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Characterization of binding between 17β-estradiol and estriol with humic acid via NMR and biochemical analysis

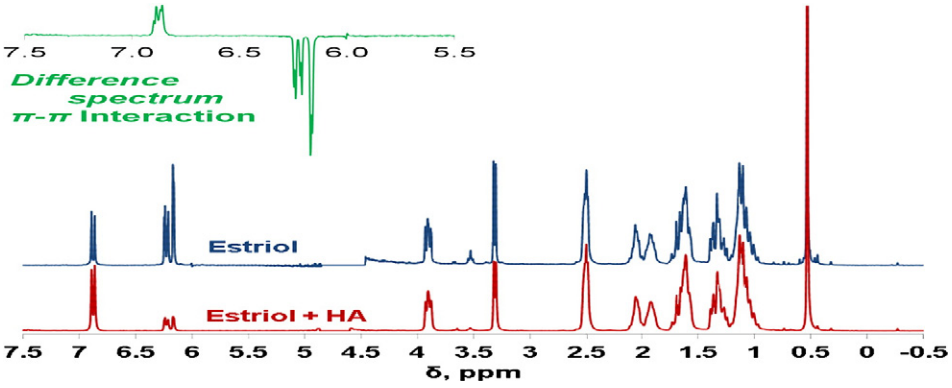
Mary Bedard [a], Kelly A. Giffear [a], Lisa Ponton [a,b], Karl D. Sienerth [a], Victoria Del Gaizo Moore [a,*]

[a] 100 Campus Drive, Department of Chemistry, Elon University, Elon, NC 27103, USA
[b] Currently at Baldwin Wallace University, Berea, OH, USA

HIGHLIGHTS

- NMR results demonstrate evidence of HA interaction at the aromatic ring of estrogen.
- As estrogen concentration increased, more binding with HA occurred.
- E2 pre-equilibrated with HA significantly decreased estrogenic activity.
- Unique correlation of results from ELISA, yeast assays and analytical NMR

GRAPHICAL ABSTRACT



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ABSTRACT

Endocrine disruptors, such as 17β-estradiol (E2) and estriol (E3), are ubiquitously found in the environment and their presence has gained recognition as a significant health risk. Sorption of estrogens by dissolved organic matter (DOM) influences their concentration and effects. Although there is some experimental evidence suggesting that sorption occurs through hydrophobic interactions, there is not a complete understanding of this association. Therefore, we sought to more extensively characterize binding between humic acid (HA), a major component of DOM, with E2 and E3 using nuclear magnetic resonance spectroscopy (NMR). Our results indicate that binding between these estrogens and HA occurs primarily at the aromatic ring, and takes place even at relatively low HA concentration. Specific interactions between E2 and HA were confirmed by assessing binding via ELISA assay, and the effect of binding on estrogenic activity was studied via a yeast bioassay. Together, these studies build upon previously theorized interactions, yielding a more comprehensive model of binding between estrogens and HA, as well as demonstrate the value of the confluence of biochemical assay methods with analytical NMR techniques.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a problem worldwide, having been detected in ecosystems of Europe [1], North America [2], and Asia [3] and in the United States. They have been identified as a major concern by the US Congress and the Environmental Protection Agency (EPA). In 1996 the EPA began screening environmental waters

* Corresponding author at: CB 2625, Elon University, Elon, NC 27244, USA. Tel.: +1 336 278 6221 (office).
E-mail addresses: mbedard@elon.edu (M. Bedard), kgiffear@elon.edu (K.A. Giffear), lponton@bw.edu (L. Ponton), sienerth@elon.edu (K.D. Sienerth), Vmoore3@elon.edu (V. Del Gaizo Moore).

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for EDCs [4,5] and estrogens, in particular, have been identified as such components [6]. Estrogens typically exhibit aqueous solubilities of less than 25 mg/L [7], thus estrogen concentrations in the environment are low. However, even trace amounts can disturb endocrine processes in animal models [6,8–11]. An estrogen concentration of 1 ng/L, for example, can alter sexual characteristics of aquatic life [12]. Many estrogen compounds are more broadly classified as pharmaceutical and personal care products (PPCPs) [13], and as such the disposal of excess or waste materials is not strictly regulated. Thus, significant amounts of such PPCPs have the potential to enter natural waters through municipal waste streams [13,14]. Understanding how estrogens interact with matter in the natural environmental is important for more precise assessment of the health risk that EDCs pose to both wildlife and humans, as well as for informing the medical and pharmaceutical industries about proper guidelines for dosage and disposal of PPCP-containing waste.

Dissolved organic matter (DOM) found in soil, water and sediments to varying degrees around the world is a consequence of natural plant decomposition. Humic substances, which represent between 50% and 80% of DOM, are comprised of the three main components: fulvic acid, humic acid (HA) and humin [15]. HA refers to a heterogeneous population of large organic molecules consisting of mainly carbon, hydrogen, oxygen, nitrogen and sulfur, although the degree of each element varies depending on location and can be significantly different between marine and freshwater [16]. HA and fulvic acid components of DOM are the most extensively dispersed, and contribute to the yellow-brown to black coloration of many natural water sources [17,18] as well as wastewater effluents [17,19]. HA may comprise up to 75% or more of the dissolved carbon in waters, such as in the Suwannee River in the United States [20], with percentages being influenced by seasons and weather events [18,20]. Research suggests that DOM influences the sorption of EDCs [21] and studies have shown that the presence of HA and nutrients in water influences the amount of biodegradation and transformation of estriol [22]. Furthermore, sorption and desorption of 17β-estradiol with the organic portion of natural sediment have been shown [23], indicating that interaction does indeed occur. Some early studies indicated that the degree of interaction with polyaromatic hydrocarbons increases with the aromaticity of the humic substances [24], and a later report demonstrated that one of the dominant modes of binding between xenoestrogens and e-receptors is through hydrogen bonding and π–π interactions via aromatic substructures [25]. Sun et al. demonstrated that the presence of estrogens affects the relative excitation–emission fluorescence spectra for a variety of humic substances [26]. Other workers used fluorescence quenching and associated methods to surmise that the interaction between estrogens and humic substances occurs primarily at the estrogenic aromatic ring [27]. The fluorescence quenching method is illustrative, but nuclear magnetic resonance (NMR) can be used to more specifically probe the binding sites among heteromolecules [28]. Therefore, while several studies have begun to explore the effect DOM has on the activity of EDCs in aqueous solution, a more concrete understanding of estrogen–humic substance interactions is of critical importance in order to guide thoughtful decisions on potential regulatory controls.

This study focuses on the binding characteristic between estrogens 17β-estradiol (E2) and estriol (E3) with HA. While it is understood that humic substances in general are far more complex than HA alone, for the purpose of clarity in the spectral studies undertaken we adopted HA as a simple but reasonable model for more complex systems. Furthermore, many studies use HA to examine the effect DOM has on the sorption of organic pollutants [29], and more recently with estrogens [30]. The molecular interaction of E2 and E3 with HA was examined using ^{1}H NMR. Then ELISA assays were utilized to measure the amount of E2 bound to HA after equilibration. Finally, to determine if their binding interferes with estrogenic activity, yeast estrogen screen (YES) bioassays comparing activity of estrogen solutions to that of pre-equilibrated estrogen and HA solution were conducted. This combination of analytical and biochemical techniques was employed to seek a more substantial understanding of binding between estrogens and HA.

2. Methods

2.1. Chemicals

The humic acid sodium salt, D-glucose, deuterium oxide 99.9%, and the selected estrogens (E2 and E3) at 98+%, and 97+% purity, respectively, were purchased from Sigma Aldrich. 3-Trimethyl sodium deuteroxide 40 wt.% solution in D_2O, 99+% atom D was purchased from Acros Organics. Both deuterium solvents were kept under nitrogen until sample preparation. E2 EIA kits were purchased from Enzo Life Science. Yeast nitrogen base powder without amino acids or carbohydrate, Drop-Out Mix Synthetic minus tryptophan and uracil, and Bacto Agar for growing the yeast were purchased from USBiological. Estrogenic activity was assayed using the Tropix® Gal-Screen® assay system from Applied Biosystems.

2.2. NMR sample preparation and spectroscopy

For NMR studies, NaOD was mixed with D_2O to a final concentration of 0.75 M and placed in a bath sonicator for at least 60 s. HA was then added to the NaOD/D_2O solution at a concentration of 0.5 mg/mL solution and the mixture sonicated for 120 s. Aliquots of 1 mL were then transferred to vials and estrogen added, resulting in saturated solutions. Samples were once again sonicated for 60 s, and then centrifuged for 5 min at 500 ×*g* to remove the excess estrogen. The resulting solution had a HA:estrogen concentration ratio of approximately 1:10 (see Results and discussion). Solutions having an HA concentration of 10 mg/mL (yielding HA:estrogen of 2:1) were prepared in a similar fashion. Aliquots of 0.25 to 0.5 mL were removed from the supernatant with a clean glass syringe and inserted into NMR tubes.

For the estimation of estrogen concentration in the saturated solutions, a bulk solution of 0.75 M NaOH was prepared, and then used as the solvent in preparation of 0.1 mg/mL, 0.5 mg/mL and 1.0 mg/mL calibration solutions. A saturated estrogen solution was prepared by adding 60 mg to 10 mL of the 0.75 M NaOH. Each solution was sonicated for 15 min and allowed to cool before being made to volume with the 0.75 M NaOH. The saturated estrogen solution was centrifuged for 2 min after which a 0.50 mL aliquot of the supernatant diluted to 10.00 mL with the NaOH solution. UV–Visible absorbance spectroscopy (0.1 cm path length) was used to prepare a calibration plot of absorbance at 298 nm vs. concentration (mg/mL).

All NMR spectra were collected on a 300 MHz Varian Mercury NMR using VNMR J 2.2d software. To produce the NMR spectra presented here in the normal mode (signal vs. ppm), raw data in the form of signal vs. frequency (Hz) was extracted from the computer and imported into Microsoft Excel™. While some preliminary spectra were collected using the pre-programmed PRESAT pulse sequence [31], the spectra represented herein were collected under standard proton conditions using 256 scans. The large H_2O peak occurring at approximately 5 ppm, which is ubiquitous in D_2O solutions that have been exposed even briefly to the atmosphere, was removed using Excel. The H_2O peak did not overlap any peaks associated with estrogen or HA but when present its magnitude overwhelmed peaks of interest. None of the spectra (HA alone, estrogens alone, mixtures) exhibited any features downfield of 7.5 ppm, so to provide better clarity in the NMR spectra, only the range of 0 to 7.5 ppm is shown.

2.3. ELISA binding studies

Immunoassays using antibodies against estrogens have been developed to take advantage of antibody–antigen binding to assess the

amount of estrogen present in a sample. E2 concentrated stock solutions were made by first dissolving the powder in a small amount of DMSO and then brought to volume in Milli-Q® water (> 16 Mohm). Samples for use in ELISA experiments were dilutions made from concentrated stocks. HA stock solution of 260 μg/mL was prepared in Milli-Q®.

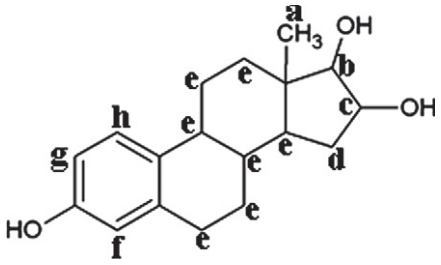
Binding reactions for each estrogen concentration were made up of equal volumes (0.5 mL each) estrogen stock solutions and HA solution to achieve final E2 concentrations of 0.1, 0.025, 0.0125 and 0.0005 mg/mL and 130 μg/mL HA, respectively, and allowed to equilibrate for 48–96 h (experimental) or 2 h (controls) at room temperature. Equilibration times for experimental reactions were chosen based on convenience of collecting data. Prior to analysis, each reaction solution was then diluted 1:100 in Milli-Q® water and processed according to the manufacturer's instructions. To account for any loss of estrogen due to degradation or sorption, the diluted estrogen solutions used in each reaction mixture were also analyzed. This control allowed the attainment of a true concentration of estrogen solution alone, so that unbound (free) estrogen used in the calculations came from a solution subjected to the same conditions (container, temperature, light exposure, etc.) Additionally, the stock HA solution was measured and used as a control to account for any background binding. Triplicate reactions for each estrogen concentration were set up, and duplicate measurements were taken for each reaction. The values analyzed and reported are means +/− standard deviations.

2.4. Yeast bioassay

Yeast co-transformed with a TRP-1 marked constitutive human ERα expression plasmid (pG/ER), a URA3-marked estrogen-inducible β-galactosidase reporter (pUCΔSS-ERE) plasmid, and the pleiotropic drug resistant gene (PDR5) (from here forward referred to as "estrogen sensitive yeast") were obtained from Dr. Heather Balsiger from the University of Texas. The four-hour yeast bioassay method described by Balsiger et al. [32] was followed. Briefly, single crystals from frozen glycerol stocks of individual recombinant estrogen sensitive yeast colonies were inoculated into sterile test tubes with 3 mL liquid media and shaken at 30 °C overnight. The next day, the cells were diluted to 10 mL with media and grown for another 24 h. On the second day estrogen:HA solutions were prepared by mixing equal volumes (typically 10 μL each) of different concentrations of E2 with HA, and equilibrated overnight. Since a recent study found that enzyme-catalyzed product formation in YES assays occurs in as little as 30 min after exposure to estrogen [33], pre-equilibration eliminated experimental differences based on order of addition or lag time between mixing of different samples. E2

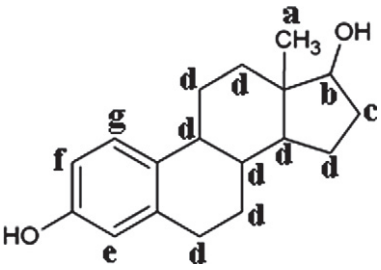
Table 1
Summary of observed peaks through NMR analysis of E2 and E3.

Estriol (E3)



Peak position (ppm)	Multiplicity	J-coupling (Hz)	Designated ^{1}H
0.54	Singlet	–	a
0.96–2.14	Series of multiplets	–	e
2.44–2.56	Multiplet (Broadened triplet)	–	d
3.31	Doublet	6.01	b
3.90	Triplet (Poss. triplet of doublets)	6.62	c
6.16	Doublet	–	f
6.23	Doublet of doublets	8.86	g
6.88	Doublet	8.86	h

17β-estradiol (E2)



0.51	Singlet	–	a
0.90–2.11	Series of multiplets	–	d
2.43–2.55	Multiplet	–	c
3.49	Triplet	8.72	b
6.16	Doublet	–	e
6.23	Doublet of doublets	8.63	f
6.88	Doublet	8.63	g

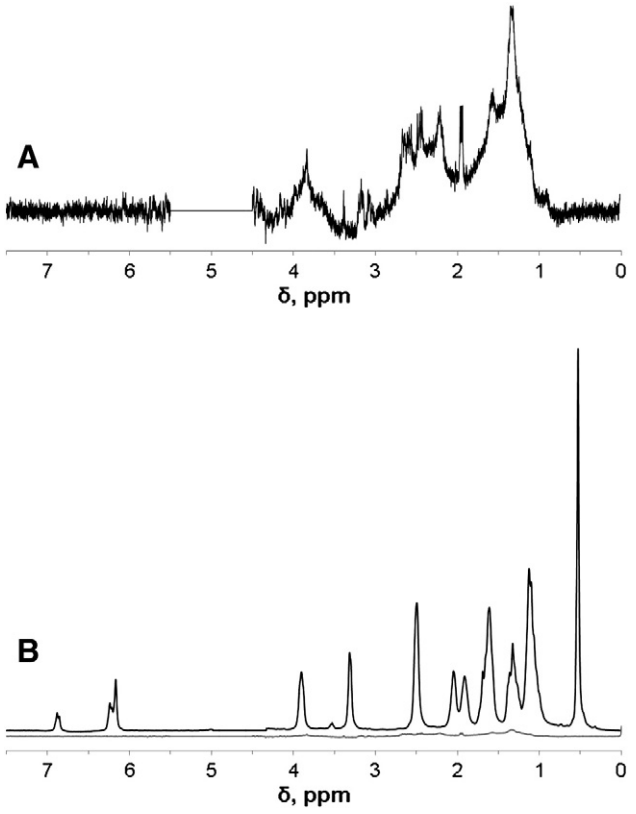


Fig. 1. NMR spectra of (A) 10 mg/mL humic acid in 0.75 M NaOD/D_2O and (B) the same HA spectrum (gray) overlaid with E3(saturated) + 10 mg/mL HA in NaOD/D_2O. The vertical scales in B are matched to demonstrate relative peak intensities.

stock solutions were purchased from Enzo Life Sciences and prepared for reactions by dilution with Milli-Q® water. The final concentrations in the reactions were 0.0150, 0.0075, and 0.00375 μg/mL E2 and 130 μg/mL HA. On the third day, the culture was diluted with media to an absorbance of OD_{600} 0.08 and incubated in a shaking incubator (200 rpm) at 30 °C until the cultures reached OD_{600} of 0.1, approximately 2.5 h later. Next, 100 μL of each growing colony was added to the appropriate amount of wells in an opaque 96-well plate. Then, 1 μL of either liquid media, estrogen solution alone, HA solution alone, or pre-equilibrated estrogen:HA solution mixture was added and the plate

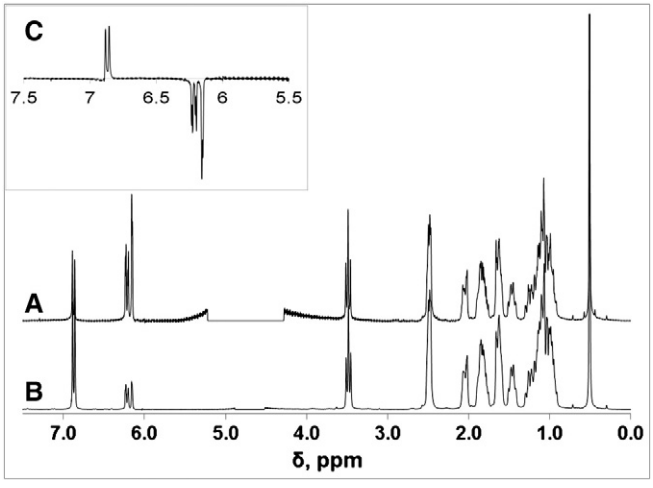


Fig. 2. ^{1}H NMR spectra in 0.75 M NaOD/D_2O (water peak removed) of (A) E2 (saturated); (B) E2 (sat) in the presence of 0.5 mg/mL HA; (C) difference of (B) − (A) in the 5.5–7.5 ppm region.

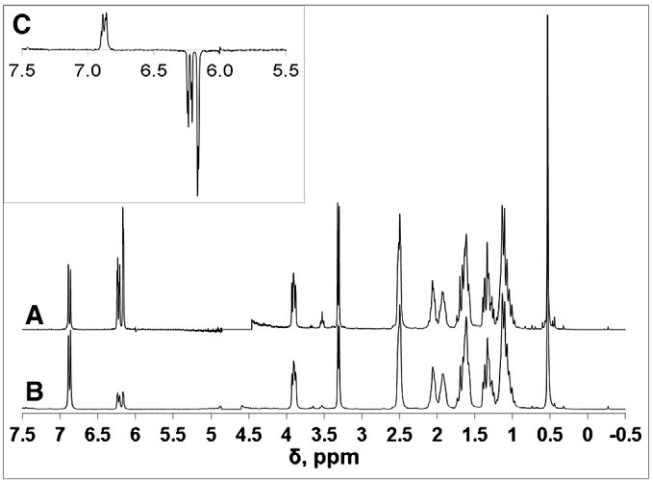


Fig. 3. ^{1}H NMR spectra in 0.75 M NaOD/D_2O (water peak removed) of (A) E3 (saturated); (B) E3 (sat) in the presence of 0.5 mg/mL HA; (C) difference of (B) − (A) in the 5.5–7.5 ppm region.

incubated at 30 °C for 2 h. Finally, 100 μL of prepared Gal-Screen Reagent was added to each well and incubated for an additional 2 h at 25 °C, at which time luminescence was read on a PerkinElmer EnSpire® multi-mode plate reader. Background luminescence was measured in wells that contained media only and wells consisting of 1:1 media and Gal-Screen Reagent. The amount of estrogenic activity in each sample was determined by comparing luminescence from experimental wells to that of wells containing original estrogen solutions as a control. All control and experimental samples were analyzed in duplicate.

2.5. Graphing and statistics

GraphPad Prism 6© was used to conduct all statistical analyses as well as make graphs for the binding assays and the yeast bioassay. Unless otherwise noted, the p values reported were derived from two-tailed, unpaired t-tests with a 95% confidence interval.

3. Results and discussion

3.1. NMR changes when estrogens are mixed with HA

NMR studies require that all materials to be analyzed are soluble, and in order to obtain spectra in a reasonable period of time, it is

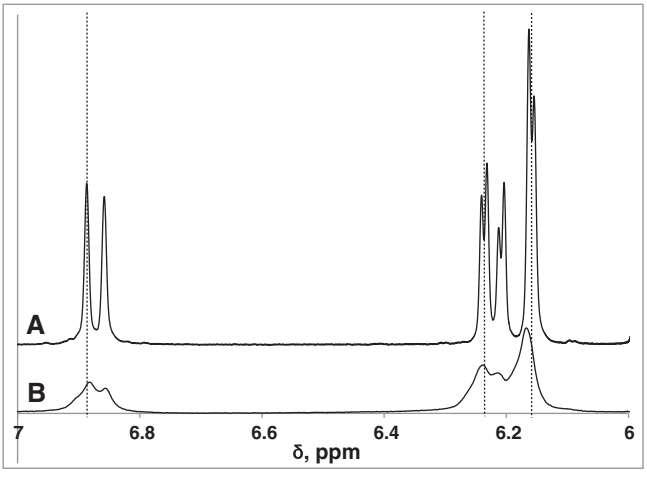


Fig. 4. ^{1}H NMR spectra in 0.75 M NaOD/D_2O of (A) E3 (saturated); (B) E3 (sat) in the presence of 10 mg/mL HA. Dashed lines are used to demonstrate peak shifts.

advantageous to use analyte concentrations in the parts-per-thousand range. To accomplish this goal, as well as to estimate the ratio of soluble estrogens and HA in the solutions used in the NMR studies, we began with adjusting the pH of the estrogen solutions. While the aqueous solubilities of estrogens are typically quite low [7], both E2 and E3 exhibit pKa_1 values of around 10.5 and so at least one hydroxyl group is deprotonated in 0.75 M NaOH (pH = 13.9), significantly increasing the solubility. Next, the UV–Visible spectrum of the E2 solution was examined and found to exhibit a strong absorption peak at 298 nm which is suitable for quantitative analysis. The calibration plot of absorbance (at 298 nm) vs. concentration for E2 yielded a zero-intercept slope of 0.998 mL/mg with $R^2 = 0.999$. A 1:20 dilution of the saturated E2 solution yielded an absorbance of 0.232 AU, and so the solubility of E2 in 0.75 M NaOH was estimated at 4.7 mg/mL. The solubility of E3 was estimated similarly (calibration slope = 0.991 mL/mg, $R^2 = 0.999$, absorbance of 1:20 dilution = 0.272) to be 5.5 g/mL. From these results, we determined that at 0.5 mg/mL HA in saturated estrogen solutions, the HA:estrogen ratio was approximately 1:10.

Next, NMR spectra were obtained for HA, E2 and E3 individually under our typical experimental conditions and a summary of the peaks observed in the estrogen spectra can be found in Table 1 [34]. Note that in 0.75 M NaOD, HA exhibits only broad, ill-defined peaks, and the observed spectrum is similar to that seen in previous studies involving aqueous solutions of HA [28]. Relative to the peaks from the saturated estrogens, HA was essentially negligible in the NMR spectra (see Fig. 1). Also, the hydroxyl protons of the estrogens are not included in Table 1, as they are not observed in 0.75 M NaOH. Spectra of E2 and E3 in the presence of 0.5 mg/mL HA were obtained and overlaid with the previously obtained NMR spectra of each estrogen alone to compare changes in the spectra (Figs. 2 and 3). Spectra were normalized relative to one another using the singlet at around 0.5 ppm in each spectrum (see Figs. 1–3); that peak corresponds to the methyl group bonded to the carbon between the 5- and 6-member rings, and it was not noticeably affected by HA concentrations even 20 times higher than that used in this study. Manual subtraction in Excel was used to produce the difference spectra, shown in the insets of Figs. 2 and 3. Since the many overlapping multiplets in the aliphatic regions (1 to 3 ppm) were non-distinctive, the three peaks in the aromatic region (6 to 7 ppm) were used to analyze the effect of added humic acid. The doublet at ~6.16 ppm and the doublet of doublets at ~6.23 were attributed to the protons ortho to the hydroxyl group on the aromatic estrogen ring, while the doublet at 6.88 ppm was attributed to the meta proton [34]. Even at relatively low HA concentration (0.50 mg/mL), a distinct difference was observed in the peaks at 6.16, 6.23 and 6.88 ppm, as displayed in the expanded difference spectra in NMR in Figs. 2 and 3. Specifically, the signals for the peaks at 6.16 and 6.23 ppm were quenched for both estrogens upon addition of humic acid even at a concentration 1/10th that of the estrogens. A uniform trend was not observed for the peak at 6.88 ppm. For E3, little change was observed in this peak. On the other hand, an increase in signal intensity for that peak was found for E2. Further, careful observation of the multiplet pattern in the expanded difference spectrum for E3 (Fig. 3, inset) indicates a slight shift in the peaks for the meta proton. The more significant change observed in the peaks at 6.16 and 6.23 ppm, corresponding to the two ortho protons, indicates that these two sites are highly affected by interactions with humic acid. Additional studies using concentrations of HA ranging up to 10 mg/mL (20 times the standard conditions) showed that the three aromatic protons became increasingly quenched with increased HA, and all other peaks were broadened to the extent that the splitting structure seen in the upfield peaks was lost entirely. Peak broadening can be attributed to more rapid relaxation of the excited nuclei, which may come about as a result of the interaction of the estrogen with HA providing a facile path for relaxation. Fig. 4 provides further evidence of interaction near the aromatic hydroxyl, showing shifting of the aromatic peaks in the E3 spectra with the highest concentration of HA (10 mg/mL): +2.6 Hz (6.16 ppm peak), +1.5 Hz (6.23 ppm peak) and −2.5 Hz (6.88 ppm peak). It is noted that these shifts are relatively small, but they were consistently reproducible over two (in the case of E2) or three (in the case of E3) trials. Further, the range of HA:estrogen ratios used (1:10 to 2:1) was small compared to those expected in natural waters, so even this minor shift potentially could be indicative of quite significant effects in the environment. Such small shifts in NMR peak positions coupled with peak broadening and quenching, particularly at high pH, have been previously reported as confirmation of noncovalent binding between small aromatic species and HA [35].

The observed changes in peaks corresponding to protons of the aromatic ring indicate an interaction between estrogens and humic acid at the aromatic group. While the specific nature of the interaction needs to be further clarified experimentally, under alkaline conditions the aromatic hydroxyl group would likely be deprotonated and the estrogen would exhibit ionic or hydrogen-bonding interaction. Previous studies have indicated potential binding through the pi networks of the interacting species [25]. Further research likely will provide clarity on this point, as in the case of Shirzadi et al. [28] in which polar bonds were found to form the primary points of humic acid contact with pesticides. While NMR spectroscopy is useful in probing possible binding interactions, it is unlikely that such methods will find notable

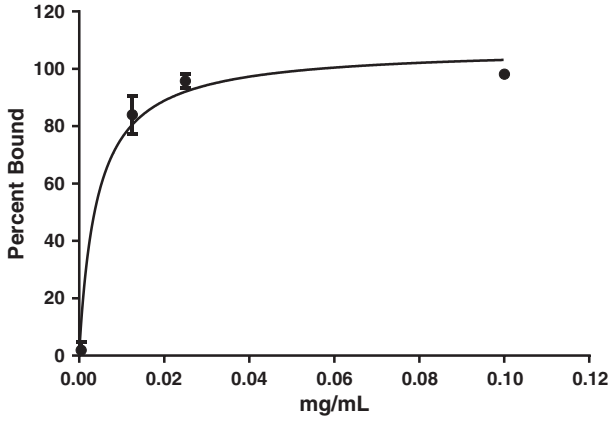


Fig. 5. E2 is able to be depleted from solution by binding to HA. Various concentrations of the estrogen were incubated with HA (130 μg/mL, final) and the amount of detectable estrogen, as determined via ELISA specific for free estrogen in a sample, was used to quantitate relative estrogen concentration of the sample. The decrease in free estrogen was then converted into percent bound estrogen. The data represent the means of n = 3 +/− SD.

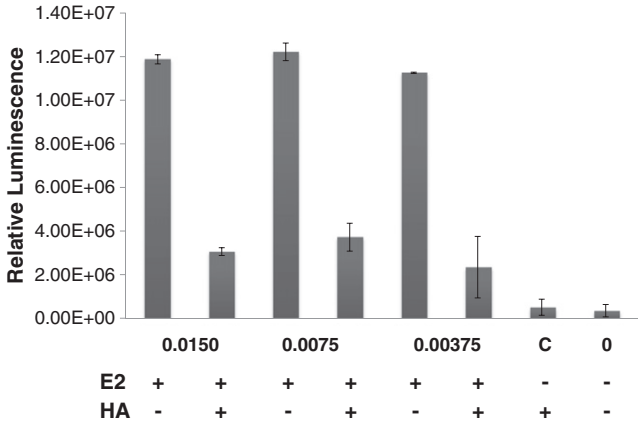


Fig. 6. Binding with HA decreases estrogenic activity of E2. Estrogen-sensitive yeast was exposed to media only (0) or various concentrations of E2 (μg/mL) alone (+,−) or pre-equilibrated with 130 μg/mL HA (+,+). The amount of estrogen available to bind ER-α was then determined by quantifying β-galactosidase expression via luminescence.

applicability in real-world analysis due to the expected need for considerable sample preconcentration and subsequent preparation prior to analysis.

Clearly the concentrations of estrogens used in the NMR study far exceed those found in nature; such concentration levels were needed in order to obtain reasonable NMR spectra. It is important to note that in other studies that investigated the interactions between estrogens and dissolved organic matter, the DOM (present as HA) was added in concentrations far exceeding those of the estrogens, up to 1000-fold excess [30,36]. Our NMR results demonstrate that even at HA concentrations 10-fold *less* than those of estrogens (i.e., 0.5 mg/mL HA), evidence of significant impact on spectral features is observed, and indicates interaction at the aromatic ring of the estrogen.

3.2. Removal of estrogen, as detectable by antibodies, when mixed with HA

Binding interactions between estrogens and DOM such as observed by the NMR data above have also been supported by data from previous studies [27,30,36–38]. To demonstrate and confirm binding between estrogens and HA in our system, an ELISA was used. E2 solutions were equilibrated with HA for 48–96 h. The amount of unbound (free) estrogen was quantified, and compared to the total amount of estrogen in the original estrogen solution so that any degradation or sorption that may have taken place during the equilibration period could be excluded. HA alone did have a small reading in ELISA assays and therefore its value was subtracted out from experimental samples during analysis. As the concentration of E2 increased in the reactions, the amount of free estrogen in the samples was detected, from which the percent bound to HA was calculated, supporting that binding is occurring (Fig. 5). Since these reactions were equilibrated for up to 96 h prior to analysis, it was important to ascertain possible influence of equilibration time on binding. Accordingly, measurements of the same solution of E2 (0.1 mg/mL, final) and HA (130 μg/mL, final) were taken at 2 h and 48 h. No significant difference between the amount of binding at the two time points was found (98.82 $\pm$ 1.64 vs. 99.19% $\pm$ 2.42 for 2 h and 48 h, respectively; $p = 0.837$).

3.3. Decrease in biological activity of estrogens in solution when mixed with HA

Yamamoto et al. (2003) have previously shown that the binding of estrogens to HA is most likely to occur at the aromatic ring containing the hydroxyl group [27]. Our NMR results also suggest that the aromatic rings of E2 and E3 (see Table 1 for structure) are the most likely binding positions with HA. Not coincidentally, these are key regions for hydrogen bonding of estrogens with the binding pocket of ER α [39]. The implication of this interaction, therefore, is that binding could prevent the estrogen from interacting with its receptor, ablating biological activity of the estrogen. To determine if the binding of estrogen to HA that had been observed via NMR and ELISA impacts estrogen's biological activity, a modified YES bioassay was employed. Solutions of E2 were pre-equilibrated with HA for 24 h, and then allowed to interact with estrogen sensitive yeast for 2 h, at which time the amount of β-galactosidase expression was quantified. We found that β-galactosidase activity of the YES system was significantly decreased for three different concentrations of E2 pre-equilibrated with HA (Fig. 6) compared to the control, unbound estrogen in the original solution, which was measured at the same time as experimental reactions. HA alone did not yield any significant luminescence above background. Such a decrease in activity implies that the estrogen bound to HA is not biologically available. These results confirm that binding is indeed occurring between the HA and estrogens used in our assays as well as corroborates the other data presented.

Other studies have found that interactions between E2 and HA have bearing on the fate of E2 conversion and biodegradation [30,38]. Furthermore, examination of estrone–HA interactions supported that their interaction limited freely dissolved estrone for adsorption onto polymer membranes [40]. Therefore our result that HA leads to lower amounts of free, biologically active estrogen is reasonable. Since the goal of this study was to first and foremost establish binding of estrogens to HA and the consequence of this binding on biological activity, the concentrations of E2 and HA used in the ELISA and yeast bioassays were also much higher than what is found in the environment. However, the large ratio of HA:E2 in the yeast bioassay is typical of what is found in environmental waters. For example, there have been reports of E2 ranging from 1 to 80 ng/L [21] and natural organic matter, made mostly of humic substances, reported from 0.1 to 20 mg/L [41] and most recently as 0.3–30 mg/L [42]. Moreover, other studies that have examined the effects of humic acid concentration on the adsorption of E2 used similar ratios 100:1 to 10,000:1 [21] and 8×10^6:1 to 125:1 [43] HA to E2, respectively. Our analyses were of pure standards, thus extraction and concentration steps usually required when using environmental samples were not necessary. Therefore future studies should utilize estrogens and DOM from environmental samples and include typical extraction and concentration procedures.

3.4. Other considerations

Humic substances are known to aggregate under certain conditions, particularly when present in high concentration and when in the presence of metal cations [44–48], and so it is important to consider the potential implications to this study. Because HAs (and DOMs in general) are complex and the term "HA" does not refer to one specific molecule, it is difficult to know exactly at what concentration aggregation might become significant. Young and von Wandruszka found that Suwanee River HA, which among those they studied appears to be most similar to that used in our work, had very little tendency to aggregate at concentrations up to 100 ppm (0.1 mg/mL) [44]. Simpson found that even at 5 mg/mL HA the aggregation involved, on average, the association of only a small number (~5–10) HA molecules [45]. From these reports, it seems unlikely that in the solutions used in the ELISA and yeast assays, having HA concentrations of 0. 13 mg/mL, significant HA aggregation would have occurred. Our NMR studies were conducted at much higher HA concentration (0.5 to 10 mg/mL), but in solutions of high pH. Jovanovic et al. found that for a variety of HAs the ability to aggregate diminished with increasing pH, and was eliminated in all cases when the pH exceeded 7 [48]. They attributed this to the deprotonation of the acidic hydroxyl groups, thus eliminating the ability of the HA molecules to undergo hydrogen bonding. It is unlikely, therefore, that HA aggregation played a significant role in the results obtained from the NMR studies.

Of course, in natural waters, the pH is likely to be somewhat lower than 7, the concentration of humic substances often will be quite large, and metal ions will be present in solution at significant concentrations. Therefore, the probability of HA aggregation in those conditions will be quite high. However, previous studies [39] have indicated that binding of aromatic species increases with aggregation of humic substances, implying an even greater influence on measured estrogen content of natural waters than our results signify.

4. Conclusions

The present study combining biochemical assay methods and analytical NMR spectroscopy builds upon previous reports, yielding a broader understanding of estrogen–humic substance interactions. The results indicated that binding occurs proximal to the hydroxyl group on the estrogen aromatic ring, that binding is specific, and that the binding decreases the biological availability of estrogens in aqueous solution. While these interactions are now more cogent, further investigations into the binding kinetics between E2 and E3 with HA, binding between other estrogens and HA, as well as estrogens and the other components of humic substances, either alone or in combination, are needed. A

comprehensive understanding of the binding and interactions between estrogens and DOM could ultimately impact regulatory controls and sampling, as well as disposal procedures. However, additional analysis is necessary to relate these results to environmental sampling conditions as well as address if binding impacts bioavailability.

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