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Is the traditional alkali extraction method valid in isolating chemically distinct humic acid?

Jianmei Zou [a,b], Huichun Zhang [b,*], Dongbei Yue [a,*], Jianzhi Huang [b]

[a] School of Environment, Tsinghua University, Beijing 100084, China
[b] Department of Civil and Environmental Engineering, Case Western Reserve University, Cleveland OH 44106, United States

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ABSTRACT

The validity of traditional humic acid (HA) extraction methods and the existence of distinct chemical structures of HA have been recently intensively debated because some researchers believe that HA only forms (humification) due to secondary synthesis during the extraction process. In this study, HAs were synthesized through abiotic humification of a polyphenol and an amino acid with and without MnO_2 as the catalyst. The synthesized HAs were first analyzed by Fourier-transform infrared spectroscopy and matrix-assisted laser desorption/ionization-time of flight mass spectrometry, and the results showed that the basic functional groups and molecular weight distribution of the synthesized HAs are similar to those of a standard peat HA from the International Humic Substance Society (IHSS). The products of homogenous abiotic humification (without MnO_2) both before and after alkali extraction were then examined by 1D ^{1}H and 2D ^{13}C-^{1}H nuclear magnetic resonance. Interestingly, the dominant features in the NMR spectra of the HAs before and after alkali extraction were largely comparable and no new structural signals were observed after the extraction, indicating that major structural features were not altered. This finding refuted the notion that the extraction method caused secondary synthesis and validated the extraction method of HA.

1. Introduction

Humic substances are natural organic products formed by extensive transformation of plant and animal residues [1]. They have long been recognized for their ability to influence soil properties [2] and the fate and transformation of contaminants in the environment [3–5], promote plant growth [6], and achieve sustainable soil N cycling [7]. Therefore, humic substances have been considered as excellent sorbents and organic amendments for environment management practices [7]. The formation of humic substances via either biotic or abiotic pathways in the environment is often referred to as 'humification' [8]. As a final stable product of humification, humic acid (HA) is a major fraction of humic substances and among the most recalcitrant fractions in soil [9]. The complex chemical structure of HA has been one of the least understood but most intriguing aspects of humus chemistry for more than 200 years [2]. To isolate HA from various sources such as soil, peat, and river before quantitative and qualitative analyses of the properties and structures of HA, the alkali extraction is the most widely employed approach [7,10].

Recently, the validity of the traditional alkali extraction approach has prompted intensive debates [7,11–13]. Based on the similarity between the nuclear magnetic resonance (NMR) spectra of biopolymers and an IHSS peat HA, Lehmann and Kleber (2015) proposed that the chemical structure of HA is a laboratory artifact [14] and HA is just a complex mixture of microbial and plant biopolymer residues [13]. They further concluded that secondary synthesis occurs during the traditional extraction process which is largely responsible for the distinct structures of HA [13,14]. Therefore, the authors suggested to discard the classical view of HA formation and proposed the Soil Continuum Model which views organic matter in the environment as "a continuum of progressively decomposing organic compounds…spanning the full range from intact plant material to highly oxidized carbon in carboxylic acids" [13].

The above opinions have faced increasing opposition from soil scientists and environmental researchers who continue to support the notion of "humification" [7,11,15–17]. So far, one of the most convincing findings to support the occurrence of humification processes is from Cao and Schmidt-Rohr (2018), who compared the spectra of the IHSS peat HA with those of four biopolymers by conducting quantitative solid-state ^{13}C NMR analyses [15]. Their results demonstrated that the structure of the HA, distinctly different from those of the biopolymers, has clear unique structural patterns with abundant oxygen-bonded nonprotonated carbons such as aryl ketone and carboxylic acid groups [15].

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* Corresponding authors.
E-mail addresses: hjz13@case.edu (H. Zhang), yuedb@tsinghua.edu.cn (D. Yue).

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However, the IHSS peat HA has to be prepared by the traditional extraction method so the findings cannot be directly used to address the claim that it was the extraction method that induced 'humification' through secondary synthesis [12]. It is important to note that the soil environment is extremely complex and heterogeneous, resulting in HA often mingled with mineral and colloidal components. Therefore, the structure detection of HA necessarily relies on strict extraction methods. For instance, some studies compared the ^{13}C NMR spectra of the extracted materials with those of the original soil materials and demonstrated no change in the structure of the humic substance during the extraction process [2,18]; however, the original soil samples were still treated with 2% HF which might have altered the materials. Unfortunately, one core issue of the debate happens to be the reliability of the extraction method. To eliminate the need for any extractions, non-invasive and in situ techniques should be explored for the analysis of the formed organic matter to provide new insight to humification processes [19].

The goal of this study was to examine whether the most commonly employed alkali extraction method can alter the structure of HA. To achieve this goal, we first need to synthesize HAs that are structurally similar to common HAs but can be characterized before and after alkali extraction. Therefore, we investigated abiotic humification [20] reaction with catechin (a plant polyphenol) and glycine (an amino acid) as the model precursors in the presence or absence of MnO_2, and compared the structural features of the obtained HAs from the reaction mixtures before and after alkali extraction. Abiotic humification was selected because of its unique controllable features, for instance, we can process humification reaction in homogenous solution so that in situ analyses of the formed organic matter become achievable. Indeed, our recent work was able to use two-dimensional (2D) correlation spectroscopy with UV–vis, a non-destructive measurement, to monitor the evolution of the humification process during the oxidative polymerization of catechin and glycine. The results proved that under the examined experimental conditions, humification did occur to form fulvic and humic acids progressively [21]. To better characterize the synthesized HAs, we used the HA produced in the presence of MnO_2 as the reference, because MnO_2 has been well known to catalyze abiotic humification reaction between polyphenols and amino acids [8,22–24]. In the above reaction, MnO_2 reportedly works mostly as a catalyst in the formation of HAs – a stable product in abiotic humification [25] – and only plays a minor role as an oxidant. Moreover, MnO_2 has been shown to mediate oxidation of fulvic acids (the intermediate product during abiotic humification) to either yield low-molecular-weight organic substrates [26–28] or continuously polymerize to form HAs [21,25,29]. The structural features of the synthesized HAs and the IHSS peat HA, a widely examined standard HA [14], were compared in terms of the functional groups and molecular size distribution based on Fourier-transform infrared spectroscopy (FTIR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), respectively. 1D ^{1}H and 2D ^{13}C-^{1}H NMR spectra were further collected to help compare the structural features of the original reaction mixture and corresponding purified HA, both being synthesized in homogeneous humification in the absence of MnO_2. Overall, this work offered new insights into the validity of the extraction method of HA. The findings will be a major milestone in the debate on the distinctive nature of HA.

2. Materials and methods

2.1. Chemicals and materials

Analytical grade catechin, glycine and sodium chloride were obtained from Sigma–Aldrich in China. γ-MnO_2, thiomersalate, sodium hydrogen phosphate and disodium hydrogen phosphate were purchased from the Sinopharm group of China with the reagent purity grade of >99%.

2.2. Incubation experiments

The abiotic humification experiments were conducted in 170-mL of autoclaved phosphate buffer (0.2 M, pH = 8) containing 0.008 M catechin and 0.02 M glycine in the presence or absence of MnO_2 in 250-mL Erlenmeyer flasks, incubated at 45 °C with constant mixing at 200 rpm for 160 h. The sample preparation was the same as in our recent work [21,25], and the standard alkali extraction method recommended by the IHSS [30] was used to purified HAs from the reaction mixtures with or without MnO_2. The above purified HAs and the un-extracted homogeneous reaction mixture were then vacuum freeze-dried and ready for characterization. A detailed description of the sample preparation can be found in Text S1 in the supporting information (SI).

2.3. Sample analysis

The freeze-dried HA samples (10 mg each) were mixed with 150 mg predried, pulverized spectroscopic-grade KBr, ground, and pressed under the irradiation of an infrared lamp to eliminate moisture in the samples. The absorbance spectra were recorded in the 4000 to 400 cm^{-1} range on a Perkin–Elmer 16F PC FTIR spectrophotometer and each spectrum was obtained after 32 scans with a 4 cm^{-1} resolution. The obtained FTIR spectra were smoothed and baseline corrected. The MALDI-TOF MS measurements were performed on a Bruker Reflex III MALDI-TOF mass spectrometer (Germany) with a nitrogen UV laser (337 nm, 3 ns) irradiation in the positive-ion reflection mode. The reflection was used with an acceleration voltage of 20.0 kV. Samples were prepared by dissolving 5 mg of freeze-dried HAs in 1 ml of 0.1 M NaOH. The matrix 2,5-dihydroxybenzoic acid (DHBA) was dissolved in 30% aqueous acetone to enhance ion formation. All spots were prepared by mixing 1 μl of the sample with 0.5 μl of DHBA. The resulting solution (1.5 μL) was evaporated and then introduced into the mass spectrometer.

From the previous research [14], we have learned that HAs in the fully protonated form can be well dissolved in dimethyl sulfoxide (DMSO). Therefore, the IHSS HA was re-dissolved in 0.1 M NaOH, and a cation exchange resin (Amberlite 1200H) was used to exchange the Na^+ ion with H^+. After being freeze-dried, samples of 50 mg HAs – including the raw reaction mixture, purified HAs, and standard IHSS HA – were thoroughly dried in a vacuum with P_2O_5, dissolved in 600 μL DMSO-*d6*, and transferred to a 5 mm NMR tube for analysis. 1D ^{1}H NMR experiments were carried out on a Bruker Avance 600 MHz instrument. 2D ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) NMR spectra were collected on a Bruker Avance 800 MHz while using the Echo/Antiecho-TPPI gradient selection in the phase-sensitive mode without sensitivity enhancement. The HSQC spectra of ^{1}H–^{13}C were collected by employing a relaxation delay of 2 s, and 2048 scans for each of the 256 increments with 2 K data points. The process used sine-squared functions with a phase shift of 90° The NMR spectra were processed using MestReNova software (version 14.1.2).

3. Results and discussion

3.1. Comparing the characteristics of the synthesized and standard HAs

The structure of HAs is complex, containing carboxyl, phenol, ester, ketone, hydroxyl, amino, and other functional groups, all of which can be identified by FTIR [31,32]. The FTIR spectra in the 4000–400 cm^{-1} region of the two synthesized HA products extracted from the reaction systems of catechin-glycine-MnO_2 (synthesized HA-p) and catechin-glycine (synthesized HA-a) and the standard IHSS HA are illustrated in Fig. 1a. The IR spectra of the two synthesized HAs are largely similar to those of the IHSS HA. They all show a broad peak at 3250–3500 cm^{-1}, indicating that the HAs contain -C-C-, –OH, –NH_2^+, –CO–NH_2^+, and –CO–NH– groups [33]. There are two strong absorption bands around 1550–1750 and 1200–1250 cm^{-1}, assigned to the –C=O of amides or quinone ketones, and the –C–O and –OH deformation bands of –COOH

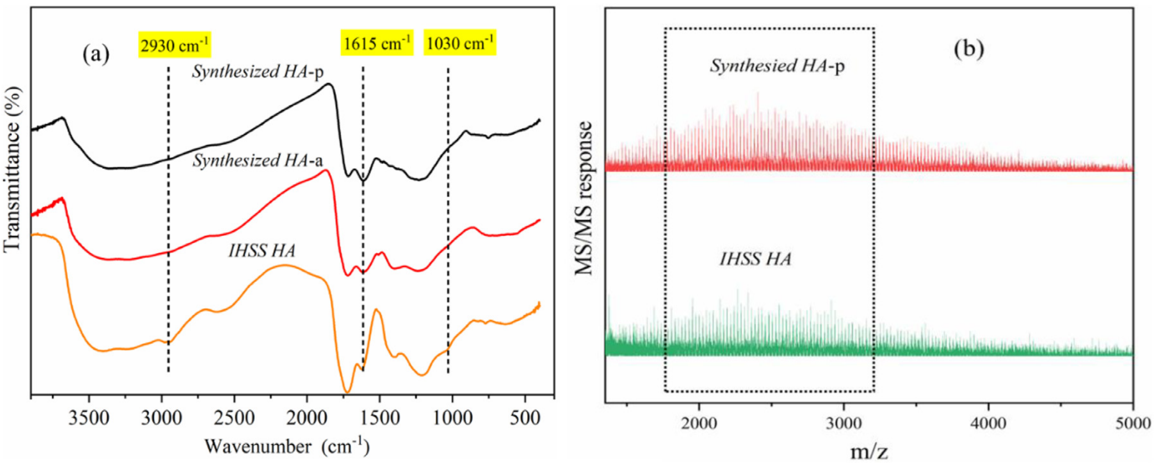

Fig. 1. Comparison of (a) the FTIR spectra of the two synthesized HAs and standard HA, and (b) the molecular weight distribution of one synthesized HA and the standard HA. Reaction conditions: catechin 0.008 M, glycine 0.02 M, initial pH 8.0, and in the presence (synthesized HA-p) or absence (synthesized HA-a) of 2 g of MnO_2 at 45 °C, reaction time 160 h.

[34]. In addition, there are some minor differences between the HAs. The IHSS HA has stronger aliphatic signals as reflected by the higher absorption intensity of the peaks at 2930 and 1030 cm^{-1}, whereas the synthesized HAs show slightly stronger absorption intensity at 1615 cm^{-1} in the aromatic region, suggesting that the synthesized HAs likely have more aromatic components than the standard IHSS HA, which is not surprising given that a polyphenol is one of the precursors in the synthesized HAs.

To further support the similar characteristics of the synthesized and standard HAs, we employed MALDI-TOF MS, a powerful tool for characterizing natural macromolecules [35], to examine the molecular weight distribution. Compared with the previous mass spectrometry studies on HA analysis by conventional electron impact ionization resulting in abundant fragmentation but little information on the overall molecular weight [36,37], the soft ionization technique in MALDI-TOF MS can overcome the above limitation. Most of the peaks of both the synthesized HA-p and standard HA were observed between 1500 and 3000 m/z (Fig. 1b). This result is comparable with a reported molecular weight range from 225 -3000 m/z [38]. As such, our results confirmed that the structure and composition of the synthesized and standard HAs are likely similar [39] so that the synthesized HAs can be used to examine whether the traditional extraction method of HA is valid, as shown below.

3.2. Comparison of the structures of HA before and after alkali extraction with ^1H NMR and 2D-NMR (HSQC)

Compared with the reaction system of catechin-glycine-MnO_2 (Figure S1), the catechin-glycine reaction system produced more abundant dissolved HA of up to 5043 mg/L. Because HA in the reaction system of catechin-glycine-MnO_2 often mixes with the MnO_2 particles which would interfere with NMR detection, the catechin-glycine reaction system was used to investigate if the extraction method of HA is valid. When interpreting the 1D ^1H NMR spectra of the synthesized HAs before and after alkali extraction (Figure S2), we noted that even though effective water suppression was conducted during the data processing, a small amount of water and DMSO solvent still produced large peaks at around 3.25 and 2.5 ppm, which suppressed the relative signals of the HAs. We then selected HA related signals, as shown in Fig. 2 (the entire spectra are shown in Figure S2). Interestingly, the 1D ^1H NMR spectra of the purified HA and reaction mixture are also comparable. Specifically, they both have three major regions in the proton spectra: 0.5 - 1.5 ppm for protons on aliphatic methyl and methylene carbons; 5.5 to 7.5 ppm for protons related to aromatic protons; and 8.5 -10.5 ppm for protons related to aldehydic and carboxylic proton [40]. Even though the signal intensity in the purified HA is much weaker than that in the reaction mixture, this still provides strong evidence that the extraction process itself did not generate any significant 1D ^1H NMR signals, which seems to suggest there is no secondary synthesis during the extraction.

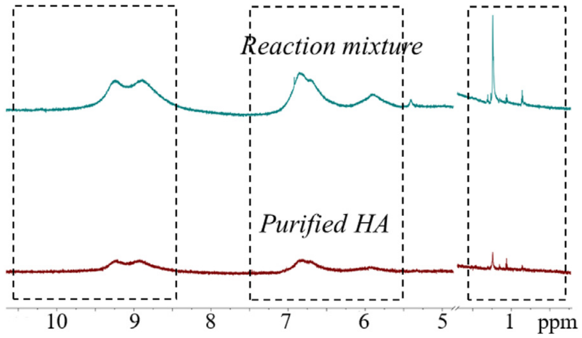

Fig. 2. Comparison of ^1H NMR spectra of the reaction mixture and purified HA between 0.4–1.7 ppm, and between 4.8–11.6 ppm.

To further validate the extraction method, we conducted 2D HSQC experiments, an advanced solution NMR approach with much better detection sensitivity, for extensive periods so that even very weak NMR signals were detected. Previous work has verified the ability of 2D HSQC spectra to reveal majority of structural features of HA [14]. A cross-peak in 2D HSQC spectra represents the chemical shifts of ^{13}C and ^1H in a ^{13}C-^1H bond, which characterizes a specific structure or a class of structural features of the HA. The spectra of the major structural units of the reaction mixture and purified HA are shown in Fig. 3a and 3b. The highlighted five regions in Fig. 3c are the basic assignments for the overly of the above two spectra: 1. aliphatic linkers and aliphatic side chain residues; 2. amino acid r-protons in peptide chains; 3. linkers between aromatic rings; 4. anomeric units; and 5. aromatic rings [14,40]. The overlay (Fig. 3c) reveals that the dominant spectra of the HA before (Fig. 3a) and after (Fig. 3b) alkali extraction have similar patterns, indicating that the major structural units of both samples are comparable. Only the highlighted area with the black box (region 2) in Fig. 3c, related to the signals of amino acid r-protons, is underrepresented in the purified HA spectrum, likely because glycine as a precursor did not fully participate in the abiotic humification reaction, which has been proven in a previous study [29]. Or, it may be related to the alkali extraction process which could partially affect some functional groups such as amino acids and aldehydes [10]. Overall, this result strongly validates the ex-

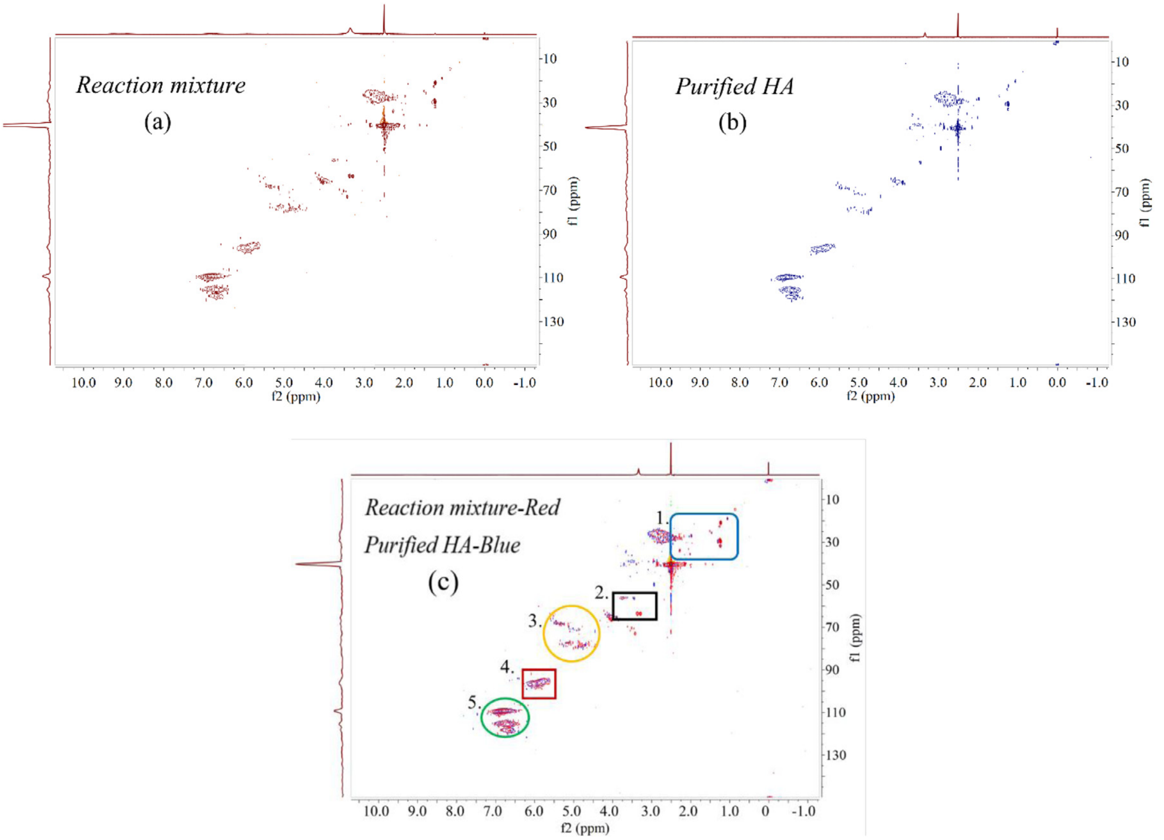

Fig. 3. 2D-NMR HSQC spectra with ^{13}C (f2) and ^{1}H (f1) chemical shifts of (a) the reaction mixture, (b) purified HA, and (c) the reaction mixture (red) overlaid on the purified HA (blue) (regions 1 – 5 in 3c are explained in the text).

traction method, and in turn challenges the idea that HA is simply a mixture of biopolymers artificially formed during improper extraction processes [12–14].

4. Conclusions

In summary, the results from FTIR spectra and MALDI-TOF MS of the synthesized HAs and the standard HA confirm that their structures and composition are likely similar, as already reported [39]. Therefore, the reaction mixture (before alkali extraction) and purified HAs (after alkali extraction) from abiotic humification reaction are suitable for examining whether the traditional extraction method of HA is valid. By comparing the 1D ^{1}H NMR and 2D HSQC NMR spectra of the reaction mixture and purified HA, we have identified that they are essentially the same. This new finding is significant in that it validates the classical extraction method which in turn challenges the idea that HA is artificially formed during improper extraction processes and is not chemically distinct.

Supporting Information

The supporting information is available free of charge.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ceja.2020.100077.

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