	HA (mg g ⁻¹)	HA (%)	FA (mg g ⁻¹)	FA (%)	Ash (%)
1. North Dakota	496	49.6	33	3.3	24
2. New Mexico	423	42.3	38	3.8	29
3. Utah Mining–Agri. Magic	113	11.3	25	2.5	58
4. Senonian compost	6	0.6	68	6.8	90
5. Live earth (raw ore)	537	53.7	89	8.9	39
6. Idaho ore	17	1.7	71	7.1	90
7. Williston, N.D.	191	19.1	66	6.6	22
8. Pine River, Canada	201	20.1	47	4.7	62

Table 5. Effect of standard and extract acidification on the humic acid concentrations of three New Mexico (NM) ore samples^a (all numbers are in mg g⁻¹)

Sample	Classical Method (mg g ⁻¹)	Nonacidified		Acidified		Acidified/cent.	
		Aldrich	NM	Aldrich	NM	Aldrich	NM
1-1	547	620	385	1003	738	858	555
1-3	461	637	402	855	580	581	478
2-3	563	809	545	1061	799	803	597

^aInitial sample weight was 0.5 g.

even greater HA values than those obtained using the original protocol (Table 5). Humic acid concentrations obtained using the New Mexico standard were less than those produced using the Aldrich HA standard and closer, in general, to the concentrations obtained using the classical procedure. Use of the New Mexico standard in combination with acidification of sample extracts and centrifugation produced the most accurate results, in comparison to the classically obtained concentrations.

The concentration of HA in the same New Mexico samples using the New Mexico standard and including the acidification and centrifugation steps are given in Table 6.

DISCUSSION

Currently the HS industry primarily relies on the colorimetric and CDFA methods to determine the HA content of their products and raw humates. The results of the work presented in this article demonstrate that both these methods, in general, overestimate the HA content compared to the values produced using the defining classical method and thus do not give an accurate measure of the HA content of humates (i.e., raw ores) or humate extracts.

The colorimetric procedure suffers from both the use of a nonrepresentative standard and the possible measurement of other

Table 6. Concentrations of HA in New Mexico ore samples using the modified colorimetric procedure

Sample ID	Classical Method (mg g ⁻¹)	Modified Colorimetric (mg g ⁻¹)
1-1	547	588 (47.2)
1-3	461	420 (40)
2-3	563	580 (28)

constituents present in the alkaline extract that may absorb at the analytical wavelength employed. We demonstrated that the accuracy of the method can be greatly improved by using a HA standard produced from the same humate from which other humate samples or humate extracts are produced and by removing acid-soluble compounds that were spectrally active at the wavelength employed (i.e., 650 nm). Offering the modified colorimetric method as presented herein would require the production of HA standards from each humate source to be tested. Because of intrasource variability, the HA standard would have to be validated on a regular basis and possibly a new standard produced as new areas of a source were excavated.

The CDFA method only differs from the classical method in that it does not employ the last purification step that removes the ash content. As illustrated in Table 2, elimination of this step can have a significant effect on the HA concentration value (e.g., HS 4 and 7). Therefore, addition of the HA purification step would improve the accuracy of the CDFA test.

The accuracy of the colorimetric test was improved greatly by using a HA standard generated from ore taken from the same location as the ore samples to be analyzed. The accuracy of the test was further improved by including acidification and washing steps to remove FA from the HA. We propose that use of the following modified procedure will improve the accuracy of the colorimetric test:

1. Prepare an extraction solution as follows: 0.2 M NaOH, 0.002 M DTPA, and 2% ethyl alcohol. For 1 L, add 8 g NaOH, 0.787 g of DTPA, and 20 mL of ethyl alcohol to a 1000-mL graduated cylinder and bring to volume with distilled water.
2. Prepare a standard curve as follows:
 - a. Weigh 0, 25, 50, 100, 200, 250, 400, and 500 μg of standard HA in 50-mL plastic centrifuge vials.
 - b. Add 20 mL of extraction medium and allow to stand for 1 h.
 - c. Add an additional 20 mL of extraction medium and let stand overnight.
 - d. Pipette 0.3 mL of undisturbed supernatant and 30 mL of H_2O into 50-mL plastic centrifuge vial and vortex.
 - e. Pipette 1 mL into plastic cuvette and read %T at 650 nm.
 - f. Construct standard curve of %T versus amount of initial weight of HA.
3. Assay samples as follows:
 - a. Measure 0.25 g of humic sample into a 50-mL plastic centrifuge tube, add 20 mL of extraction medium, and vortex. Prepare three tubes for each sample.

- b. After 1 h, add an additional 20 mL of extraction medium, vortex, and let stand overnight.
- c. Acidify the medium to pH 2 with concentrated HCl, centrifuge, pour off supernatant, and resuspend precipitate by vortexing in 40 mL deionized H₂O adjusted to pH 1 with concentrated HCl.
- d. Centrifuge for 20 min, resuspend in 40 mL of extraction medium, and centrifuge for 20 min.
- e. Transfer 0.3 mL of undisturbed supernatant and 30 mL of deionized H₂O into a 50-mL plastic centrifuge tube and vortex.
- f. Pipette 1 mL into plastic cuvette and read %T at 650 nm.
- g. Determine the humic matter content (i.e., $\mu\text{g/g}$) using the standard curve.

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