
**Fertilizers and soil conditioners —
Determination of humic and
hydrophobic fulvic acids
concentrations in fertilizer materials**

*Engrais et amendements minéraux basique - Détermination des
acides humique et fulvique*





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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 134, *Fertilizers and soil conditioners*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Humic substances are present in all ecosystems: oceans, rivers, lakes, and top soils. Quantifying the amount of humic material present in these systems is essential for academic research and commercial applications, specifically agricultural soil and plant management.

The increased use of humic substances in agriculture has generated intense interest among producers, consumers, and regulators for a reliable method for quantification of the active ingredients in raw humic ores and commercial fertilizer products; specifically humic and fulvic acids. As both commercial trade and regulation of humic products are based on percentage (%) of the humic and fulvic acids in commercial humic products, use of % units instead of SI units is warranted, therefore incorporated into this standard.

This document establishes a method for the determination of humic acids (HA) and acidic hydrophobic fulvic acids (HFA). The method is based on an existing preparative procedure use by the International Humic Substances Society (IHSS) for extracting high purity HA and HFA from soil samples^[1], which is a modified form of the “classical” technique described in detail by Stevenson^[2]. The “classical” methods and the IHSS method were developed as preparative methods for the fractionation of soil organic matter; they were not intended to be used as quantitative analytical methods. The classical method of extracting humic acids and *fulvic acids* from soil humus utilize a “strong base” to extract the alkaline soluble materials, and then the alkaline extract solution is acidified to flocculate the humic acids, which appear to precipitate out of solution. The remaining substances in solution after alkaline and acid treatment were called *fulvic acids*.

This method modifies the “classical” technique in a number of ways:

- it determines the quantity of humic substances on an “ash free” basis (mineral salts excluded);
- the alkali extraction is done under anoxic conditions to reduce oxidation of the analytical sample during extraction;
- it defines the materials that are soluble in both alkali and acid as the Fulvic Fraction;
- it can differentiate products containing certain non-humic materials that some manufactures claim to contain humic substances;
- it further defines HFA as materials of low sulfur content^[3] that bind to a hydrophobic resin at pH 1^[4]^[5]^[6], instead the classical, and perhaps more common definition, for *fulvic acids* as materials that are defined as soluble in both acid and alkali solution. This stricter definition is necessary to distinguish HFA from mineral salts, polysaccharides, amino sugars, amino acids, proteins, acids, and carbohydrates that are extracted along with humic substances when using the “classical” method^[1]^[4].

See [Annex B](#) for information on ISO/CD 19822 interlaboratory study.

Fertilizers and soil conditioners — Determination of humic and hydrophobic fulvic acids concentrations in fertilizer materials

1 Scope

This document specifies the procedure for the analysis of humic acids and hydrophobic fulvic acids which is applicable to dry and liquid materials used as ingredients in commercial fertilizers, soil amendments, and geological deposits.

2 Normative References

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1 hydrophobic fulvic acids HFA

materials composed of less than 0,75 % elemental sulfur (S) that are soluble in aqueous alkaline and acid solution and are adsorbed at pH 1 onto a polymeric adsorbent resin of moderate polarity. The resin is of a type designed for adsorption of amphiphilic compounds having molecular weights typical of fulvic acids

3.2 fulvic fraction

alkali extracted portions of humic substances that are soluble in both alkali and acid aqueous solutions

3.3 humic acids HA

alkali extracted humic substances that are insoluble in strongly acidic solution and will precipitate from the alkali extract in acid solutions of pH 1

3.4 humic substance

major organic constituent of natural organic matter consisting of complex heterogeneous mixtures of carbon-based substances formed by biochemical reactions during the decay and transformation of plant and microbial remains

3.5 lignosulfonates

amorphous light to dark brown powder or liquid derived from the sulfite pulping of softwoods. The lignin framework is a sulfonated random polymer of three aromatic alcohols: coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol, of which coniferyl alcohol is the principle unit

4 Principles

4.1 This method determines ash-free quantities of HA and HFA gravimetrically after separation from their matrix.

4.2 The method of extracting HA and HFA utilizes a strong base to extract the alkaline-soluble materials, and then, after removal of non-soluble components, the alkaline solution is acidified to flocculate the HA.

4.3 The liquid supernatant remaining after the removal of the HA is called the Fulvic Fraction. The Fulvic Fraction, which can contain Hydrophobic Fulvic Acids (HFA), is treated to determine the quantity of HFA in the Fulvic Fraction by selective adsorption onto a methacrylic-ester resin designed to separate the HFA from non-humic compounds. The material retained by the hydrophobic resin is referred to in the literature as the hydrophobic acid fraction of soluble organic matter^[3].

5 Warnings

5.1 Requirements

5.1.1 Good laboratory practices

Related standards (e.g. ISO/IEC 17025) should be followed at all times in regards to personal protective equipment (safety glasses, handling strong acids, hydrochloric acid) and alkali (sodium hydroxide).

5.1.2 Moisture control

Humic and fulvic acids are hygroscopic materials; it is critical to prevent absorption of moisture during the handling of dried materials.

5.3 Lignosulfonates

Lignosulfonates will damage the hydrophobic resin. This analytical method cannot differentiate between hydrophobic fulvic acids and lignosulfonates, therefore pre-screening for the presence of lignosulfonates is recommended for liquid products of unknown origin.

See [Annex A](#).

5.4 Temperature control

Do not exceed 65 °C when drying the humic and fulvic analytes. The analytes are subject to decomposition at higher temperatures.

6 Reagents

6.1 Sodium hydroxide solution, 0,1 M, dilute 3,99 g of 99,99 % purity NaOH in 1 l of deionized water.

6.2 Sodium hydroxide solution, 0,5 M, dilute 19,99 g of 99,99 % purity NaOH in 1 l of deionized water.

6.3 Hydrochloric acid solution, 6 M, dilute 12 M HCl with an equal part of deionized water.

6.4 Hydrochloric acid solution, 1 M, dilute 83,3 ml of 12 M HCl with 1 l of deionized water.

6.5 Hydrochloric acid solution, 0,1 M, perform a 1:10 dilution using 1 M HCl prepared in [6.4](#) with final volume of 1 l using deionized water.

6.6 Nitrogen gas (UN1066) 99,9 % purity.

6.7 Methacrylic-ester resin, 40–60 mesh, approximately $0,79 \text{ ml}\cdot\text{g}^{-1}$ pore volume, $225 \text{ \AA}$ mean pore size, $160 \text{ m}^2\cdot\text{g}^{-1}$ surface area, for adsorption of materials up to 150 000 MW, e.g. Supelite DAX-8 Resin, or any other available resin meeting equivalent properties.

6.8 Amberlite IR-120 strong cation exchange resin, hydrogen form.

6.9 Deionized water^[8].

6.10 Acetone.

7 Apparatus

7.1 Analytical balance with draft guard: capacity 210 g, with readability to $\pm 0,0001 \text{ g}$.

7.2 Drying oven, capable of $120 \text{ }^\circ\text{C}$, precision $\pm 3 \text{ }^\circ\text{C}$.

7.3 Centrifuge, minimum relative centrifugal force $1\,500 \times g$, capable of $3\,900 \times g$.

7.4 4 ml to 50 ml or 250 ml polyethylene or HDPE centrifuge tubes, or heavy duty high temperature resistant centrifuge tubes capable of $600 \text{ }^\circ\text{C}$ (for example; Kimble-Chase, catalog number 45212-50 KIMAX).

7.5 4 ml to 100 ml wide-form crucibles (for example: Fisher Scientific catalog number FB-965-M).

7.6 Rotary evaporator 400 ml capacity.

7.7 Magnetic stir plates and 5 cm to 7 cm long magnetic stir bars.

7.8 pH meter and electrode.

7.9 Electrical conductivity meter with probe having a calibrated cell constant of approximately one, as determined using standard protocols.

7.10 Spectrophotometer, capable of measuring $\pm 0,005$ absorbance units at 350 nm .

7.11 Peristaltic pump with a minimum flow rate of $1,2 \text{ ml}\cdot\text{min}^{-1}$ and tubing.

7.12 Muffle furnace.

7.13 Rotating shaking mixer.

7.14 Desiccator with silica gel (or its equivalent) as desiccant.

7.15 Erlenmeyer flask, 1 000 ml.

7.16 Beaker, 4 l.

7.17 Graduated cylinder, 1 000 ml.

7.18 Glass chromatography column, 4 cm × 25 cm for DAX-8 resin.

7.19 Glass chromatography column, 5 cm × 60 cm for IR120 H+ exchange resin.

7.20 Ceramic mortar and pestle.

7.21 Sieve, 74 µm (#200 US mesh).

7.22 Parafilm®¹⁾.

8 Preparing crucibles, drying, and weighing samples

8.1 Preparing crucibles

8.1.1 If using new crucibles, first wash them with acetone and then dry them in an oven at 105 °C for 2 hours.

8.1.2 Prepare previously used crucibles by washing in acetone, then firing them in a muffle furnace at 500 °C for 2 hours. Cool the crucibles in a desiccator to room temperature. Remove from desiccator when cool, record weight of the crucibles to four decimal places.

8.2 Drying and weighing solid analytical samples

8.2.1 If the analytical sample is a solid material, crush and screen approximately 5 g of the analytical sample to ≤75 µm making sure that the powder becomes well homogenized.

8.2.2 Transfer approximately 5 g analytical sample to a 100 ml crucible prepared according to [8.1](#).

8.2.3 Place the analytical sample in a drying oven for 24 h at a temperature of 62 ± 3 °C. (do not exceed 65 °C). If any clumping of the sample occurs during drying, break up the clumps with a glass rod. Continue drying until the sample is dried to a constant weight. This can take up to 24 h;

8.2.4 After achieving constant weight, remove the analytical sample from the drying oven and immediately place in a desiccator to cool.

NOTE Both humic and fulvic acids are hygroscopic materials, it is critical to prevent absorption of moisture during the handling of these materials.

8.2.5 Weigh out approximately 2,5 g test portion from the dried analytical sample in a pre-weighed crucible prepared according to [8.1](#), taking precautions to prevent moisture adsorption while handling. Record the test portion + crucible weight to four decimal places. Proceed to [9.1](#) immediately or return the crucible with test portion to the desiccator.

8.2.6 Determine the weight of the test portion by subtracting the weight of the crucible from the test portion + crucible; record the result as the Test Portion Dried Weight.

1) Parafilm® is a trademark of Bemis NA, Neenah, Wisconsin, USA. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results. It is commonly used for sealing or protecting vessels. It is a ductile, malleable, waterproof, odorless, translucent and cohesive thermoplastic.

8.3 Drying and weighing liquid samples

8.3.1 For liquid analytical samples, thoroughly homogenize the analytical sample by shaking the sample for one minute in the container in which the liquid was delivered. Weigh out approximately 5 g test portion from the liquid analytical sample to four decimal places. Record this as the Liquid Test Portion Weight.

For liquid analytical samples with an expected HFA content <1 %, use a test portion weight of 10 g.

8.3.2 Follow steps [8.2.2](#) to [8.2.6](#) for drying the sample and weighing the test portion of the sample.

9 Extraction procedure

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

Transfer the prepared test portion of the analytical sample to a 1 l Erlenmeyer flask containing a 5 cm to 7 cm long magnetic stir bar. Add 0,1 M NaOH, constantly stirring on a stir plate, to a final volume of 1 l. Evacuate the head space with N₂ and cover with parafilm (or seal the flask with a similar material). Then mix vigorously on a stir plate (e.g. 300 rpm to 400 rpm). Stir liquid samples for 1 hour; stir the solid samples for 16 hours to 18 hours.

NOTE For solid samples, it can be convenient to perform this step late in the day so the solid samples can be stirred overnight.

9.1.1 After stirring, remove the flask from the stir plate, transfer to suitable centrifuge tubes and centrifuge the entire volume at $3\,900 \times g$ for 30 minutes to separate any insoluble material from the dissolved materials in the alkaline extract. Carefully transfer the alkaline extract to a clean 1 l Erlenmeyer flask containing a magnetic stir bar. Discard the insoluble materials.

9.1.2 While gently stirring the solution, adjust the pH of the alkaline extract solution to flocculate the HA from the acid soluble materials by adding 6 M HCl (1:1) drop-wise to the alkaline extract, until $1,0 \pm 0,1$ pH is reached.

9.1.3 Cover the flask with parafilm and mix for 1 h. After 1 hour, check the pH and readjust to $\text{pH } 1,0 \pm 0,05$ with additional 6 M HCl, if necessary. If the pH falls below 0,95, adjust the pH back to $1,0 \pm 0,05$ with 0,5 M NaOH solution. Continue mixing the acidified extract until it stabilizes at $\text{pH } 1,0 \pm 0,05$ for exactly 5 minutes. Do not let the acidified extract sit for longer than 5 minutes after the pH stabilizes. Remove the pH electrode.

9.2 Separation of HA

9.2.1 Once the pH is stable, remove the flask from the mixer, and cover the flask with parafilm. Allow the pH-adjusted extract to sit undisturbed for 4 hours ± 5 minutes (do not exceed 4 hours). This stage is critical to prevent further partitioning of the HA and HFA constituents. The flocculated HA will drop out of solution.

9.2.2 Immediately centrifuge the extract solution at $3\,900 \times g$ for 30 minutes using pre-weighed 50 ml centrifuge tubes to recover the flocculated humic acids portion. Decant the supernatant (Fulvic Fraction), being careful not to include any of the flocculated HA. Typically, about 500 ml of the clarified extract can be decanted without disturbing the flocculated HA. If hydrophobic fulvic acids (HFA) analysis is to be performed, decant the fulvic fraction into a clean 1 l Erlenmeyer flask. [Alternatively: using heavy duty high temperature centrifuge tubes instead of plastic centrifuge tubes eliminates the need to transfer the flocculated HA to a crucible, decreasing labor and increasing precision.]

9.2.3 Centrifuge the tubes containing the flocculated HA again at $1,500 \times g$ for 20 minutes to 30 minutes to further separate the flocculated HA that appears to precipitate from the liquid fulvic fraction. If HFA analysis is intended, add this supernatant to the decanted fulvic fraction supernatant in [9.2.2](#).

9.2.4 If using high temperature centrifuge tubes, place the tubes containing the flocculated HA in a drying oven at 62 ± 3 °C. If using plastic or low temperature centrifuge tubes, carefully scrape all of the flocculated HA from the centrifuge tubes into 100 ml wide-form crucible(s) prepared according to [8.1](#). After scraping out the flocculated HA, add a minimal amount of deionized water^[8] to the centrifuge tube, secure the caps on the tubes, shake the capped tubes vigorously to get all the flocculated HA out of the tube, adding this deionized water/flocculated HA mixture to the crucible(s). Regardless of using either heavy duty tubes or crucibles, dry the flocculate HA to constant weight (typically overnight) in an oven at 62 ± 3 °C. Break up any clumps that form during drying using a glass rod, taking care to avoid removal of any material from the tubes. Depending on the source of the HA, the drying process can take up to 24 hours.

NOTE The high temperature centrifuge tubes can be supported in the furnace using 50 ml beakers.

9.3.2 Once the flocculated HA is dried to constant weight, remove the tubes/crucibles from the drying oven and immediately place them in a desiccator to cool. Once cooled to room temperature in the desiccator, reweigh the tubes/crucibles containing the flocculated HA. Take precautions to reduce adsorption of moisture during this step. Record the combined weight of the dried flocculated HA + tubes/crucibles, subtract the weight of the tubes/crucibles; record this weight as the Dried Flocculated HA Weight.

10 Determination of ash content

10.1 At this stage, the dried flocculated HA contains some residual ash. The ash content is determined by combusting the dried flocculated HA in either heavy duty centrifuge tubes or crucibles (prepared according to [8.1](#)) in a muffle furnace for 4 h at 500 °C. Wait until weight is constant. If any solid clumps form during ashing, carefully break up the clumps using a glass rod. Do not use a metal rod.

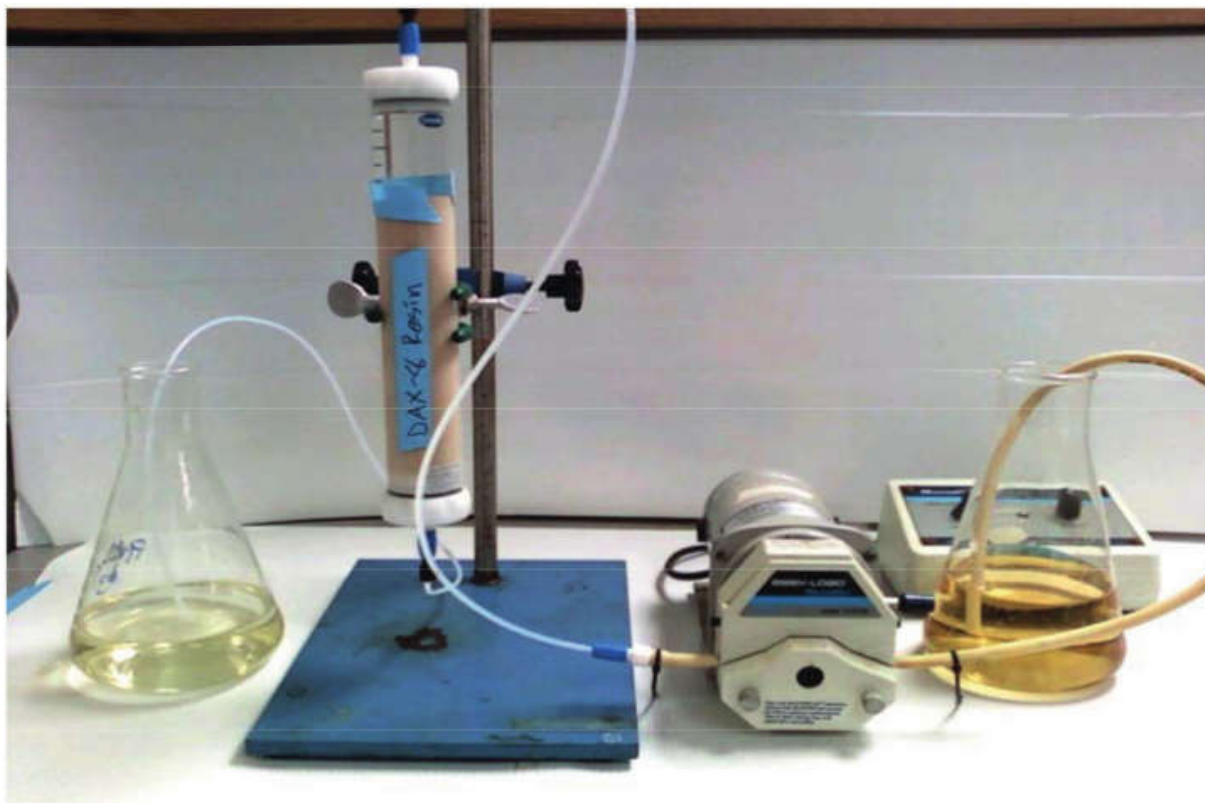
10.2 After achieving constant weight, remove the tubes/crucibles containing the flocculated HA from the muffle furnace and place them in a desiccator to cool to room temperature.

10.3 Once cool, determine the weight of the dried ash by subtracting the weight of the tube/crucible from the combined weight of the tube/crucible + ash. Record the result as Flocculated HA Ash Weight.

11 Separation of Hydrophobic Fulvic Acids (HFA)

11.1 Hydrophobic fulvic acids (HFA) are separated from other acid soluble substances in the Fulvic Fraction by selective adsorption to an acidified hydrophobic resin (i.e. Supelite DAX-8), to which hydrophilic acid soluble components do not bind and can thus be removed. This is accomplished using a 4 cm × 25 cm glass chromatography column partially filled with 280 ml of resin soaked in deionized water^[8] to prevent air pockets. Maintain an optimum space of approximately 2,5 cm from the top of the wet resin to the top of the column (see [Figure 1](#)). The intent is to leave sufficient space above the resin to visually monitor the flow rate through the column. If using new resin, clean the resin prior to use according to [14.1](#). After using the chromatography column resin, regeneration of the column resin is performed according to [14.1.2](#). When finished using the resin at the end of the day, store in methanol. Before reusing the resin, rinse the methanol from the resin with deionized water^[8] by using a slurry technique in a large beaker until the resin is free of methanol (less than 2 mg·l⁻¹ dissolved organic carbon). If the resin becomes discolored, or has been used about 15 times, perform a quality control test

using a quality control material. If necessary, clean the resin according to [14.1](#) or discard and replace with new resin.



NOTE The fulvic fraction is in the Erlenmeyer flask on the right side of the photo.

Figure 1 — Example of apparatus used to separate hydrophobic fulvic acids from the fulvic fraction

11.2 The Fulvic Fraction solution is passed through the column via the top of the column using a peristaltic pump set at just enough pressure so that the flow rate is sufficient to completely cover the resin in the column without overflowing the column or allowing the resin to be exposed to air. The suggested flow rate is about $4 \text{ ml} \cdot \text{min}^{-1}$ to $5 \text{ ml} \cdot \text{min}^{-1}$. Do not allow the solution level to drop below the top of the resin. Discard this effluent.

11.3 The column is then washed with deionized water^[8] using a peristaltic pump with enough pressure so that the flow rate (about $4 \text{ ml} \cdot \text{min}^{-1}$ to $5 \text{ ml} \cdot \text{min}^{-1}$) is sufficient to maintain complete coverage of resin. Continue washing until the UV absorbance $A = 0,015$ (350 nm) or after eluting 2 column volumes, whichever occurs first. Do not exceed 2 column volumes. Discard the effluent.

11.4 The HFA is then desorbed from the resin by back elution (i.e. influent introduced into the bottom of column) with 0,1 M NaOH, which is pumped using the peristaltic pump (flow rate about $4 \text{ ml} \cdot \text{min}^{-1}$ to $5 \text{ ml} \cdot \text{min}^{-1}$). Capture the HFA-containing effluent. The HFA has been desorbed when the absorbance of the effluent is $A = 0,030$ (350 nm) using a spectrophotometer or after eluting 3 column volumes, whichever occurs first. Do not exceed 3 column volumes.

12 Hydrogen ion exchange

12.1 Using a *hydrogen form* H^+ exchange resin prepared according to [14.2](#). Pour 500 ml of the prepared H^+ exchange resin into a $5 \text{ cm} \times 50 \text{ cm}$ chromatography column according to [14.2.1](#). Pump the HFA-

containing solution into the top of the column allowing it to flow through the column by gravity only. Repeat this step for a total of 2 passes through the column. This step protonates the HFA by exchanging Na^+ on the HFA with H^+ .

12.2 Wash the column with 500 ml of deionized water^[8]. This wash water effluent is added to the protonated HFA solution from [12.1](#).

12.3 Concentrate the HFA to a volume of approximately 50 ml to 60 ml by using a rotary evaporator set at 65 °C. Do not exceed 65 °C.

12.4 Dry the HFA concentrate using a crucible that has been prepared according to [8.1](#). Record the weight of the crucible. Transfer the remaining HFA concentrate to the prepared crucible. Dry the HFA concentrate at 62 ± 3 °C to constant weight in a drying oven, breaking up any clumps that have formed. Do not exceed 65 °C.

12.5 After drying to constant weight, place the crucible with HFA concentrate in a desiccator to cool to room temperature. Record the total weight of the crucible + dried HFA concentrate. Subtract the weight of the crucible from the total weight; this is the Weight of Dried HFA.

12.6 Determine the residual ash content of HFA. The procedure for determining ash content is the same as that described in [Clause 10](#) for HA, using either a prepared crucible or heavy duty centrifuge tubes.

12.7 Calculate % of HFA analyte using the calculations in [13.2](#) to [13.4](#).

13 Calculations

13.1 Determination of ash-free HA weight

$\% \text{ Ash} = (\text{Flocculated HA ash weight} \div \text{Dried flocculated HA weight}) \times 100$

$\text{Weight of ash-free HA} = \text{Dried flocculated HA weight} \times (1 - \% \text{ Ash})$

13.2 Determination of ash-free HFA weight

$\% \text{ Ash} = (\text{HFA ash weight} \div \text{Dried HFA weight}) \times 100$

$\text{Weight of ash-free HFA} = \text{Dried HFA weight} \times (1 - \% \text{ Ash})$

13.3 Determination of % analyte in solid analytical samples

$\% \text{ Analyte in a solid sample} = (\text{Weight of ash-free analyte} \div \text{Test portion dried weight}) \times 100$

13.4 Determination of % analyte in liquid samples

$\% \text{ Analyte} = (\text{Weight of ash-free analyte} \div \text{Liquid test portion weight}) \times 100$

14 Resin regeneration and column preparation

14.1 General

14.1.1 Preparation of chromatography resin

It is paramount that this preparation precedes the use of a new resin or discoloured used resin. Soxhlet-extract sequentially for 8 hours with methanol, diethyl ether, acetonitrile, and methanol at 2 hour intervals. If the resin will not be used immediately after preparation, store it in methanol.

14.1.2 Chromatography column preparation

Before column packing, rinse the methanol from the resin with deionized water^[8] using a slurry technique in a large beaker until free of methanol (less than 2 mg·l⁻¹ dissolved organic carbon). After packing 280 ml of the resin into a column, rinse 3 times, alternating from 0,1 N NaOH to 0,1 N HCl; this removes impurities which could otherwise be incorporated into the resin.

14.1.3 Chromatography Resin regeneration

Regeneration is accomplished by using a 4 cm × 25 cm chromatography column containing 280 ml of resin, rinse the resin in the column by pumping 2 column volumes of deionized water^[8] with a peristaltic pump into the top of the column, allowing the water to flow down by gravity only. Then pass 1 column volume of 0,1 M HCl through the bottom of the column.

14.2 Regeneration of hydrogen form H⁺ exchange resin

14.2.1 The *hydrogen form* H⁺ exchange resin is regenerated using a batch process by pouring a minimum of 500 ml of the resin into a 4 l beaker, pour off any liquid if present, cover the resin with 1 M HCl. Let it stand for a minimum of 30 minutes, stirring once every 5 min. Remove the excess HCl from the resin by pouring off the acid and covering the resin with deionized water^[8]. Stir vigorously with a stirring rod for 15 seconds, then let the resin and rinse water stand for 5 minutes. Pour off the water, cover the resin again with deionized water^[8] and stir vigorously.

14.2.2 Load 500 ml of the regenerated H⁺ exchange resin into a 5 cm × 50 cm column. Once loaded, wash with deionized water^[8] until the rinse water tests negative for the presence of Cl⁻ using AgNO₃.

Annex A (informative)

Procedure to confirm the presence lignosulfonates

A.1 General

The materials extracted from humified substances that are both alkali and acid soluble meet the classical definition for *fulvic acids*. However, this document defines the alkali and acid soluble materials as the *fulvic fraction* because there are numerous substances that fit that generalized operational definition. The *fulvic fraction* possibly will or possibly will not contain the analyte of interest; *hydrophobic fulvic acids*. As some materials in the market place are represented as *fulvic acids*, especially lignosulfonates, pre-screening for the presence of lignosulfonates might be necessary.

CAUTION — The hydrophobic absorbent resin used in this document is not compatible with lignosulfonates. Lignosulfonates do not quantitatively desorb from the resin and can permanently damage the resin.

A.1.1 Humic substances are either odorless or have a slight petroleum-like smell. Lignosulfonates have a distinctive sulfurous odor because they are derived from wood lignins treated with sulfites. As organoleptic inspection is not conclusive, pre-screening for lignosulfonates is accomplished initially by analyzing for elemental sulfur(S).

A.2 Sulfur concentration

Total sulfur concentration in lignosulfonates is typically $\approx 5\%$. As the concentration of sulfur in humic substances is typically $<1\%$, with an average of $\approx 0,6\%$. Any product that exceeds $0,75\%$ total elemental sulfur (S) can be tested by Fourier Transform Infrared Spectroscopy (FTIR) analysis to confirm the absence or presence of lignosulfonates.

A.3 FTIR analysis

Fourier Transform Infrared Spectroscopy (FTIR) is based on the principle that absorption of infrared light causes chemical bonds to bend and stretch. Mid-FTIR records the energy released as wavenumbers between $4\,000\text{ cm}^{-1}$ and 400 cm^{-1} . The observed absorption peaks of the infrared spectrum of a substance correspond to the frequencies of vibrations between the bonds of the atoms making up a material. As no two compounds produce the same exact infrared spectra, FTIR analysis is commonly used to positively identify substances. Numerous commercial laboratories offer FTIR spectral analysis and will provide interpretation reports.

A.3.1 Spectra of sulfonate bonds

The sulfur-oxygen bonds in lignosulfonates demonstrate characteristic symmetric stretching of sulfoxide single S=O bonds at $1\,030\text{ cm}^{-1}$ to $1\,041\text{ cm}^{-1}$, symmetric stretching of O=S=O at $1\,150\text{ cm}^{-1}$ to $1\,200\text{ cm}^{-1}$, and asymmetric stretching of O=S=O sulfonate bonds at approximately $1\,330\text{ cm}^{-1}$ to $1\,430\text{ cm}^{-1}$. The presence of lignosulfonates is difficult to detect by Mid-FTIR at concentrations $<500\text{ mg}\cdot\text{kg}^{-1}$ in complex mixtures. However, lignosulfonates lack the distinct sharp peak of C=O stretch of aliphatic carboxylic acids at $1\,700\text{ cm}^{-1}$ to $1\,730\text{ cm}^{-1}$, and the slightly weaker peak of C=O stretching at $1\,680\text{ cm}^{-1}$ to $1\,710\text{ cm}^{-1}$ of aromatic carboxylic acids typically associated with carboxylic acids. Therefore, the absence of these peaks can be used to rule out the presence of hydrophobic fulvic acids rather than confirm the presence of lignosulfonates. See [A.3.4](#) for FTIR spectra of lignosulfonates.

A.3.2 Spectra of fulvic acids

Both humic acids and hydrophobic fulvic acids display similar spectral bands, with strong double peaks at approximately $1\,620\text{ cm}^{-1}$ and $1\,720\text{ cm}^{-1}$. Wavenumbers in the range of $1\,600\text{ cm}^{-1}$ to $1\,710\text{ cm}^{-1}$ correspond to aromatic C=O stretching, and $1\,700\text{ cm}^{-1}$ to $1\,730\text{ cm}^{-1}$ are from aliphatic C=O stretching. The wave number at around $1\,720\text{ cm}^{-1}$ is much stronger in fulvic acids than humic acids because of the occurrence of more -COOH groups. These bands are either very weak or absent in spectra for lignosulfonates. Typically there are two weaker but distinct peaks at about $1\,220\text{ cm}^{-1}$ and $1\,400\text{ cm}^{-1}$ generated by C-O stretching ($1\,210\text{ cm}^{-1}$ to $1\,320\text{ cm}^{-1}$) and O-H plane bending ($1\,395\text{ cm}^{-1}$ to $1\,440\text{ cm}^{-1}$) for carboxylic acids. See A.3.5 for spectra of hydrophobic fulvic acids.

A.3.3 Specifications for FTIR analysis

Sample media: Water^[8]

Spectral wavenumber Range: Mid-IR ($4\,500\text{ cm}^{-1}$ to 600 cm^{-1})

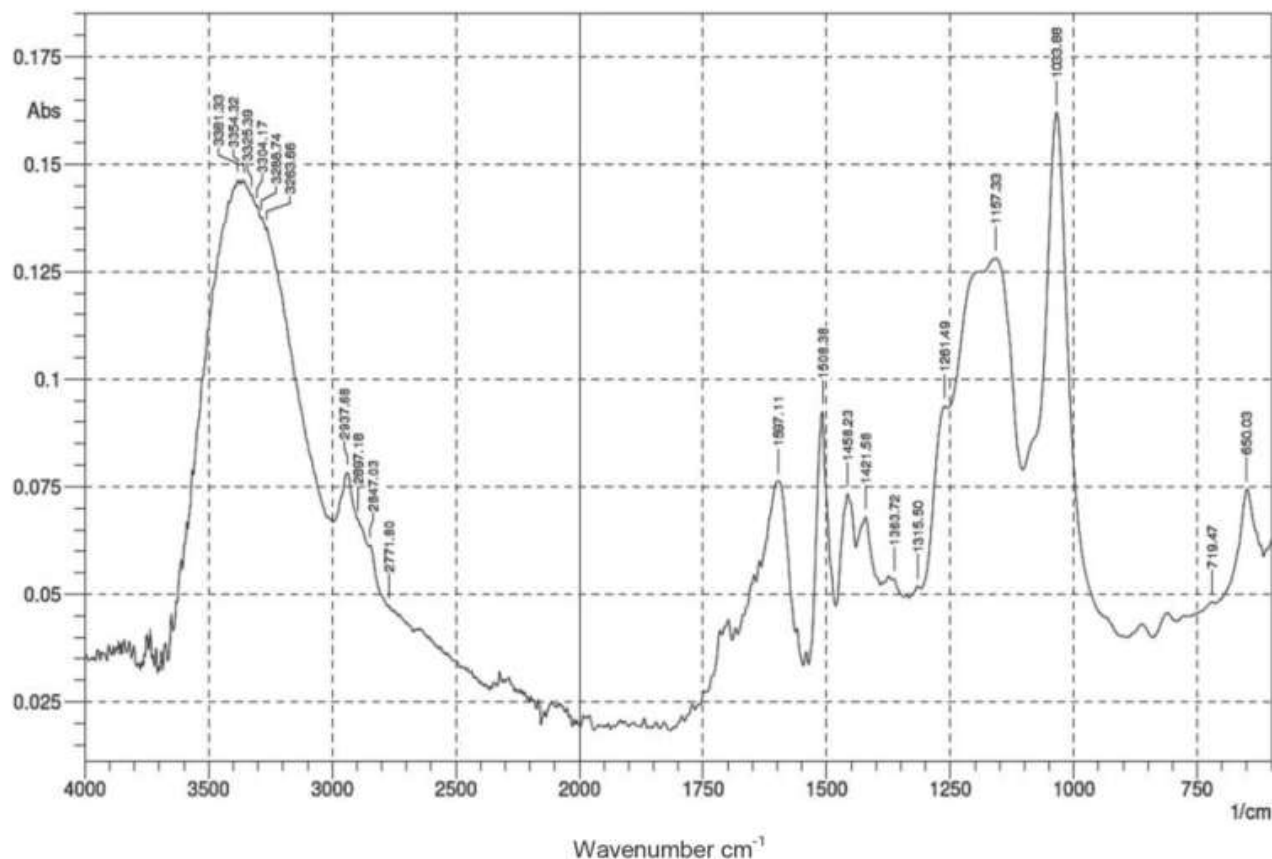
Apodization: Happ-Genzel

Resolution: 4 cm^{-1}

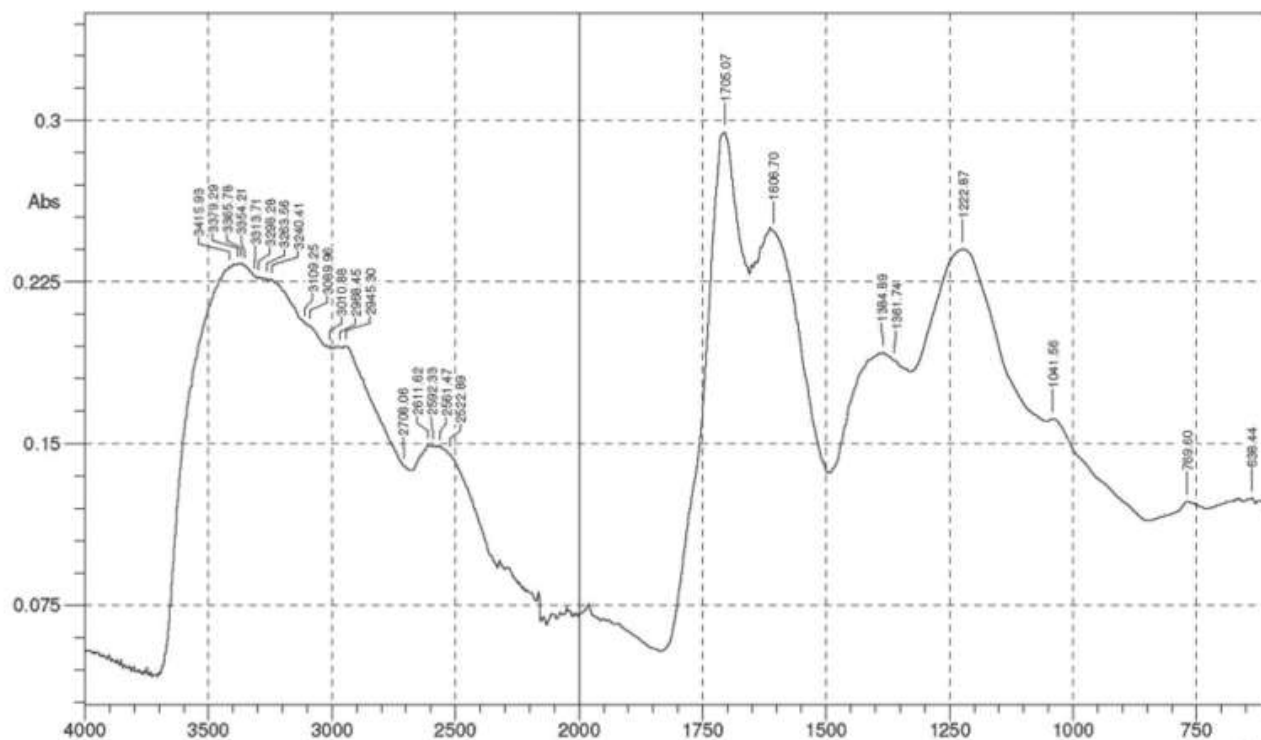
Number of Scans: 16 to 64

Temperature: 296 K

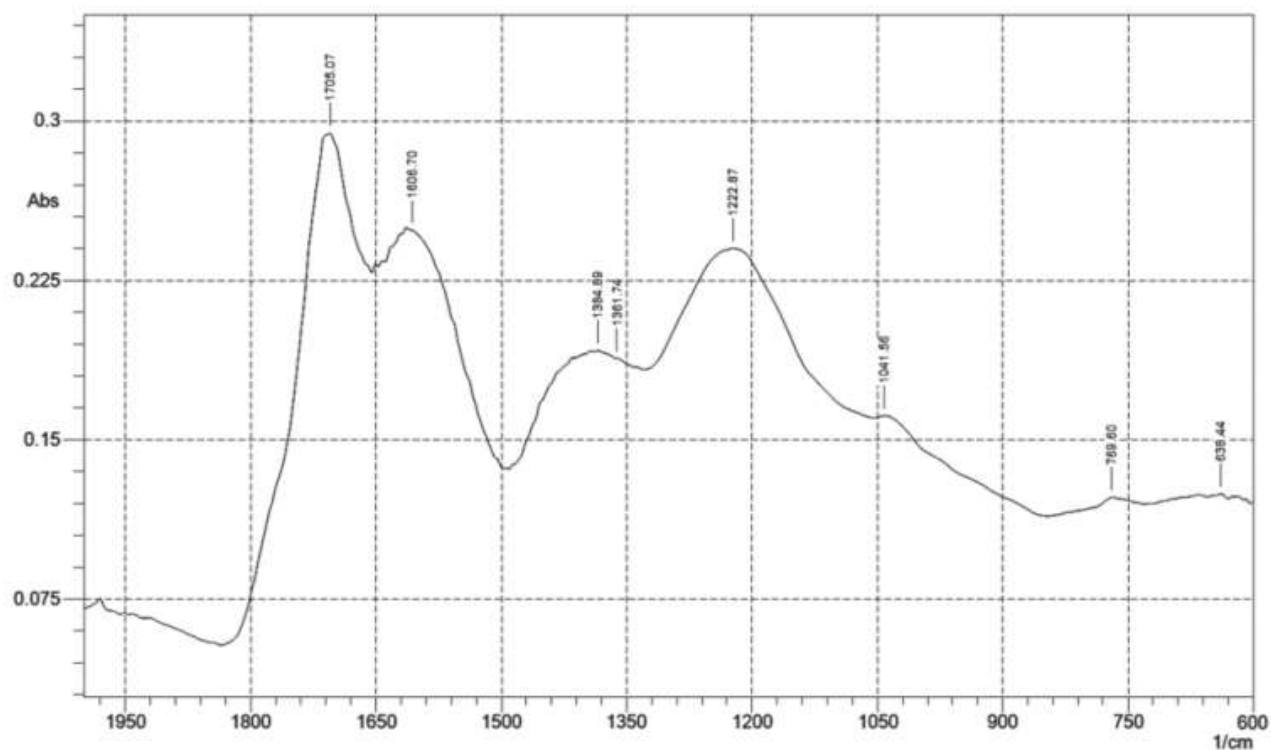
A.3.4 Representative spectra of a commercial lignosulfonate



A.3.5 Spectra of International Humic Substances Society 2S103F Suwannee River Fulvic Acid Standard



A.3.6 Detail of Mid-FTIR Spectra 600 cm⁻¹ to 1 900 cm⁻¹ of International Humic Substances Society 2S103F Suwannee River Fulvic Acid Standard



Annex B (informative)

ISO/CD 19822 interlaboratory study

The purpose of the ISO/CD 19822 interlaboratory test was to statistically analyze the data generated from the 11 participating laboratories for precision of the method as per ISO 5725-2:1994. "Precision" refers to the closeness of agreement between test results from the participating laboratories using the same method on the same materials to determine what difference in results can occur under actual testing conditions.

In February of 2016, letters of invitation were sent out to numerous laboratories asking them to participate in an interlaboratory study of an ISO analytical method for analyzing humic and hydrophobic fulvic acids in commercial products. 12 laboratories from 6 countries agreed to participate in the study and adhere to the following criteria:

- a) all essential apparatus, chemicals and other requirements specified in the method shall be available in their laboratory when the study begins;
- b) specified "timing" requirements, such as starting date and finishing date of the program, shall be rigidly met;
- c) the analytical protocols shall be strictly adhered to without any modifications
- d) a qualified operator shall perform the analysis;
- e) each analysis shall be carried out under repeatability conditions, i.e. on different days by the same operator using the same equipment;
- f) participants were instructed to report the results from the 4 replicated analyses without discarding or reworking the analyses regardless of how inconsistent the data might appear.

The interlaboratory study commenced in June of 2016 starting with a pre-study Phase 1.

Phase 1 consisted of analyzing *pre-study practice samples* using the ISO/CD 19822 procedure. This phase allowed laboratories to familiarize themselves with the method and to communicate any technical issues to the Study Director. The samples were supplied to the laboratories at no cost by the Humic Products Trade Association of the United States.

Phase 1 involved analyzing two sample materials; a solid mined material and a commercial liquid product. As the solid was previously homogenized and as the liquid product could be homogenized simply by shaking the sample in its container prior to removing an aliquot, sampling variability was reduced substantially. Sufficient materials were supplies to allow for numerous analyses.

Each laboratory was assigned a confidential laboratory number. Each laboratory reported the results of 4 replicate analyses for the two samples, along with comments and observations, to the Study Director. After subjecting the pre-study data to statistical analyses, the data was shared with all of the participating laboratories.

Preliminary statistical analyses of the Phase 1 pre-study results revealed high variability of the analyte reported. After reviewing the reported moisture in the solid sample and the ash reported for humic acids (HA), hydrophobic fulvic acids (HFA) in both samples, column volumes for elution, and quantity of DAX-8 resin used by each lab, some procedures were revised. The revisions were standardization of:

- Moisture determination of solid samples;
- Ash determination of humic acids (HA) and hydrophobic fulvic acids (HFA);

- HFA equilibration based on spectrophotometer absorbance;
- The number of column volumes for elution;
- Volume of DAX-8 resin packed into the chromatography column;
- Volume of Amberlite ion exchange resin;
- An option to use heavy duty high temperature centrifuge tubes in place of plastic tubes, eliminating the need to transfer materials to heat resistant crucibles.

The comments from the laboratories were compiled and distributed to the participating laboratories. The participants' technical recommendations were reviewed and incorporated into the draft standard to further refine the analytical procedure.

On July 15, 2016, Phase 2 of the interlaboratory study commenced.

Phase 2 involved the analysis of 4 commercial humic products with varying concentrations of moisture, ash, humic acids, and hydrophobic fulvic acids. The participants were instructed to perform 4 replicated analyses on different days using the same equipment for each sample performed by the same chemist. As the solid samples were crushed, screened, and homogenized by the study director, the participants were instructed to use the solid samples as received without further preparation. The liquid samples were homogenized by the chemist performing the analyses by shaking the samples for one minute in preparation for analysis.

Four different samples were shipped to the participants:

- The same solid mined material (QCM 1.1) that was used in Phase 1;
- A solid mined material (D2) from another mine with a reportedly lower concentration of HA than the QCM 1.1;
- A liquid extract (L2);
- A liquid extract (L3) with a reportedly 2× higher concentration of HA and HFA content than sample L2.

The Study Director applies statistical analyses to the data submitted by the laboratories and the compiled data along with statistical analysis was sent to all the participating laboratories for their inspection.

Results

The data reported by the participating labs were initially subjected to the same statistical analyses as were performed on the data from Phase 1 using the same statistical analytical software program to determine outliers. Possible near outliers were identified as observations further than $-1,5 \times \text{IQR}$ from the 1st quartile, and further than $+1,5 \times \text{IQR}$ from the 3rd quartile. Possible far outliers were defined as data further than $3,0 \times \text{IQR}$ from the quartiles. By comparison, the Phase 2 data for analyte concentration revealed substantially less variation than the Phase 1 data, suggesting the analytical chemists became more familiar with the procedure. [Table 1](#) below summarizes the mean (average) of for all the parameters tested, excluding outliers. The data and justification for determining outliers were presented to the ISO Technical Committee 134, Working Group 2 in Linyi, China, 15 November, 2016.

Repeatability and reproducibility were subsequently calculated using the ISO 5725-2, Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method loaded into a spreadsheet utilized by the ISO/TC 134 Working Group 2. The statistical analyses were transmitted to the working group via email in early 2017, and the comments generated by the ISO/TC 137 Working Group 2 were discussed at the next meeting in Giraju, Brazil, 11 June 2017.

[Table 2](#) lists the precision and repeatability of the analyte data from the interlaboratory study.

Table 1 — Mean concentration of moisture, ash, and ash-free analyte concentration in four sample materials

Sample I.D.	Moisture %	Ash in HA extract %	Ash in fulvic frac- tion extract %	Humic acids %	Hydrophobic fulvic acids %
QCM 1.1	9,03	15,28	11,11	59,08	2,37
D2	12,79	18,94	37,56	56,02	2,00
L2	—	15,32	50,76	5,76	0,69
L3	—	15,79	34,9	8,91	1,28

Table 2 — Precision as *r* (repeatability) and *R* (reproducibility)

Sample ID	Humic acids				Hydrophobic fulvic acids			
	<i>r</i>	<i>R</i>	<i>r</i> %	<i>R</i> %	<i>r</i>	<i>R</i>	<i>r</i> %	<i>R</i> %
QCM 1.1	4,08	9,98	6,91	16,9	0,55	2,63	23,26	110,9
D2	2,64	15,94	4,71	28,45	0,95	1,62	47,38	81
L2	0,78	1,42	13,61	24,63	0,5	0,96	72,9	139,2
L3	1,1	2,05	12,3	22,98	0,65	1,63	50,86	127,1

Bibliography

- [1] SWIFT R.S. 1996. Organic Matter Characterization. In SPARKS D.L. (eds.) *Methods of Soil Analysis. Part 3. Chemical methods*. Soil Science Society of America, Madison, Wisconsin, pp. 1018-1020
- [2] STEVENSON F.J. 1994. *Humus Chemistry; Genesis, Composition, Reactions*, 2nd Edition. John Wiley and Sons, Inc., New York
- [3] LAMAR R.T., OLK D.C., MAYHEW L., BLOOM P.R. 2014. A new standardized method for quantification of humic and fulvic acids in humic ores and commercial products. *Journal of AOAC International* **97**: 721-730
- [4] HAYES M.H.B., & GRAHAM C.L. 2000. Procedures for the isolation and fractionation of humic substances. In: GHABBOUR E.A., & DAVIES G. (eds.), *Humic Substances: Versatile Components of Plants, Soil and Water*. Royal Society of Chemistry, Cambridge, UK. p106
- [5] LEENHEER J. A., & CROUÉ J.P. 2003. Characterizing dissolved aquatic organic matter. *Environmental Science & Technology* **37**: 18A-26A
- [6] LEENHEER J. A. 2009. Systematic approaches to comprehensive analyses of natural organic matter. *Annals of Environmental Science* **3**: 1-130
- [7] SMITH B. 1999. *Infrared Spectral Interpretation: A Systematic Approach*. CRC Press, Boca Raton, FL
- [8] ISO 3696, *Water for analytical laboratory use — Specification and test methods*

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