Interactions between stepwise-eluted sub-fractions of fulvic acids and protons revealed by fluorescence titration combined with EEM-PARAFAC

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HIGHLIGHTS

• Fluorescence titrations of FA sub-fractions with proton were visited by EEM-PARAFAC.
• Carboxylic/phenolic/protein-like chromophores were identified in PARAFAC components.
• pK_{a1} and pK_{a2} of fulvic-like components were 2.43–4.13 and 9.95–11.27, respectively.
• pK_{a1} and pK_{a2} of protein-like components were 3.33–4.22 and 9.77–10.13, respectively.

GRAPHICAL ABSTRACT

ABSTRACT

In aquatic environments, pH can control environmental behaviors of fulvic acid (FA) via regulating hydrolysis of functional groups. Sub-fractions of FA, eluted using pyrophosphate buffers with initial pHs of 3.0 (FA3), 5.0 (FA5), 7.0 (FA7), 9.0 (FA9) and 13.0 (FA13), were used to explore interactions between the various, operationally defined, FA fractions and protons, by use of EEM-PARAFAC analysis. Splitting of peaks (FA3 and FA13), merging of peaks (FA7), disappearance of peaks (FA9 and FA13), and red/blue-shifting of peaks were observed during fluorescence titration. Fulvic-like components were identified from FA3-FA13, and protein-like components were observed in fractions FA9 and FA13. There primary compounds (carboxylic-like, phenolic-like, and protein-like chromophores) in PARAFAC components were distinguished based on acid-base properties. Dissociation constants (pK_{a}) for fulvic-like components with proton ranged from 2.43 to 4.13 in an acidic pH and from 9.95 to 11.27 at basic pH. These results might be due to protonation of di-carboxylate and phenolic functional groups. At basic pH, pK_{a} values of protein-like components (9.77–10.13) were similar to those of amino acids. However, at acidic pH, pK_{a} values of protein-like components, which ranged from 3.33 to 4.22, were 1–2 units greater than those of amino acids. Results presented here, will benefit understanding of environmental behaviors of FA, as well as interactions of FA with environmental contaminants.

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Keywords: Binding, Functional groups, Fulvic-like components, Protein-like components, Dissociation constant

1. Introduction

Fulvic acid (FA) is a more mobile hydrophobic acid fraction of dissolved organic matter (DOM) ubiquitously existing in water and soil environments (Bai et al., 2015; Lehmann and Kleber, 2015). FA has...
potential effects on transport and bioavailability of nutrients, metals, polycyclic aromatic hydrocarbons, and other contaminants (Cabaniss and Shuman, 2002; Cai et al., 2016; Dai et al., 2006; Giesy et al., 1978; Giesy et al., 1983; Leversee et al., 1983; Wang et al., 2016; Xia et al., 2009). In various aquatic systems, factors including pH, temperature, and salinity can affect physical and chemical behaviors of FA (Gao et al., 2015; Pace et al., 2012; Sun et al., 2016; Yu et al., 2010b). Effects of increasing or decreasing pH on molecular structures, functionalities, conformations, and intermolecular interactions of FA with metal ions have been reported (Giesy, 1983; Giesy et al., 2010; Giesy et al., 1986; Midorikawa and Tanoue, 1998; Pace et al., 2012; Timko et al., 2015; Tipping, 2005; Yan et al., 2013). For example, based on fluorescence properties, it can be demonstrated that pH can affect molecular orbitals of excitable electrons and spherico-colloidal configurations of FA (Pace et al., 2012; Yan et al., 2013). The pH can affect molecular sizes of FA by partial disassembly of small groups at lower pHs, and formation of additional inter-/intra-molecular hydrogen bonds at neutral pH (Pace et al., 2012; Piccolo et al., 1999; Romera-Castillo et al., 2014). Dissociation constants of fluorescent ligands of DOM binding with protons are associated with the protonation of carboxylates, which, based on pH-dependence of fluorescence, are candidate ligands (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001).

Although mechanisms of interactions between FA and proton have been studied, due to the structural heterogeneity and variety of compositions, as well as the challenged operational separation of FA, it is not well understood (Bai et al., 2015; Lehmann and Kleber, 2015). Fractionating FA into sub-fractions can reduce their heterogeneity, which facilitates exploration of origin, structure and evolution, as well as interactions with chemical species. Successful isolation of standard sub-fractions of FA derived from Chinese sources was accomplished by use of stepwise elution from XAD-8 with pyrophosphate buffers. Detailed descriptions of isolation, fractionation and characterization of FA sub-fractions have been reported previously (Bai et al., 2015). Enrichment of protein-like substances was also reported in FA sub-fractions; however the possible contributions was also reported (Bai et al., 2015). Enrichment of protein-like sub-fractions were then diluted with KClO4 solution to 10.0 mg/L as stock solutions (Bai et al., 2015). The pH values of solutions were controlled sequentially at 0.5 pH units from 2.5 to 11.5 by adding small amounts of HClO4 or KOH. Each solution was continuously stirred by magnetic stirrer during fluorescence titration. All the solutions were purified for 15 min using nitrogen to avoid static quenching caused by oxygen and buffering effect of carbonate species during fluorescence spectra detection (Ryan and Weber, 1982). All chemicals were analytical reagent grade unless otherwise noted. All solutions were prepared in Milli-Q water then filtered through 0.45 μm filter membranes (Whatman, UK) before use.

2.2. Fluorescence spectral analysis

Fluorescence spectra of sub-fractions were measured using a fluorescence spectrometer (Hitachi F-7000, Tokyo, Japan) with a 1 cm path-length quartz cuvette at room temperature. EEM spectra were obtained by subsequently scanning emission (Em) wavelengths from 250 to 550 nm and excitation (Ex) wavelengths from 200 to 450 nm, both stepped by 2 nm intervals. Slit widths were 5 nm for both Ex and Em and scanning speed was set at 2400 nm-min⁻¹.

2.3. Parallel factor analysis

The three-way data of EEMs can be statistically reduced into trilinear terms and a residual array by using PARAFAC modeling (Stedmon and Bro, 2008) (Eq. (1)).

\[
x_{ijg} = \sum_{n=1}^{N} a_{ijg} b_{nj} c_{ng} + e_{ijg}, \quad i = 1, ..., I; \quad j = 1, ..., J; \quad g = 1, ..., G
\]

where: for EEM data, \(x_{ijg}\) represents fluorescence intensity of the \(i\)th sample measured at \(g\)th Ex wavelength and \(j\)th Em wavelength. Parameters \(a\), \(b\), and \(c\) represent concentration, Em spectra, and Ex spectra of fluorophores, respectively. \(N\) is the number of components and \(f\) represents the individual component in the samples. The \(e_{ijg}\) represents variability unexplained by the model.

PARAFAC modeling was separately performed on five sets of EEMs (19 samples for FA3, FA5, and FA13, respectively; 16 samples for FA5 and FA7, respectively) for the FA3-FA13 at various pH values by utilizing the DOMfluor (version 1.7) toolbox in MATLAB software (Stedmon and Bro, 2008). In addition, several important preprocessing steps were applied to minimize the effects of scatter lines of the EEMs. The EEMs of the KClO4 blank were subtracted from each sample EEMs. The region of no fluorescence (Ex ≪ Em) were inserted a series of zero values. The residual Rayleigh and Raman scatterings were regulated using interpolation methods derived from Bahram et al. (2006) (Bahram et al., 2006; Maqbool and Hur, 2016; Wu et al., 2011; Xu et al., 2013; Yu et al., 2010a). The PARAFAC models with 2–7 components were computed.
for the EEMs. Ex and Em loadings were constrained non-negative values (Lee et al., 2015; Maqbool and Hur, 2016; Murphy et al., 2013). The maximum fluorescence intensity (F) derived by use of the PARAFAC decomposition method, represented the relative concentration or intensity of the PARAFAC component (Maqbool and Hur, 2016).

2.4. Determination of dissociation constant

Binding parameters based on EEMs were successfully obtained to characterize interactions between DOM and environmental contaminant (Avendano et al., 2016; Cabaniss, 2002; Cabaniss and Shuman, 2002; Guo et al., 2015; Yamashita and Jaffé, 2008). Also, a 1:1 stoichiometric model has been employed previously to describe binding affinities between DOM and proton, as well as metal ions (e.g., Cu\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\)) (Midorikawa and Tanoue, 1998; Ryan et al., 1983; Ryan and Weber, 1982; Wu and Tanoue, 2001). Therefore, values for \(K_{a1}\) and \(K_{a2}\), calculated from reactions between PARAFAC components (L) and proton (H) at acidic or basic pH range, respectively, can be quantitatively described with 1:1 stoichiometric model (Eq. (2)).

\[
\log \frac{F_{H\text{-}\text{pH}} - F}{F_{L\text{-pH}}} = pK_{a} - pH
\]

where: quantities \(F_{H\text{-pH}}\) and \(F_{L\text{-pH}}\) obtained from MATLAB software fitting were \(F\) at the high and low values of pH in specific pH ranges, respectively. The \(pK_{a1}\) and \(pK_{a2}\) values were calculated with MATLAB software at acidic and basic pH range, respectively (detailed derivational information of Eq. (2) see Supporting Information Appendices A and B).

3. Results and discussion

3.1. General EEM fluorescence spectral of FA sub-fractions

EEMs have been provided for compositions of FA, associated with humic-like, fulvic-like, protein-like substances (Chen et al., 2003; Sun et al., 2016). EEMs and spectral parameters of FA sub-fractions are shown (Table 1 and Fig. 1). For EEMs of FA9 and FA13 at pH 3.0, two main peaks were observed at Ex/Em wavelengths of 290–320/410–450 nm (Peak A) and 230–270/400–450 nm (Peak B) (Table 1 and Fig. 1a and b). Peak B of FA9 tended to be resolved into two peaks at neutral (i.e. pH 7.0) or basic conditions (i.e. pH 11.0) (Table 1 and Fig. 1a2–a3). Three main peaks including Peak A, Peak B and Peak C (Ex/Em: 220–235/400–420 nm), were observed in EEMs of FA9 at pH 3.0 (Fig. 1c1). Peaks B and C of FA9 tended to merge into a single peak under basic conditions (i.e. pH 11.0) (Table 1 and Fig. 1c3). Peak D (Ex/Em: 260–275/310–320 nm) and weaker, less distinct peaks appeared at pH 5.0–9.0 for the EEMs of FA9. The similar four peaks for FA9 at pH 5.0–9.0 have been reported previously (Bai et al., 2015). Four peaks, Peak A, B, D and E (Ex/Em: 215–225/300–310 nm), were identified from the EEMs of FA9 and FA13 (Fig. 1d1–d3, e1–e3). Peak D and Peak E of FA9 and FA13 disappeared at basic pH range (i.e. pH 10.5–11.5). In addition, Peak B was broken into two peaks for FA13 under basic conditions (i.e. pH 10.0–11.5) (Table 1 and Fig. 1d3, e3). The splitting of peaks (FA9 and FA13) and merging of peaks (FA13) might be associated to the red/blue shifts of peaks and the various affinities of FA sub-fractions to proton (Chen et al., 2002; Wang et al., 2009). These phenomena should be investigated in the further study.

More detailed analyses showed that the Ex/Em of peak A of FA9 was 10–19/13–29 nm longer than that of FA13, and the Em of peak B of FA9 was 6–27 nm longer than that of FA13 for corresponding pH values (Table 1). The longer Ex and Em observed were related to greater amounts of conjugated aromatic π-electron systems with electron-withdrawing groups, such as carbonyl containing substituents and carboxyl constituents (Chen et al., 2002; Senesi et al., 1991; Wang et al., 2009). These results observed and presented here were consistent with the results of NMR analysis in previous study, indicating that FA9 contained more carbonyl/carboxyl groups than did FA13 (Bai et al., 2015).

Shifts in locations of peaks were complicated with changes in pH. With pH increasing from 3.0 to 11.0, blue shifts were observed for Peak A of FA3 (Ex for 9 nm and Em for 7 nm), Peak A of FA7 and FA9 (both Ex for 7 nm), Peak B of FA13 (Ex for 22 nm and Em for 4 nm), Peak B of FA9 (Ex for 6 nm) and FA13 (Ex for 6 and 32 nm); while red shifts were observed for Peak A of FA7 (Em for 5 nm), Peak A of FA9 (Em for 13 nm), Peak B of FA9 (Em for 22 nm), Peak A of FA13 (Em for 7 nm) and Peak B of FA13 (Em for 9 and 19 nm) (Table 1). These inconsistent shifts of peaks were likely related to conformational changes of compositions for FA sub-fractions at various pH values. Red and blue shifts were also observed for EEMs of standard HAs from the International Humic Substance Society and extracellular polymeric substances as a function of pH (Mobed et al., 1996; Sheng and Yu, 2006). Maximum peak intensities were observed under neutral conditions (Table 1).

3.2. PARAFAC analysis of FA sub-fractions

Spectra of individual components were successfully deconvoluted by use of PARAFAC analysis on the EEMs of each sub-fraction at various pH values. Appropriate numbers of individual components were identified by PARAFAC models residual analysis and split half analysis (Fig. S2) (Maqbool and Hur, 2016; Stedmon and Bro, 2008; Wu et al., 2015).

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flourescence spectral parameters of FA sub-fractions at pH 3.0, 7.0 and 11.0.</td>
</tr>
<tr>
<td>Samples</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>FA13</td>
</tr>
<tr>
<td>7.0</td>
</tr>
<tr>
<td>11.0</td>
</tr>
<tr>
<td>FA13</td>
</tr>
<tr>
<td>7.0</td>
</tr>
<tr>
<td>11.0</td>
</tr>
</tbody>
</table>

- data not available.

* Int.: fluorescence intensity with arbitrary unit (a.u.).
Fig. 1. Fluorescence EEMs of FA sub-fractions with arbitrary unit at pH 3.0, 7.0 and 11.0: FA9 (a1-a3); FA9 (b1-b3); FA7 (c1-c3); FA9 (d1-d3); FA13 (e1-e3).
The residual analysis and split half analysis have been widely used to determine the appropriate numbers of PARAFAC components derived from DOM (Wu et al., 2011; Xu et al., 2013). Three PARAFAC components were determined for FA3, FA5 and FA7, and four PARAFAC components were identified for FA9 and FA13 (Figs. 2 and S3).

Distributions of locations of peaks, component categories and primary compounds of PARAFAC components are shown (Table 2). Individual components identified by use of PARAFAC analysis were referred as Component 1 (C1, Ex/Em: 225–305/410–425 nm), Component 2 (C2, Ex/Em: 210–360/465–485 nm), Component 3 (C3, Ex/Em: 210–330/300–455 nm) and Component 4 (C4, Ex/Em: 220–275/320–325 nm) (Table 2). Based on distributions of Ex/Em of PARAFAC components reported previously (Chen et al., 2003; Wei et al., 2016; Zhang et al., 2011), the C1 (FA3-FA13), C2 (FA3, FA7 and FA13) and C3 (FA3-FA7) were categorized as fulvic-like components, and C3 and C4 of both FA9 and FA13 were categorized as protein-like components (Table 2).

Values of $F$ for each PARAFAC component at various pH values are presented (Figs. 3 and S4). For C1 (FA3-FA13), C2 (FA3, FA7 and FA13) and C3 (FA3) among fulvic-like components, $F$ values were directly proportional to pH in the range of 2.5–4.5 and reached a plateau at pH 4.5–9.5, then were inversely proportional to pH in the range of 10.0–11.5. The rapid increase and decrease of fluorescence intensities at certain pH ranges were also reported during measurements of fluorescence titration of DOM (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001; Yan et al., 2013). Due to deprotonation of carboxylic-like and phenolic-like chromophores, intensity of fluorescence increased from pH 2.0 to 5.0 then decreased from 8.0 to 10.0 (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001; Yan et al., 2013). Therefore, increasing values of $F$ at pH 2.5–4.5 were attributed to deprotonation of carboxylic-like chromophores, and decreasing values of $F$ values at pH 10.0–11.5 were due to ionization of phenolic-like chromophores of fulvic-like components. According to the theory of Yan et al. (2013), C1 (FA3–FA13), C2 (FA3, FA7 and FA13) and C3 (FA3) were primarily composed of both carboxylic-like and phenolic-like chromophores (Table 2).

Based on acid-basic properties, the same primary compounds were also reported for Suwannee River FA and Nordic Reservoir DOM (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001; Yan et al., 2013). The $F$ values increased insignificantly as pH was increased from 2.5 to 8.0, and then decreased rapidly as pH was increased from 8.5 to 11.5 for C3 (FA3). It can thus be speculated that C3 (FA3) was primarily composed of phenolic-like chromophores. Values of $F$ of C2 (FA3 and FA9) increased at pH values between 2.5 and 4.5, but exhibited less clear transitions at basic ranges (Fig. 3). Thus, C2 (FA5 and FA9) was primarily composed of carboxylic-like chromophores. C3 of FA7 was composed of other unknown compounds, which were also reported previously (Yan et al., 2013) (Fig. S4).

For protein-like components, values of $F$ of both C3 and C4 of FA9 exhibited four stages with pH 2.5–11.5, including a plateau at pH 2.5–3.5, a rapid increasing at pH 3.5–5.5, a plateau at pH 5.5–8.5, and a decreasing at pH 8.5–11.5 (Fig. 3). Values of $F$ values for both C3 and C4 of FA13 increased insignificantly at pH 2.5–8.5, and then decreased rapidly at pH 8.5–11.5 (Fig. S4). The C3 and C4 (FA9 and FA13) were likely associated to peptide bond of protein-like components. The insignificant
Table 2
Summary of fluorescence parameters of PARAFAC components.

<table>
<thead>
<tr>
<th>Components</th>
<th>Ex/Em locations</th>
<th>Component categories</th>
<th>Primary compounds in term of acid-base properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA3</td>
<td>C1 (235,265,300 nm)/425 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C2 (265,360 nm)/485 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C3 (210,260,330 nm)/450 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td>FA3</td>
<td>C1 (240,305 nm)/425 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C2 (210,250,350 nm)/475 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like</td>
</tr>
<tr>
<td></td>
<td>C1 (210,255 nm)/455 nm</td>
<td>Fulvic-like</td>
<td>Phenolic-like</td>
</tr>
<tr>
<td>FA7</td>
<td>C1 (225,255,300 nm)/410 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C2 (210,250,300 nm)/465 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C3 (225,260 nm)/405 nm</td>
<td>Fulvic-like</td>
<td>Other components</td>
</tr>
<tr>
<td>FA9</td>
<td>C1 225 nm/410 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C2 (210,260,340 nm)/465 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like</td>
</tr>
<tr>
<td></td>
<td>C3 220 nm/300 nm</td>
<td>Protein-like</td>
<td>Protein-like</td>
</tr>
<tr>
<td></td>
<td>C4 275 nm/325 nm</td>
<td>Protein-like</td>
<td>Protein-like</td>
</tr>
<tr>
<td>FA13</td>
<td>C1 (235,280 nm)/415 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C2 (210,265,350 nm)/470 nm</td>
<td>Fulvic-like</td>
<td>Carboxylic-like and phenolic-like</td>
</tr>
<tr>
<td></td>
<td>C3 (230,275 nm)/325 nm</td>
<td>Protein-like</td>
<td>Protein-like</td>
</tr>
<tr>
<td></td>
<td>C4 220 nm/320 nm</td>
<td>Protein-like</td>
<td>Protein-like</td>
</tr>
</tbody>
</table>

Increasing $F$ values of C3 and C4 for FA13 indicated that FA13 contained lesser amounts of carboxylic-like chromophores than FA9, which was consistent with recent results detected by NMR analysis (Bai et al., 2015). Also, for the $F$ values of FA9, the plateau stage (pH 2.5–3.5) was also observed in changes of fluorescence peak intensities with pH changing reported by Midorikawa and Tanoue (1998) (Fig. 3). The $F$ values of C1 (FA3 and FA9), C2 (FA3), C3 (FA9) and C4 (FA9) at pH 7.0 were lower than that on both sides. This phenomena was also observed by Sheng and Yu (2006) (Fig. 3), and it should to be further researched.

3.3. Determination of dissociation constants of PARAFAC components

The $pK_a$ of FA-proton had previously been determined by use of fluorescence titration at acidic pH (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001), however $pK_a$ values of PARAFAC components with protons have not been investigated. The $F$ values of PARAFAC components at acidic and basic pH ranges were fitted by Eq. (2), respectively (\(R^2 = 0.97–0.99\)). The $pK_{a1}$ and $pK_{a2}$ values of PARAFAC components with protons ranged from 2.43 to 4.22 and 9.77 to 11.27, respectively (Table 3).

At acidic pH, $pK_{a1}$ values of fulvic-like components (2.43–4.13) were comparable to that of FA-proton (2.28–4.42) based on fluorescence peak intensities (Midorikawa and Tanoue, 1998; Wu and Tanoue, 2001). The $pK_{a1}$ values of fulvic-like components were also close to those of di-carboxylate molecules (2.5–4.3), except for maleic acid (Martell and Smith, 1975; Smith and Martell, 1989). These results indicated that fulvic-like components were likely predominated by di-carboxylate functional groups. Moreover, conditional stability constants for PARAFAC components of DOM for Cu(II), Cd(II) and Hg(II), as determined by use of the Ryan-Weber equation, were $4.48–6.32$, $4.64–5.10$ and $3.92–6.76$, respectively (Guo et al., 2015; Wu et al., 2011; Yamashita and Jaffé, 2008). The lower $pK_{a1}$ values for binding of protons to fulvic-like components suggested that protons were bound relatively weaker than were some specific metal ions. At basic pH, $pK_{a2}$ values of fulvic-like components were in the range of 9.95–11.27, which were similar to those of hydroxybenzenes, which had a range of 8.0–10.0 (Midorikawa and Tanoue, 1998). This result indicated that the phenolic groups in fulvic-like components were likely to play a major role in binding of protons by FA at basic pH. The conditional stability constants value of the second ligand class in DOM (<9.0), which was obtained using fluorescence quenching titration with Cu(II) at pH 8.15 (Midorikawa and Tanoue, 1998), was lesser than $pK_{a2}$ values of fulvic-like components.

DOM with greater contents of carboxyl-like chromophores exhibited lesser $pK_{a1}$ values for protons (Midorikawa and Tanoue, 1998). Therefore, for fulvic-like components, the $C1$ (FA3, FA9, FA5 and FA13), $C2$ (FA5, FA13) and $C3$ (FA5) exhibited lesser $pK_{a1}$ values (2.43–3.40), which indicated more carboxyl-like chromophores. In addition, the $C1$ (FA9 and FA13) and $C3$ (FA9) among fulvic-like components with lesser $pK_{a2}$ values (9.95–10.12) suggested greater content of phenolic-like chromophores. These conclusions were consistent with the results of

![Image](image-url)
Table 3

Dissociation constants of PARAFAC components at acidic and basic pH.

<table>
<thead>
<tr>
<th>Components</th>
<th>At acidic pH range</th>
<th>At basic pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_\text{pH-max}$</td>
<td>$F_\text{pH-min}$</td>
</tr>
<tr>
<td>FA3 C1</td>
<td>527.0</td>
<td>328.6</td>
</tr>
<tr>
<td>FA3 C2</td>
<td>259.8</td>
<td>175.6</td>
</tr>
<tr>
<td>FA3 C3</td>
<td>228.3</td>
<td>179.8</td>
</tr>
<tr>
<td>FA3 C4</td>
<td>349.7</td>
<td>294.2</td>
</tr>
<tr>
<td>FA7 C1</td>
<td>108.7</td>
<td>87.9</td>
</tr>
<tr>
<td>FA7 C2</td>
<td>128.0</td>
<td>117.6</td>
</tr>
<tr>
<td>FA7 C3</td>
<td>386.7</td>
<td>356.4</td>
</tr>
<tr>
<td>FA7 C4</td>
<td>209.4</td>
<td>176.4</td>
</tr>
<tr>
<td>FA13 C1</td>
<td>379.3</td>
<td>17.9</td>
</tr>
<tr>
<td>FA13 C2</td>
<td>284.2</td>
<td>239.6</td>
</tr>
<tr>
<td>FA13 C3</td>
<td>108.5</td>
<td>18.0</td>
</tr>
<tr>
<td>FA13 C4</td>
<td>418.3</td>
<td>189.7</td>
</tr>
<tr>
<td>FA13 C5</td>
<td>277.7</td>
<td>134.5</td>
</tr>
<tr>
<td>FA13 C6</td>
<td>307.0</td>
<td>240.4</td>
</tr>
<tr>
<td>FA13 C7</td>
<td>109.3</td>
<td>88.0</td>
</tr>
<tr>
<td>FA13 C8</td>
<td>177.5</td>
<td>157.7</td>
</tr>
<tr>
<td>FA13 C9</td>
<td>135.2</td>
<td>115.2</td>
</tr>
</tbody>
</table>

+ data cannot be accurately estimated;  
$F_\text{pH-max}$: the maximum $F$ values with arbitrary unit of individual components derived from PARAFAC in specific pH range;  
$F_\text{pH-min}$: the minimum $F$ values with arbitrary unit of individual components derived from PARAFAC in specific pH range.

References


Supporting Information

Interactions between stepwise-eluted sub-fractions of fulvic acids and protons revealed by fluorescence titration combined with EEM-PARAFAC

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Supplementary caption

**Fig. S1.** Extraction and fractionation procedures of FA.

**Fig. S2.** Residual analysis and split half analysis for PARAFAC model validation for FA subfractions: FA$_3$:a1-a2; FA$_5$:b1-b2; FA$_7$:c1-c2; FA$_9$:d1-d2; FA$_{13}$:e1-e2.

**Fig. S3.** Identified PARAFAC components of FA$_5$, FA$_7$ and FA$_{13}$ with arbitrary unit: FA$_5$ (a1-a3); FA$_7$ (b1-b3); FA$_{13}$ (c1-c4).

**Fig. S4.** $F$ values of each PARAFAC component with arbitrary unit at various pH values for FA$_5$, FA$_7$ and FA$_{13}$: FA$_5$ (top); FA$_7$ (middle); FA$_{13}$ (bottom).

**Appendix A and Appendix B.** Detailed derivational information of equation (2) in manuscript.
**Fig. S1.** Extraction and fractionation procedures of FA.

1. **Extraction**
   - Extract FA from soils with 0.1 mol·L\(^{-1}\) NaOH
   - Acidify the FA extractant with 6 mol·L\(^{-1}\) HCl
   - Apply the supernatant to XAD-8 resin
     - Adsorbed fraction
     - Elute the resin with 0.1 mol·L\(^{-1}\) NaOH
       - Eluted fraction
         - Acidify with 6 mol·L\(^{-1}\) HCl
         - Purify the eluted and acidified FA
           - FA
             - Apply the FA to XAD-8 resin
               - Adsorbed fraction
                 - Elute the resin with 0.1mol-L\(^{-1}\) Na\(_2\)P\(_2\)O\(_7\) (pH 3.0)
                   - Adsorbed fraction
                     - Eluted sub-fraction
                       - Elute the resin with 0.1mol-L\(^{-1}\) Na\(_2\)P\(_2\)O\(_7\) (pH 5.0)
                         - Adsorbed fraction
                           - Eluted sub-fraction
                             - Elute the resin with 0.1mol-L\(^{-1}\) Na\(_2\)P\(_2\)O\(_7\) (pH 7.0)
                               - Adsorbed fraction
                                 - Eluted sub-fraction
                                   - Elute the resin with 0.1mol-L\(^{-1}\) Na\(_2\)P\(_2\)O\(_7\) (pH 9.0)
                                     - Adsorbed fraction
                                       - Eluted sub-fraction
                                         - Elute the resin with 0.1mol-L\(^{-1}\) Na\(_2\)P\(_2\)O\(_7\) (pH 13.0)
                                           - Eluted sub-fraction

2. **Fractionation**
   - Remove minerals (HF/HCl), pass through H\(^+\)-type cation exchange resin
   - Pass through H\(^+\)-type cation exchange resin, and freeze-dry (FA\(_3\))
   - Pass through H\(^+\)-type cation exchange resin, and freeze-dry (FA\(_5\))
   - Pass through H\(^+\)-type cation exchange resin, and freeze-dry (FA\(_6\))
   - Pass through H\(^+\)-type cation exchange resin, and freeze-dry (FA\(_3\))
Fig. S2. Residual analysis and split half analysis for PARAFAC model validation for FA subfractions: FA₃:a₁-a₂; FA₅:b₁-b₂; FA₇:c₁-c₂; FA₉:d₁-d₂; FA₁₃:e₁-e₂.
For FA\textsubscript{3}-FA\textsubscript{7}, the residual analysis showed a great improvement of fit from two to three component model, while little enhancement was observed from three to four component model, indicating three components were adequate for these data (a\textsubscript{1}, b\textsubscript{1}, c\textsubscript{1} in Fig. S2). For FA\textsubscript{9} and FA\textsubscript{13}, the residual analysis showed a great improvement of fit from three to four component model, while little enhancement was observed from four to five component model, indicating four components were adequate for these data (d\textsubscript{1}, e\textsubscript{1} in Fig. S2).

In addition, the similar curves of each pair of halves based on the split half analysis (a\textsubscript{2}, b\textsubscript{2}, c\textsubscript{2}, d\textsubscript{2}, e\textsubscript{2} in Fig. S2) further validated that three was the appropriate number of components for FA\textsubscript{3}-FA\textsubscript{7} and four was the appropriate number of components for FA\textsubscript{9} and FA\textsubscript{13}.
Fig. S3. Identified PARAFAC components of FA5, FA7 and FA13 with arbitrary unit: FA5 (a1-a3); FA7 (b1-b3); FA13 (c1-c4).
Fig. S4. $F$ values of each PARAFAC component with arbitrary unit at various pH values for FA5, FA7 and FA13: FA5 (top); FA7 (middle); FA13 (bottom).
Appendix A

Dissociation constants (pK$_{a1}$) of PARAFAC components were estimated from the increasing maximum fluorescence intensity ($F$) with the increase of pH at acid conditions. At acidic pH range, the reactions between PARAFAC component ($L_1$) and proton ($H$) can be quantitatively described with 1:1 stoichiometric model (Equations A1 and A2).

$$HL_1 \leftrightarrow L_1 + H$$  \hspace{1cm} A1

The dissociation constant, $K_{a1}$, can be expressed:

$$K_{a1} = \frac{[L_1][H]}{[HL_1]}$$  \hspace{1cm} A2

During fluorescence titration, the $F$ is associated with concentration ($C$) of PARAFAC component ($L_1$) by using the molar fluorescence coefficient ($\varepsilon$) (Equations A3-A5).

$$F_{H-pH} = \varepsilon_{L_1} \cdot C_{L_1}$$ at high pH  \hspace{1cm} A3

$$F_{L-pH} = \varepsilon_{HL_1} \cdot C_{L_1}$$ at low pH  \hspace{1cm} A4

$$F = \varepsilon_{L_1} \cdot [L_1] + \varepsilon_{HL_1} \cdot [HL_1]$$ at middle pH  \hspace{1cm} A5

$F_{H-pH}$ is for $L_1$, dissociated at high pH; $F_{L-pH}$ is for $HL_1$, dissociated at a low pH.

From mass balance of $C_{L_1} = [L_1] + [HL_1]$,

$$F_{H-pH} - F = (\varepsilon_{L_1} - \varepsilon_{HL_1}) \cdot [HL_1]$$ at high pH  \hspace{1cm} A6

$$F - F_{L-pH} = (\varepsilon_{L_1} - \varepsilon_{HL_1}) \cdot [L_1]$$ at middle pH  \hspace{1cm} A7

At acidic pH range, the $K_{a1}$ can be quantitatively described (Equation A8):

$$\frac{F_{H-pH} - F}{F - F_{L-pH}} = \frac{[HL_1]}{[L_1]} = \frac{[H]}{K_{a1}}$$  \hspace{1cm} A8

Thus,

$$log \frac{F_{H-pH} - F}{F - F_{L-pH}} = -log K_{a1} - pH = pK_{a1} - pH$$  \hspace{1cm} A9

The pK$_{a1}$ values were calculated by MATLAB software.
Appendix B

Dissociation constants \((pK_{a2})\) of PARAFAC components were estimated from decreasing maximum fluorescence intensity \((F)\) with the increase of pH at basic condition. At basic pH range, the reactions between PARAFAC component \((L_2)\) and hydroxyl \((OH)\) can be quantitatively described with 1:1 stoichiometric model (Equations B1 and B2).

\[
HL_2 + OH \leftrightarrow L_2 + H_2O \tag{B1}
\]

The conditional stability constant, \(K_t\), and dissociation constant, \(K_{a2}\), can be expressed:

\[
K_t = \frac{[L_2]}{[HL_2][OH]} = \frac{K_{a2}}{K_{ow}} \tag{B2}
\]

During the fluorescence titration, the \(F\) is associated to the concentration \((C)\) of PARAFAC component \((L_2)\) by using the molar fluorescence coefficient \((\varepsilon)\) (Equations B3-B5).

\[
F_{H-pH} = \varepsilon_{L_2} \cdot C_{L_2} \quad \text{at high pH} \tag{B3}
\]

\[
F_{L-pH} = \varepsilon_{HL_2} \cdot C_{L_2} \quad \text{at low pH} \tag{B4}
\]

\[
F = \varepsilon_{L_2} \cdot [L_2] + \varepsilon_{HL_2} \cdot [HL_2] \quad \text{at middle pH} \tag{B5}
\]

\(F_{H-pH}\) is for \(L_2\), dissociated at high pH; \(F_{L-pH}\) is for \(HL_2\), dissociated at low pH.

From mass balance of \(C_{L_2} = [L_2] + [HL_2]\),

\[
F - F_{H-pH} = (\varepsilon_{HL_2} - \varepsilon_{L_2}) \cdot [HL_2] \tag{B6}
\]

\[
F_{L-pH} - F = (\varepsilon_{HL_2} - \varepsilon_{L_2}) \cdot [L_2] \tag{B7}
\]

At basic pH range, the \(K_{a2}\) can be quantitatively described (Equation B8).

\[
\frac{F-F_{H-pH}}{F_{L-pH}-F} = \frac{[HL_2]}{[L_2]} = \frac{K_{ow}}{K_{a2}[OH]} = \frac{[H]}{K_{a2}} \tag{B8}
\]

Thus,

\[
\log \frac{F-F_{H-pH}}{F_{L-pH}-F} = -\log K_{a2} - pH = pK_{a2} - pH \tag{B9}
\]

The \(pK_{a2}\) values were calculated by MATLAB software.