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Preparation of Entrapment-Based Microcolumns for Analysis of Drug-Humic Acid Interactions by High-Performance Affinity Chromatography

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Abstract

Reversible interactions between drugs and humic acid in water can be an important factor in determining the bioavailability and effects of these pharmaceuticals as micropollutants in the environment. In this study, microcolumns containing entrapped humic acid were used in high-performance affinity chromatography (HPAC) to examine the binding of this agent with the drugs tetracycline, carbamazepine, ciprofloxacin, and norfloxacin. Parameters that were varied to optimize the entrapment of humic acid within HPLC-grade porous silica included the starting concentration of humic acid, the mass ratio of humic acid vs silica, and the method of mixing the reagents with the support for the entrapment process. The highest retention for the tested drugs was obtained when using supports that were prepared using an initial humic acid concentration of 80 mg mL^{-1} and a humic acid vs silica mass ratio of 600 mg per g silica, along with preincubation of the humic acid with hydrazide-activated silica before the addition of a capping agent (i.e., oxidized glycogen). Characterization of the humic acid support was also carried out by means of TGA, FTIR, SEM, and energy-dispersive X-ray spectroscopy. The binding constants measured by HPAC for the given drugs with entrapped Aldrich humic acid gave good agreement with values reported in the literature under similar pH and temperature conditions for this and other forms of humic acid. Besides providing valuable data on the binding strength of various drugs with humic acid, this work illustrates how HPAC may be used as an analytical tool for screening and characterizing the interactions of drugs and man-made contaminants with humic acid or related binding agents in water and the environment.

Keywords: High-performance affinity chromatography; Entrapment; Affinity microcolumn; Humic acid; Drug binding; Micropollutant

1. Introduction

The extensive use of pharmaceutical products in animal feed and human healthcare has led to the presence of low concentrations of around 24 therapeutic classes of drugs in environmental water, such as non-steroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, lipid regulators, and antibiotics [1-9]. The occurrence of these drugs in surface and groundwater, wastewater, and drinking water has made these compounds a topic of growing concern [2-4,6,9]. These pharmaceuticals are often referred to as "emerging contaminants" or "micropollutants" and are believed to contribute to such problems as toxicity to the aquatic ecosystem, the development of microorganisms with antibiotic resistance, and potential health effects in humans [1,2,4,10,11]. These concerns have, in turn, led to a need for new analysis methods that can measure and study these contaminants in water [12,13].

Humic acid is produced by the breakdown of plant matter and is an important component of dissolved organic matter in soils, sediments, and water [14-17]. Humic acid is a large heterogeneous set of organic polymers (see general structure in Fig. 1) with a molar mass that can range from 2 to 1300 kDa [14,15]. The structure of humic acid typically contains quinones and carboxylic, phenolic, or enolic groups; some types of humic acid may also contain peptides and sugars in their structure, depending on the source of this material [14,15].

It is known that some pharmaceuticals (see examples in Fig. 1) can undergo reversible, non-covalent interactions with humic acid [8,16-21]. This binding can influence the bioavailability, transportation, and solubility of these micropollutants, along with the persistence and degradation rates of such organic contaminants in the environment [8,16-22]. Previous techniques that have been employed to examine the interactions of drugs with humic acid have included fluorescence spectroscopy, UV-Vis spectroscopy, equilibrium dialysis, and solid-phase

extraction combined with methods such as liquid chromatography-mass spectrometry [7,8, 16,17,19,21,23-26]. However, these methods have been found to have several disadvantages for such work, such as the need for relatively large sample volumes and/or long equilibration or extraction times [7,19-21].

This report used high-performance affinity chromatography (HPAC) as a new and alternative method to examine the binding of humic acid with pharmaceuticals. HPAC has been used in the past to characterize the interactions of drugs and other solutes with biological agents such as serum proteins [27-30]. These interactions are analogous to those seen between pharmaceuticals and humic acid, suggesting that HPAC could also be used to screen and characterize binding by drugs with this form of dissolved organic matter [31-33]. Potential advantages of this method, as noted in prior work with drug-protein systems, are its need for only small sample volumes and its ability to obtain precise and accurate binding data in short periods of time (i.e., minutes per sample injection) [27-30].

Commercial preparations and natural isolates of humic acid have previously been covalently immobilized to supports such as silica, cellulose, hyperbranched polytriazine, and styrene-divinylbenzene copolymers for use as stationary phases in liquid chromatography or solid-phase extraction [7,34-38]. In this study, an alternative immobilization approach based on non-covalent entrapment, as illustrated in Fig. 2, was used to place humic acid within porous HPLC-grade silica [31,39]. In this approach, the silica was first converted into a form that had active hydrazide groups on its surface [32,33,40]. A solution of humic acid was then combined with this activated support along with mildly oxidized glycogen (i.e., a high-mass capping agent that contained some aldehyde groups). As these reagents were combined, a stable hydrazone bond was formed between hydrazide groups on the support and aldehyde groups on the capping agent. It

was expected that this process would result in humic acid being entrapped and immobilized in a soluble and unmodified form that was still accessible for interacting with small solutes (e.g., drugs). This strategy has been successfully employed in recent work to entrap serum proteins for use in drug binding studies [32,33,40]. However, this method has not yet been employed for humic acid or other forms of natural, dissolved organic matter.

This study will seek to prepare supports that contain entrapped humic acid for use in HPAC and binding studies with model drugs that may be found as micropollutants in water. A preparation of Aldrich humic acid, which is often used as a model for binding studies with humic acid [7,19], will be used for this work along with HPAC microcolumns (i.e., columns with volumes in the low-to-mid μL range) [31-33]. Once the humic acid supports have been made and characterized, they will be evaluated for their binding to model drugs known to interact with humic acid and that represent a broad range of affinities. These drugs, as shown in Fig. 1, will include the antibiotics tetracycline, ciprofloxacin, and norfloxacin, as well as the anticonvulsant carbamazepine [8,19,20,23,24]. The results of these binding studies will be compared to prior data from the literature on drug-humic acid interactions. This study should also provide valuable information concerning the use of HPAC as a new analytical tool for measuring and studying the effects of humic acid on the bioavailability, biological or toxicological effects, and persistence of pharmaceuticals and other emerging contaminants in the environment.

2. Experimental

2.1. Reagents

The humic acid ("Aldrich humic acid", product 53680; ~20% inorganic residue), glycogen (bovine liver, type IX, product G0885; total glucose ≥ 85% on a dry basis), periodic acid reagent (H_5IO_6, purity 99%), oxalic dihydrazide (98%), tetracycline (≥ 98.0%), ciprofloxacin (≥ 98.0%),

and norfloxacin (≥ 98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The carbamazepine (≥ 99.0%) was obtained from Tocris Bioscience (Minneapolis, MN, USA). All other reagents were of the purest grades available. Nucleosil Si-300 silica (7 µm particle diameter, 300 Å pore size) was acquired from Macherey-Nagel (Duren, Germany). Amicon Ultra centrifugal filter units (30 kDa cutoff; EMD Millipore Sigma, Burlington, MA, USA) were used for purification of the oxidized glycogen. All buffers and aqueous solutions were prepared by employing purified water from a Milli-Q system (EMD Millipore Sigma). Buffers were filtered through 0.22 µm GNWP nylon membrane filters (Fischer Scientific, Pittsburgh, PA, USA) and were degassed by sonication under vacuum for at least 30 min before use.

2.2. Instrumentation

A Prep 24 pump from ChromTech (Apple Valley, MN, USA) was used for packing the affinity microcolumns and corresponding control microcolumn. The chromatographic analysis was carried out using an HPLC system consisting of a PU-2080 Plus pump, an AS-2057 Plus autosampler, a UV-2075 Plus absorbance detector, and a DG-2080-54 degasser from Jasco (Easton, MD, USA). A Jasco HV-2080-01 column selection unit was utilized to regulate buffer and sample flow through the microcolumns. A temperature of 25.0 (± 0.1) °C was maintained during all the experiments using a Jasco CO-2067 Plus column oven. The HPLC system was controlled using ChromNAV v1.18.04 software and LCNet from Jasco. Automatic baseline subtraction and fitting of the chromatograms were performed using the progressive, linear, and exponentially modified Gaussian (EMG) functions, respectively, of PeakFit v4.12 software (Jandel Scientific, San Rafael, CA, USA). The data were also analyzed using Excel for Office 365 (Microsoft, Redmond, WA, USA). Other equipment used for characterizing the supports prepared in this study are described in Section 2.5.

2.3. Preparation of humic acid supports

The humic acid supports were prepared through physical entrapment of humic acid into porous HPLC-grade silica, as illustrated in Fig. 2. This method began by first converting Nucleosil Si-300 silica into diol-bonded silica [31,33]. This support was then converted into an aldehyde form and reacted with oxalic dihydrazide as the activating agent, as described in prior entrapment studies with proteins ranging in size from 26.5-66.5 kDa (e.g., human serum albumin, concanavalin A, and alpha$_1$-acid glycoprotein) [31-33,40].

Humic acid has a pH-dependent solubility in water. This substance is readily soluble at a basic pH; however, it can also be soluble at a neutral pH, depending on the humic acid's composition [41,42]. For this reason, the humic acid used for entrapment (i.e., at initial concentrations of 40-80 mg mL^{-1}) was initially dissolved in pH 11.0, 0.10 M potassium phosphate buffer and then adjusted to a pH of 6.0 by gradually adding pH 2.5, 0.10 M potassium phosphate buffer. The initial conditions used for the entrapment of humic acid were adapted from those described for human serum albumin [31]. In these early studies, a 40 mg mL^{-1} solution of the humic acid solution was prepared in pH 11.0, 0.10 M potassium phosphate buffer, which after adjustment to pH 6.0 reached a final concentration of about 13.3 mg mL^{-1}. A 2.25 mL portion of this pH 6.0 humic acid solution was then combined with 0.070 g of hydrazide-activated silica, giving a slurry that contained 400 mg humic acid per g of hydrazide-activated silica. This slurry was degassed for 10 min to remove any trapped air within the support.

Next, 0.31 mL of a 4.25 mg mL^{-1} solution of mildly oxidized glycogen in pH 6.0, 0.10 M potassium phosphate buffer was added to the slurry, giving a mixture that contained 18 mg oxidized glycogen per g silica. Before this mixture was prepared, the glycogen had been oxidized in pH 5.0, 20 mM sodium acetate buffer that contained 15 mM sodium chloride and periodic acid.

This oxidation was carried out by placing 17 mg glycogen and 135 mg periodic acid into 4 mL of the pH 5.0 acetate buffer and allowing the mixture to react in the dark at room temperature for 16 h. These conditions have previously been shown to oxidize ~ 0.5% of the glucose monomers in glycogen [31,33]. After this oxidation step, the oxidized glycogen was washed and purified by using ultrafiltration and placed into pH 6.0, 0.10 M potassium phosphate buffer for use in entrapment (typical volume, 4 mL containing 4.25 mg oxidized glycogen per mL) [32].

The slurry volume containing the activated silica, humic acid, and oxidized glycogen was adjusted to 3.0 mL by adding pH 6.0, 0.10 M potassium phosphate buffer. This entrapment solution was mixed, by using a wrist action shaker, for 18 h at room temperature. The final stage of entrapment involved adding 50 μL of a 1 mg mL^{-1} oxalic dihydrazide solution, prepared in pH 6.0, 0.10 M potassium phosphate buffer, to the slurry for removing any remaining unreacted aldehyde groups on the glycogen or the support [31]. The slurry containing oxalic dihydrazide was allowed to react for 2 h at room temperature. Control supports were prepared in the same manner, but with only pH 6.0, 0.10 M potassium phosphate buffer being employed in place of the humic acid solution that was used for the entrapment step.

Several conditions for entrapment were altered in this study to adjust and increase the amount of humic acid that could be placed within the supports, as discussed in more detail in Section 3.2. One modification was to vary the initial concentration of humic acid (i.e., up to 80 mg mL^{-1}), as was done before adjusting the pH of the humic acid solution to 6.0 and modifying the relative volume of this final humic acid solution vs the total reaction solution volume (i.e., to give a consistent humic acid-to-silica mass ratio). The ratio of humic acid vs silica was also varied in some experiments. Finally, the entrapment process was conducted by using a split incubation step instead of a single step for slurry preparation. In the split incubation approach, the activated

support was preincubated with a humic acid solution, with the oxidized glycogen being added later in a separate step for entrapment (see Section 3.2.3).

2.4. Chromatographic studies

Each humic acid support or control support was placed into a separate 10 mm long and 2.1 mm inner diameter (I.D.) stainless steel column. A pH 7.4, 0.067 M potassium phosphate buffer was employed as the packing solution. The microcolumns were downward slurry packed at 4000 psi (28 MPa). When not in use, all supports and microcolumns were stored at 4 ºC in pH 7.4, 0.067 M potassium phosphate buffer.

The chromatographic studies were carried out in replicate (n = 4) by injecting 20 µL of each drug solution at flow rates of 0.10, 0.25, and 0.50 mL min^{-1} onto the humic acid microcolumns and corresponding control microcolumn. A mobile phase consisting of pH 7.4, 0.067 M potassium phosphate buffer was used for the application and elution of each drug, as well as for preparing the drug solutions (Note: pH 7.4 was a convenient intermediate value in the range of pH 7-8 that has been used in prior studies of binding by humic acid with the model drugs considered in this work [8,19,23]; this pH has also often been used for examining drug binding processes in biological systems) [28,30]. The microcolumns used in this report were equilibrated with this buffer at 25.0 (± 0.1) ºC and 0.25 mL min^{-1} for 2-2.5 h prior to any sample injection; the drug samples were kept in the autosampler at 25 ºC for 30-60 min prior to injection.

The concentration of the injected tetracycline and carbamazepine solutions were 10 µM, while 20 µM solutions of ciprofloxacin and norfloxacin were employed. The void volumes of the HPLC system and microcolumns were obtained by injecting a 10 µM solution of sodium nitrate as a non-retained marker [28,31]. The detection wavelengths were as follows: tetracycline, 276 nm; carbamazepine, 286 nm; ciprofloxacin, 276 nm; norfloxacin, 273 nm; and sodium nitrate, 205

nm. The drug solutions were stored at 4 °C when not in use and were used within one week of preparation.

The average retention time of each drug or solute was determined by employing a zonal elution format and linear elution conditions [27,28,31,32]. The retention factor of a drug on a humic acid microcolumn ($k_{humic\ acid}$) was determined by using the measured retention time of the drug (t_R), the total void time of the system with the microcolumn present (t_M), and void time of the chromatographic system with no microcolumn present (t_o), as shown in eq. (1) [27,28,31,32]. The retention factors of the drugs on a control microcolumn (k_{control}) were obtained in the same way.

$$k_{humic\ acid} \quad \text{or} \quad k_{control} = \frac{t_R - t_M}{t_M - t_o} \tag{1}$$

Although a correction for the system void time t_o is not normally made during the calculation of a retention factor in work with traditional column sizes, such as a correction is needed with microcolumns to obtain accurate values for the true column void time and corresponding retention factor (Note: t_o is not included in the numerator of eq. (1) because it is already a component of both t_R and t_M and is eliminated by taking the difference in these two values) [27,32]. The specific retention factor (k') of each drug on a given humic acid microcolumn was determined from the difference in the total retention factors of the drug on the humic acid microcolumn and corresponding control microcolumn, as shown in eq. (2) [27,28, 31,32].

$$k' = k_{humic\ acid} - k_{control} \tag{2}$$

2.5. Characterization of humic acid supports

The overall structure and elemental composition of the supports prepared in this study were examined by using SEM and energy-dispersive X-ray spectroscopy (EDS), respectively. This work was performed using an FEI Nova NanoSEM 450 system (FEI, Hillsboro, OR, USA) that was operated at 5.0 kV. For these analyses, dried samples of the supports were pretreated by gold

sputtering using a Cressington 208HR High Resolution Sputter Coater (Watford, England, UK) prior to the SEM and EDS analysis.

FTIR was performed on the humic acid, humic acid silica, and control support by using a Nicolet iS50 FTIR system (Thermo Scientific, Waltham, MA, USA), which was controlled by OMNIC v9.7.46 software. The FTIR spectra were acquired over wavenumbers spanning from 3800 to 1300 cm^{-1}, with a spectral resolution of 4 cm^{-1} obtained over 16 scans.

TGA of the supports was performed using a TGA 550 system (Waters, New Castle, DE, USA) equipped with nitrogen flow for inert analysis conditions and controlled by TRIOS v5.1.1.46572 software. In this analysis, the samples were initially heated from room temperature to 110 °C at 5 °C min^{-1}. The sample was then held at 110 °C for 20 min to eliminate any moisture and to condition the sample; the mass recorded at this stage was used as the 'initial weight'. The temperature was subsequently increased from 110 °C to 650 °C at 20 °C min^{-1}. For modified silica samples like those used in this study, a previous report has shown that the weight decrease reaches a plateau at around 650 °C [43], so the weight at this temperature was used to provide the final weight for the samples in this current study. This same upper temperature limit, or a similar value, has also been employed in prior work using TGA to examine the organic content of humic acid samples [44,45]. During the TGA analysis, nitrogen at a flow rate of 20 mL min^{-1} was introduced into the analysis chamber to purge oxygen from the system.

The humic acid content of the humic acid silica was estimated by comparing the relative change in mass obtained by TGA for samples of the humic acid silica and a control support (i.e., with no humic acid present). This was determined by using the relative difference in weight that was seen for each of these samples between 110 °C and 650 °C. These differences were then used along with information on the inorganic content of the humic acid (as provided by the supplier) to

find the weight fraction (*w/w*) of humic acid that was entrapped in the humic acid silica (see Supplementary Material). Multiplying this fraction by 1000 gave a value in parts-per-thousands, or the equivalent to expressing the humic acid content in units of mg humic acid per g support.

3. Results and Discussion

3.1. Selection of initial conditions for preparing and using entrapped humic acid microcolumns

The feasibility of preparing and utilizing microcolumns that contained entrapped humic acid was first examined in this study. This was done by using entrapment conditions adapted from those used in prior studies for serum proteins with molar masses in the same range as is common for humic acid [31-33,40]. This early work utilized a microcolumn that was prepared using HPLC-grade silica with an average pore size of 300 Å, along with a starting humic acid concentration of 40 mg mL^{-1} and a humic acid vs silica mass ratio of 400 mg g^{-1}. Other conditions for entrapment were the same as described in Section 2.3. A slurry-based entrapment method was used in this work instead of on-column entrapment, as employed in some prior reports with proteins [31,39], because the humic acid solutions tended to give high column backpressures for the latter method during the process of microcolumn preparation. The humic acid content of the final support that was prepared by the initial slurry entrapment conditions was 22.9 mg humic acid per g silica, as determined by TGA. Some typical results for such an analysis are provided in Fig. 3.

The presence of entrapped humic acid in the prepared support was confirmed by placing this material into a microcolumn and injecting some model drugs that were known to interact with humic acid. Tetracycline was one drug used for this purpose because it is known to bind with humic acid in water and with the commercial preparation of Aldrich humic acid that was used in this study [19,20]. Small 20 μL injections of this drug at sample concentrations of 5-10 μM were made at 0.25 mL min^{-1} onto both the humic acid microcolumn and a control microcolumn. These

chromatograms gave a peak for tetracycline on the humic acid microcolumn that eluted with a peak maximum at about 3.3 min and a mean retention time of 4.4 min, as shown in Fig. 4. No appreciable retention (i.e., <5-6% of that measured on the humic acid microcolumn) was seen for tetracycline on a control microcolumn prepared in the same manner as the humic acid microcolumn but with no humic acid being added during the entrapment process. It was further noted that consistent retention was seen over the range of sample concentrations that were used, indicating that linear elution conditions were present [27]. Based on these results, a sample concentration of 10 μM was used in the remaining studies to provide consistent retention and a reasonable peak size for detection.

The injection of tetracycline onto a control microcolumn gave a peak that appeared near the estimated void time of the microcolumn and system (i.e., a difference of only 4-5 s). It was further determined that any non-specific binding by tetracycline to the support, as measured using the control microcolumn (i.e., which contained all the components used for entrapment other than the humic acid) made up only 1.3-2.6% of the total retention seen for tetracycline on the humic acid microcolumn. These results confirmed that humic acid was present in the support that was prepared by entrapment and that this humic acid support was capable of binding and retaining tetracycline.

3.2. Optimization of humic acid entrapment

3.2.1. Effect of varying humic acid concentration and solution volume

Once it had been determined that humic acid could be placed within a silica support by entrapment, several factors were considered for increasing the amount of humic acid that could be entrapped. The effect of these factors was screened by looking at how they altered the retention of tetracycline on the humic acid supports. One factor considered was the initial concentration of

humic acid, and the corresponding volume of humic acid solution, that was added to the silica for entrapment while keeping the mass of added humic acid constant. Previous studies with proteins have found that the amount of entrapped binding agent can be increased by reducing the protein solution volume that is used for entrapment while keeping the mass or moles of added protein constant (i.e., as can be done by also increasing the concentration of protein added to the reaction slurry) [31]. It has been proposed that this effect occurs because these conditions increase the ratio of the support's pore volume versus the protein's solution volume, resulting in a more effective utilization of the protein for entrapment [39]. In this current study, the initial concentration of the humic acid was varied from 40 to 80 mg mL^{-1} while the final volume of the added humic acid solution was decreased proportionally (i.e., from 2.26 mL to 0.91 mL); these changes made it possible to keep the total mass of added humic acid constant at approximately 29 mg (range, 28.8 to 29.9 mg; average, 29.4 mg). During these studies, the humic acid vs silica mass ratio was also kept constant at 400 mg g^{-1}. Examples are shown in Fig. 5(a) of chromatograms that were obtained at 0.10 mL min^{-1} for tetracycline with the humic acid supports that were made in this manner.

It was found that a change in the original concentration and volume of humic acid that was used for entrapment, while keeping the total mass of added humic acid the same, resulted in only small variations in the final retention that was observed for tetracycline. For instance, the retention measured for tetracycline at 0.10 mL min^{-1} on microcolumns made using initial humic acid concentrations of 60 vs 80 mg mL^{-1} gave a retention factor that increased by only 3.7%. This apparent change in retention was comparable to the precision (i.e., level of random variations) of the retention measured for tetracycline on these microcolumns (i.e., ± 0.6-3.8% for 1 S.D. at $n = 4$ and over 0.10-0.50 mL min^{-1}). However, results from TGA and data obtained in later studies indicated that supports made using an initial humic acid concentration of 80 mg mL^{-1} (and added

humic acid solution volume of only 0.91 mL) provided about a 2.1-fold improvement in the overall humic acid content vs supports made using 40-60 mg mL^{-1} humic acid. Thus, an initial humic acid concentration of 80 mg mL^{-1} was used for entrapment in all further work in this report.

3.2.2. Effect of varying amount of added humic acid vs silica

A second factor that was considered was the mass ratio of humic acid vs silica that was used to prepare the humic acid support. Previous studies with proteins have found that the mass ratio of binding agent vs support is a key factor that can be varied to increase the amount of protein that can be immobilized within porous silica through entrapment [31]. In these experiments, the initial concentration of the humic acid was kept constant at 80 mg mL^{-1} while the humic acid vs silica mass ratio was varied from 400 to 600 mg per g silica. Fig. 5(b) shows some chromatograms for tetracycline that were obtained at 0.10 mL min^{-1} with microcolumns that contained these supports.

Only a small apparent change of 3% was seen in the retention of tetracycline as the humic acid vs silica mass ratio for making the supports and microcolumns was raised from 400 to 500 mg humic acid per g silica. This change in retention, which had an uncertainty of ± 4.4% (1 S.D. for n = 3, as measured at 0.10-0.50 mL min^{-1}), was not significant at the 95% confidence level. However, further increasing the humic acid vs silica mass ratio from 500 to 600 mg per g silica gave an increase in retention for tetracycline at flow rates ranging from 0.10 to 0.50 mL min^{-1} of ~17-26% (average, 20 (± 5)% for n = 3), a change which was significant at the 95% confidence level. This observed change in retention was consistent with an increase of 19% that was measured by TGA for the final humic acid content of the same supports when going from 500 to 600 mg humic acid per g silica. This shift in retention was much greater than the precision of the retention factors, which was ± 0.7-4.0% (n = 4) for data acquired for tetracycline at 0.10 to 0.50 mL min^{-1}

on microcolumns that contained these supports. Based on these results, a mass ratio of 600 mg humic acid per g silica (e.g., 42 mg humic acid per 70 mg silica, with one 10 mm × 2.1 mm I.D. microcolumn containing around 16 mg silica) was employed in most of the rest of this study.

3.2.3. Effect of preincubation of humic acid with support

The previous sections used humic acid microcolumns that were prepared by mixing the humic acid, oxidized glycogen, and hydrazide-activated silica in a single step. The slurry entrapment method was next modified by first preincubating the humic acid with the hydrazide-activated silica for 3.5 h before the addition of the oxidized glycogen to this slurry. This modification was used to allow humic acid to enter the pores of the activated support before oxidized glycogen was coupled to the hydrazide groups. The other conditions used for entrapment in these experiments were the same as optimized in Sections 3.2.1-3.2.2 (i.e., an initial humic acid concentration of 80 mg mL^{-1} and a mass ratio of 600 mg humic acid per g silica).

The effect of this preincubation and split mixing on the retention of drugs is illustrated in Fig. 6. Several drugs in addition to tetracycline were now examined in this part of the study. Two of these drugs were ciprofloxacin and norfloxacin, which have known interactions and binding strengths with some types of humic acid [8,46]. A third drug that was included was carbamazepine, which is a common marker for microcontaminants in water and is also known to bind to humic acid [23,24].

The chromatograms in Fig. 6 were obtained at 0.50 mL min^{-1} for ciprofloxacin and norfloxacin on 10 mm × 2.1 mm I.D. humic acid microcolumns that were made with and without preincubation of the support with humic acid prior to entrapment. There were 1.4- to 1.5-fold and 1.1- to 1.2-fold increases in the retention factors for ciprofloxacin and norfloxacin, respectively, as measured at 0.10-0.50 mL min^{-1}, when going from the use of a single step for entrapment to the

use of preincubation of humic acid with the support. These changes were statistically significant at the 95% confidence level and were much larger than the relative precisions and random variations of the retention factors for these same drugs (1 S.D. of ± 0.7-1.2% for ciprofloxacin and ± 0.2-0.5% for norfloxacin at n = 4). Similar increases in retention up to 1.1-fold and up to 1.5-fold were seen for tetracycline and carbamazepine (1 S.D., ± 0.7-1.9% for tetracycline and ± 0.4-1.2% for carbamazepine at n = 4). Based on these results, preincubation was used for the entrapment of humic acid throughout the remainder of this study.

3.3. Characterization of humic acid supports

As mentioned in Section 3.1, the absolute or relative amount of humic acid that was entrapped in the chromatographic supports was examined by TGA and/or using the measured retention of these supports for drugs known to bind humic acid (see examples in Figs. 3-6). The humic acid contents that were found by TGA for the various supports made in Section 3.2 ranged from 9.7 to 47.6 mg g^{-1} silica, with the final support that was used in most of the remainder of this study having a content of 46.6 mg g^{-1} silica. These measured humic acid contents gave a good correlation with the retention factors that were initially measured for carbamazepine and several of the other model drugs on microcolumns containing these materials. For instance, the retention factors measured for carbamazepine at flow rates of 0.10, 0.25, or 0.50 mL min^{-1} gave correlation coefficients of 0.887-0.936 (n = 4) when compared to the humic acid content that was determined by TGA.

The entrapment of the humic acid was further examined qualitatively by using attenuated total reflectance FTIR. Some typical FTIR spectra are provided in the Supplemental Material for humic acid, a support containing entrapped humic acid, and a control support that was made by entrapment but with no humic acid added. One indication that entrapped humic acid was present

in the humic acid support was the increase in absorbance (and decrease in %transmittance) that was seen for the broad peak centered around ~3300 cm^{-1}, which was due to O-H plus N-H stretching modes [47,48]. The presence of humic acid in the support made by entrapment was also demonstrated by an increase in absorbance and/or appearance of additional peaks at approximately 1610 and 1651 cm^{-1}, as expected from the C=C stretch of aromatic groups and the C=O stretch from carboxylic acid groups in humic acid, respectively [47,48].

SEM images were also acquired to determine if any changes in the morphology or microstructure of the supports occurred due to the entrapment process or due to the presence of the humic acid. The images that were obtained (see Supplementary Material) demonstrated that no aggregation or cross-linking occurred for the silica particles because of their modification during the entrapment process. An elemental analysis by EDS was also carried out on these particles (see Supplementary Material). The carbon and oxygen contents for the humic acid support were found by this analysis to increase by 3.0-fold and 3.3-fold, respectively, versus the control support with no humic acid present. The aromatic and aliphatic groups of humic acid contributed to the increased carbon content, and the increase in oxygen content could be attributed to the carboxylic, quinone, and/or phenol groups in the structure of humic acid. A 2-fold increase in nitrogen content was further noted upon the entrapment of humic acid vs the control support, due the presence of groups such as amines and amides in the structure of humic acid [14].

The stability of the entrapped humic acid stationary phases was also characterized. This was evaluated by making repeated injections of carbamazepine onto 10 mm × 2.1 mm I.D. humic acid microcolumns. Some typical results that were obtained at 0.50 mL min^{-1} are provided in the Supplementary Material. In the example given, a maximum variation of only 16% was seen in the specific retention factor for carbamazepine over injections made during the application of 380 mL

of mobile phase (~1.1 × 10^4 column volumes). The backpressure across the microcolumns under these conditions ranged from only 2.7 to 4.0 MPa (392-580 psi). Similar behavior was seen with humic acid microcolumns that were prepared under the final optimized conditions, some of which provided good retention over several hundred injections and more than a year of use. These results indicated that the humic acid microcolumns were quite stable under these flow rate conditions and suitable for repeated and long-term use in drug binding studies.

3.4. Estimation of binding constants between humic acid and drugs

The final section of this work used the humic acid microcolumns to estimate the equilibrium binding constants for the selected drugs with the entrapped humic acid. The first way this was done was by using the measured retention factor for a given drug along with the humic acid content of the microcolumn, as obtained by TGA (see Section 3.3). For instance, it was known from prior work with biological agents that the specific retention factor (k') of an injected drug should be directly proportional to the global affinity constant (nK'_a, expressed in units of L mol^{-1}) of the same drug with an entrapped form of a binding agent, as shown by eq. (3) [27,32].

$$k' = \frac{(nK'_a)\, m_L}{V_M} \qquad (3)$$

Other terms in this equation include the void volume of the microcolumn (V_M) and the moles of active binding agent (m_L) [32,39,40]. The global affinity constant can be used in this situation as a general equilibrium constant for the interaction of a drug with a binding agent that has n independent sites (i.e., as may occur for humic acid) [8]. For a binding agent that has a single binding site for a drug (n = 1), the term nK'_a in eq. (3) can be replaced with the association equilibrium constant (K_a) [32,39,40].

Another, equivalent way of describing the binding of a drug with an entrapped agent is to relate *k'* to 1) the mass-per-volume amount of binding agent in a microcolumn (i.e., m_g/V_M, in units

such as g L^{-1}) and 2) the distribution equilibrium constant for the drug with the binding agent (K_D, also sometimes referred to as an "adsorption coefficient" or "partition coefficient") [19,22,23,49]. The expression that can be used in this situation is shown in eq. (4),

$$k' = \frac{(K_D)\, m_g}{V_M} \tag{4}$$

where m_g is the mass of binding agent present, and V_M is the void volume of the microcolumn.

Prior work with humic acid and dissolved organic matter have reported binding constants for these agents with drugs by using either nK'_a or K_D [19,21-23,49]. The advantage of using a global affinity constant (nK'_a) for this purpose is this is a standard way of describing drug interactions with other binding agents, such as proteins [32,39,40]. An advantage of instead using K_D is this provides an equilibrium constant for a system in which the molar mass of the binding agent may not be known. If an estimate of the average molar mass (or "molecular weight", M_w) for the binding agent is available, it is possible to convert between the values of nK'_a (or K_a) and K_D. This conversion can be done by using the relationship $nK'_a = K_D M_w$ (or $K_a = K_D M_w$) and converting from g to kg to give K_D in units of L kg^{-1}.

Fig. 7 shows some typical chromatograms that were obtained when evaluating the binding of various drugs with entrapped humic acid (i.e., as prepared under the final optimized conditions described in Section 3.2.3). As is illustrated in this figure, it was possible to quickly compare the relative binding strengths of these drugs with the entrapped humic acid by comparing the elution order and retention times of the drugs. In this case, tetracycline and carbamazepine had the weakest binding to the entrapped humic acid at the pH and temperature that were used in this particular experiment, followed by ciprofloxacin or norfloxacin with much higher retention times. This relative order of this retention agrees, to an initial approximation, with the types of forces that are thought to dominate these interactions. For instance, carbamazepine is believed to undergo

weak hydrophobic interactions with humic acid [22,23], and tetracycline is thought to take part in hydrogen bonding or electrostatic interactions [19,22]. However, both ciprofloxacin and norfloxacin have been proposed to take part in a combination of hydrogen bonding, ion exchange, and cationic bridging during their binding with humic acid [8,22,49].

A more specific ranking of binding strengths could be obtained by determining the specific retention factor (*k'*) of each drug with the humic acid, as was done by using eq. (2) and correcting for any non-specific retention with the support (i.e., as measured on a control microcolumn). Table 1 lists the specific retention factors that were measured for each drug at several flow rates (0.10, 0.25, and 0.50 mL min^{-1}) and the average values that were obtained over this flow rate range. These specific retention factors were consistent across the range of flow rates that were examined, confirming that a local equilibrium was present at the positions of the peak centers for these drugs under these chromatographic conditions [27]. The relative precisions for the average *k'* values over all the listed flow rates ranged from ± 1.2-2.4% (1 S.D. for $n = 4$). Small apparent decreases of 4-10% in the retention factors were seen in going from 0.10 to 0.50 mL min^{-1} for carbamazepine, ciprofloxacin, and norfloxacin; up to a 12-24% change was seen for tetracycline. These observed changes were due to peak tailing that was present from some of the drugs, which was probably caused by slow adsorption/desorption or slow mass transfer kinetics (e.g., as can be seen in affinity columns with binding agents that have moderate-to-high affinities for a target) [50-52].

It was determined from these *k'* values and eqs. (3-4) that tetracycline had a binding constant (i.e., nK'_a or K_D) for the entrapped humic acid that was 1.9-fold higher than that of carbamazepine (i.e., the drug with the lowest *k'* in the group of drugs that were studied). Ciprofloxacin and norfloxacin had binding constants that were 24-fold and 32-fold higher than for carbamazepine, respectively. These differences were significant at the 95% confidence level, and

this order agreed with previous observations that have been made with the same drugs during their interactions with humic acid or related binding agents [8,19,23].

The numerical values of K_D and nK'_a were both estimated from the specific retention factors. For instance, K_D was obtained from k' by using eq. (4) and the measured humic acid content of the support, along with the known packing density of this support (0.45 g mL^{-1}, as listed by the manufacturer of the silica that was used as the starting material). Together, these last two factors provided the value of m_g/V_M in eq. (4). In a similar manner, nK'_a was obtained from K_D by multiplying this latter value by an estimate for the average molar mass of the humic acid (~35 kDa). The values of K_D and nK'_a that were obtained through this process are provided in Table 2. The model drugs used in this study gave K_D and nK'_a values that spanned over almost two orders of magnitude and over the general ranges of $10^3 - 10^5$ L kg^{-1} and $10^4 - 10^6$ L mol^{-1}, respectively. The average precision of these binding constants was ± 1.7% (range, ± 0.9-2.7%).

The values that were listed in Table 2 for K_D and nK'_a showed good agreement with previous literature values that were acquired at the same temperature (25 °C) and approximate pH (i.e., pH 7-8). For instance, the K_D of 1.84 (± 0.05) × 10^3 L kg^{-1} that was determined for tetracycline with the Aldrich humic acid at pH 7.4 in this study differed by only 2% and was statistically equivalent (at the 95% confidence interval) to a prior estimate obtained for the same drug and type of humic acid at pH 7 [19]. Similar agreement, with results equivalent at the 95% confidence level, was seen between a nK'_a of 3.49 (± 0.05) × 10^4 L mol^{-1} that was calculated for carbamazepine with Aldrich humic acid at pH 7.4 vs a nK'_a of 3.8 (± 0.5) × 10^4 L mol^{-1} that has been measured at pH 7 for this drug with Amherst humic acid [23] (i.e., a preparation with similar properties and composition to Aldrich humic acid) [14,53]. In addition, the K_D in Table 2 for carbamazepine with Aldrich humic acid at pH 7.4 and 25 °C is the same order of magnitude as a prior value in the

range of 10^2 – 10^3 L kg^{-1} (at an unspecified pH and temperature) that has been reported for this system [24].

As was expected from the differences in retention seen in Fig. 7 and Table 1, ciprofloxacin and norfloxacin gave binding constants that were one-to-two orders of magnitude higher than were noted for carbamazepine and tetracycline. For instance, the K_D values for ciprofloxacin and norfloxacin with Aldrich humic acid at pH 7.4 were found to be 2.34 (± 0.04) × 10^4 and 3.15 (± 0.03) × 10^4 L kg^{-1}, respectively. These values were consistent with a K_D range spanning from 10^4 – 10^5 L kg^{-1} that has been reported for these drugs at pH 7 with another form of humic acid (i.e., Pahokee peat) [8] as well as for ciprofloxacin with Aldrich humic acid under more acidic pH conditions [46].

4. Conclusion

In this study, a non-covalent entrapment technique was used to prepare a porous hydrazide-activated silica support that was capped with oxidized glycogen and contained humic acid as a stationary phase for HPAC. The conditions needed to prepare this material were optimized, and the support was characterized in terms of its humic acid content and retention for several model drugs (i.e., carbamazepine, tetracycline, ciprofloxacin, and norfloxacin). The highest retention of these drugs was obtained when using a support that was prepared by entrapment using an initial humic acid concentration of 80 mg mL^{-1} and a mass ratio of 600 mg humic acid per g silica. Preincubation of the humic acid with the activated silica, followed by the addition of oxidized glycogen to the slurry, was found to further improve the humic acid content of this material.

This support was used in affinity microcolumns to compare the retention of the selected drugs with the entrapped humic acid and to determine the values of the binding constants for these interactions. It was shown how this binding could be described in terms of a distribution

equilibrium constant (K_D, in units of L kg^{-1}) or a global affinity constant (nK'_a, in units of L mol^{-1}). The results were in good agreement with previous observations and measurements made for the binding of these drugs with humic acid under similar pH and temperature conditions [8,19,23].

There were several advantages noted for using HPAC and affinity microcolumns made by entrapment to examine the binding of drugs with humic acid. First, the microcolumns could be prepared using only a small amount of humic acid (i.e., about 10 mg humic acid to make the support for a 10 mm × 2.1 mm I.D. microcolumn) and allowed this same preparation of humic acid to be used over hundreds of sample injections. Second, this humic was immobilized and entrapped in a fully soluble and non-modified form that should have been a good mimic of this form of dissolved organic matter in environmental samples such as water. When these microcolumns were used in drug binding studies, they required only small injection volumes and provided, within a matter of minutes, a precise means of comparing the retention of a series of drugs and for estimating their binding strengths with the humic acid.

The same approach can be used in future work to study the binding of other drugs or with other types of humic acid and related forms of dissolved organic matter. This analytical method and the information it provides should be valuable in characterizing the forces that are involved in the binding of drugs with humic acid (e.g., by varying the pH and temperature) [8,20,23,46], as well as for studying the bioavailability and biological or toxicological effects associated with pharmaceutical agents as micropollutants in water and the environment [22,49]. In addition, the strong retention of a variety of drugs and good selectivity for this new class of humic acid supports should be useful for other applications. Examples could include the use of such supports for the isolation or separation of drugs that bind humic acid in solid-phase extraction or in mixed-mode separations based on HPLC [7,37,38,54].

5. Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgments

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Figure Legends

Fig. 1. General structure of a humic acid and examples of drugs that bind to humic acid: tetracycline, carbamazepine, ciprofloxacin, and norfloxacin. The structure of humic acid is based on information from Ref. [11].

Fig. 2. Scheme for the preparation of a silica-based support containing entrapped humic acid.

Fig. 3. Typical results obtained by TGA for a humic acid support that was prepared by entrapment (solid line) and a control support that was prepared by the same method but with no humic acid present (dashed line). The TGA results for the original sample of humic acid are provided within the inset for reference. These results are for the humic acid support that was prepared by the final entrapment method using preincubation of the humic acid with the support, an initial humic acid concentration of 80 mg mL^{-1}, and a slurry concentration of 600 mg humic acid per g silica.

Fig. 4. Typical chromatograms obtained for 20 μL injections of 5 or 10 μM tetracycline onto (a) a 10 mm × 2.1 mm I.D. humic acid microcolumn that was prepared by entrapment (see conditions given in the text) or (b) a control microcolumn of the same size that was prepared under the same conditions but with no humic acid added. The results were obtained at 0.25 mL min^{-1} and 25 ºC using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase.

Fig. 5. Typical chromatograms obtained at 0.10 mL min^{-1} for 20 µL injections of 10 µM tetracycline onto 10 mm × 2.1 mm I.D. humic acid microcolumns prepared by (a) using an initial humic acid concentration of 60 mg mL^{-1} or 80 mg mL^{-1} (final humic acid solution volumes: 1.36 and 0.91 mL, respectively) and a humic acid vs silica mass ratio of 400 mg per g silica or (b) using a single starting concentration of humic acid (80 mg mL^{-1};) while varying the humic acid vs silica mass ratio from 400 to 500 or 600 mg per g silica, and with a corresponding change in the final humic acid solution volume from 0.91 to 1.08 or 1.30 mL, respectively. All these chromatograms were obtained in presence of pH 7.4, 0.067 M potassium phosphate buffer at 25 °C.

Fig. 6. Typical chromatograms obtained at 0.50 mL min^{-1} for 20 µL injections of (a) 20 µM ciprofloxacin or (d) 20 µM norfloxacin onto 10 mm × 2.1 mm I.D. humic acid microcolumns prepared with no preincubation of the humic acid with the support (dashed line) or with preincubation (solid line). These results were obtained in presence of pH 7.4, 0.067 M potassium phosphate buffer at 25 °C.

Fig. 7. Typical chromatograms obtained at 0.50 mL min^{-1} for 20 µL injections of 10 µM carbamazepine or tetracycline and 20 µM ciprofloxacin or norfloxacin made onto 10 mm × 2.1 mm I.D. humic acid microcolumns, as prepared under the final optimized conditions described in Section 3.2.3. These results were obtained at 25 °C in presence of pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase.

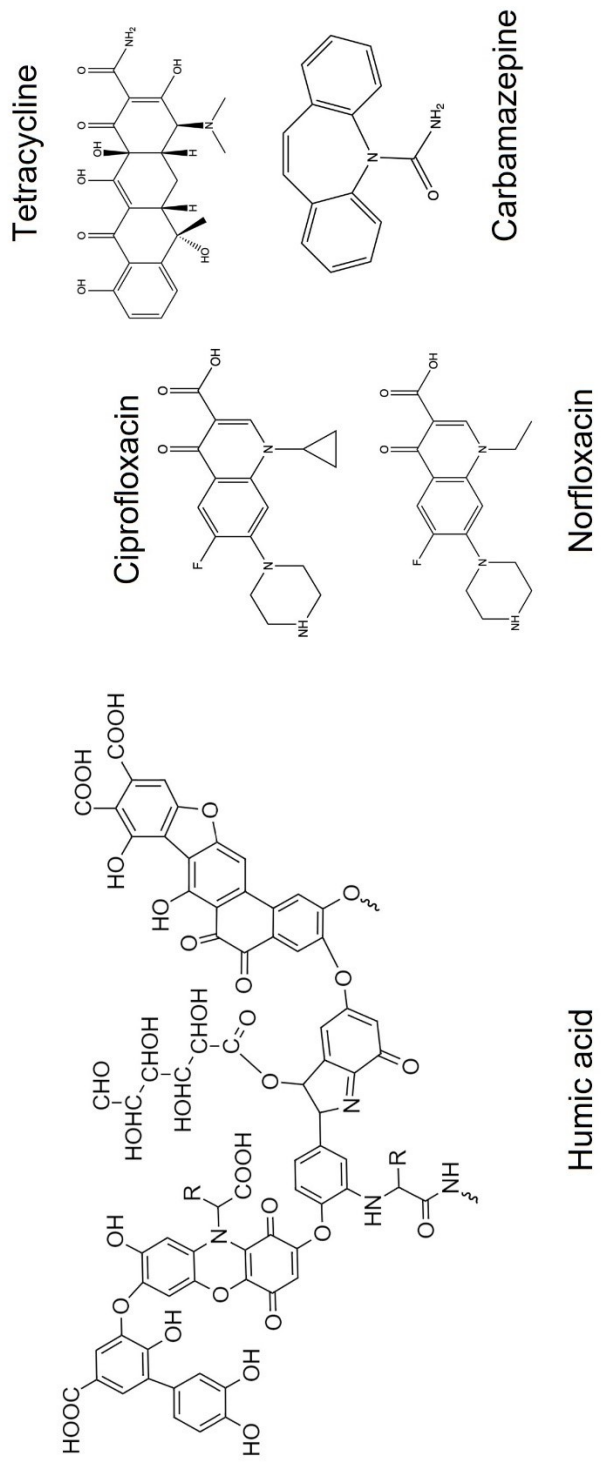
Tetracycline
Carbamazepine
Ciprofloxacin
Norfloxacin
Humic acid

Figure 1.

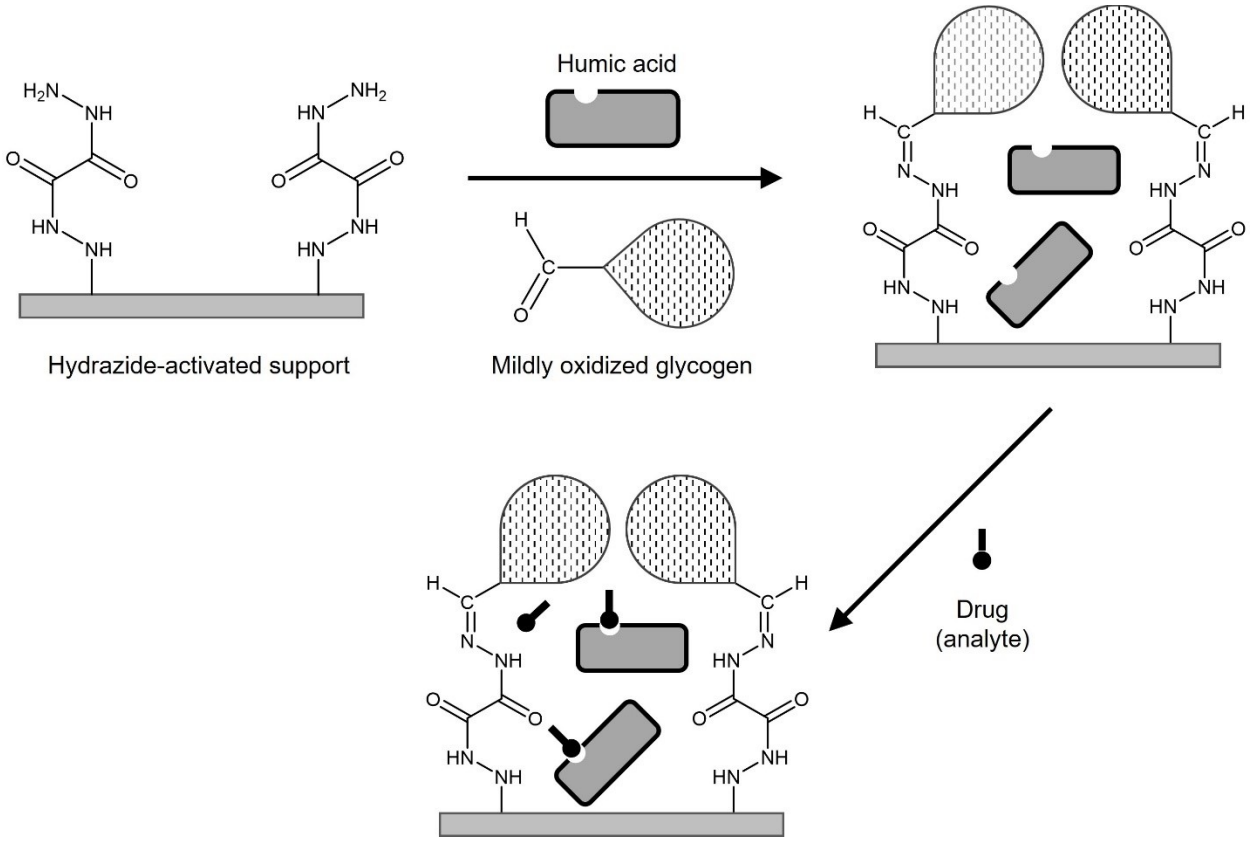
Humic acid
Hydrazide-activated support
Mildly oxidized glycogen
Drug
(analyte)

Figure 2.

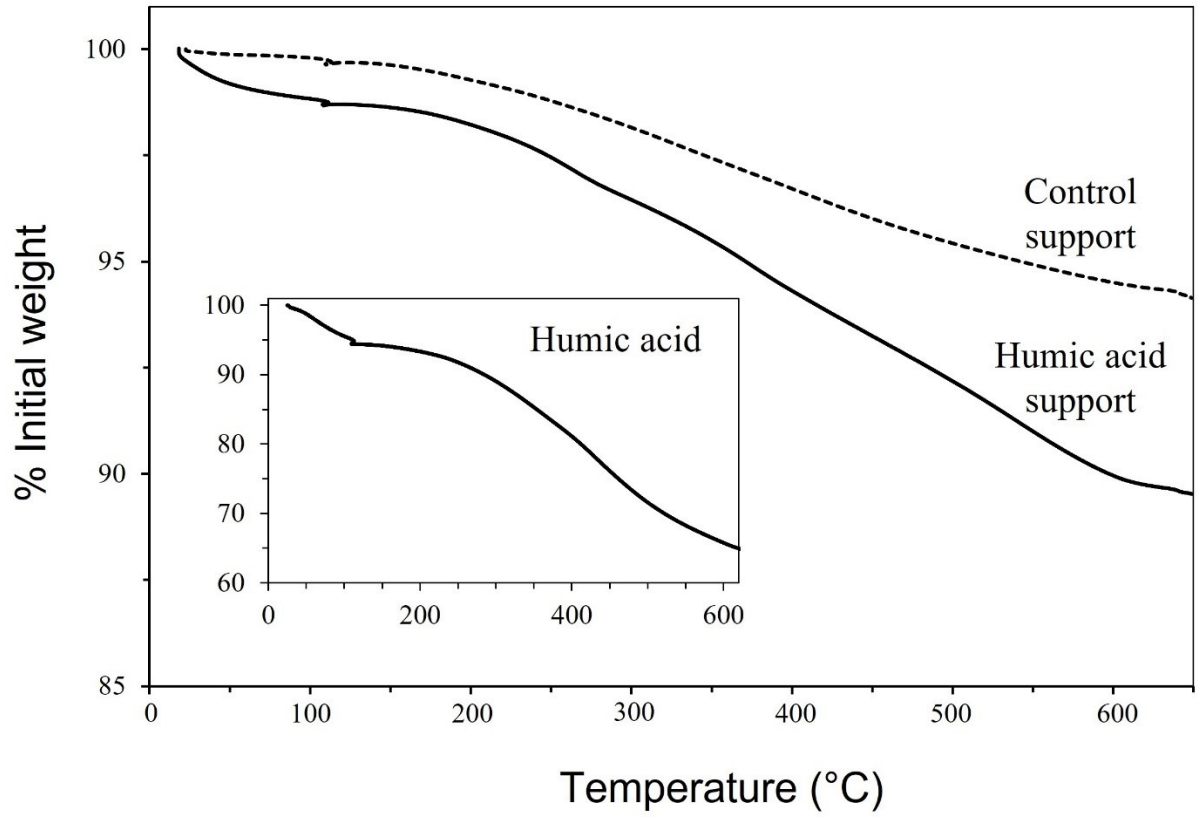
100
95
90
85
% Initial weight
0
100
200
300
400
500
600
Temperature (°C)
Control
support
Humic acid
support
Humic acid
100
90
80
70
60
0
200
400
600

Figure 3.

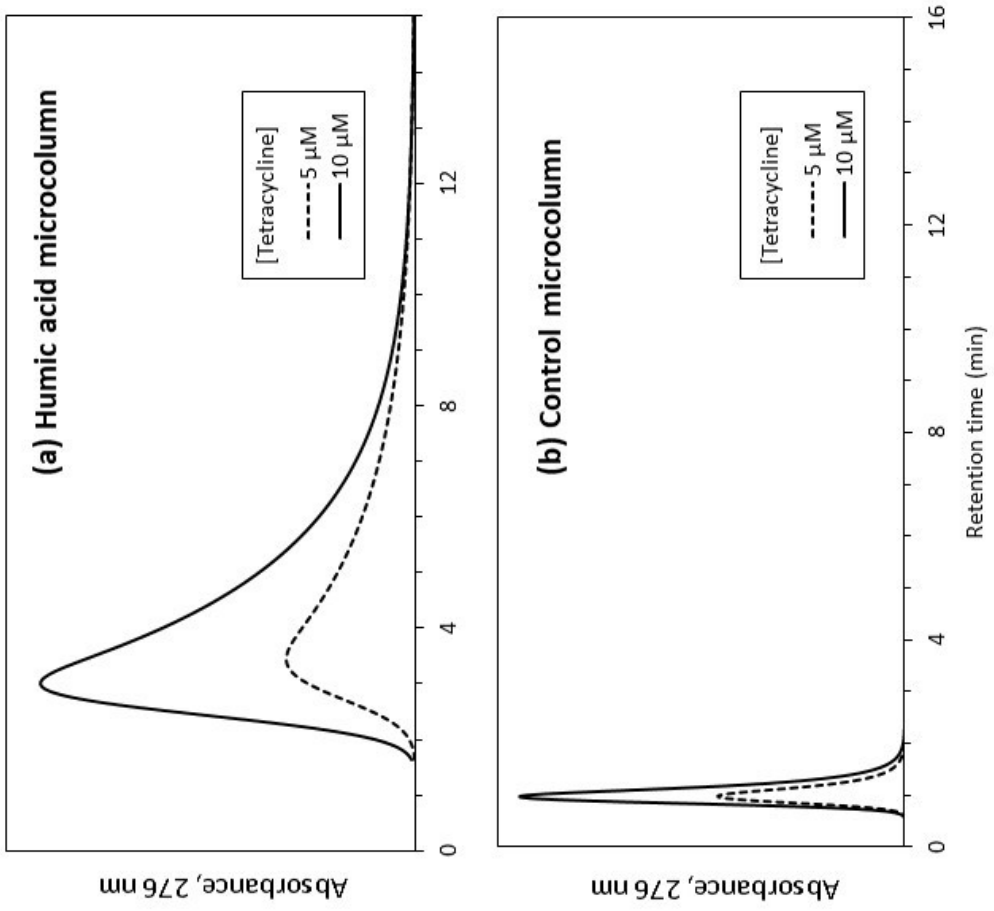
(a) Humic acid microcolumn
[Tetracycline]
5 µM
10 µM
Absorbance, 276 nm
0
4
8
12
(b) Control microcolumn
[Tetracycline]
5 µM
10 µM
Absorbance, 276 nm
0
4
8
12
16
Retention time (min)

Figure 4.

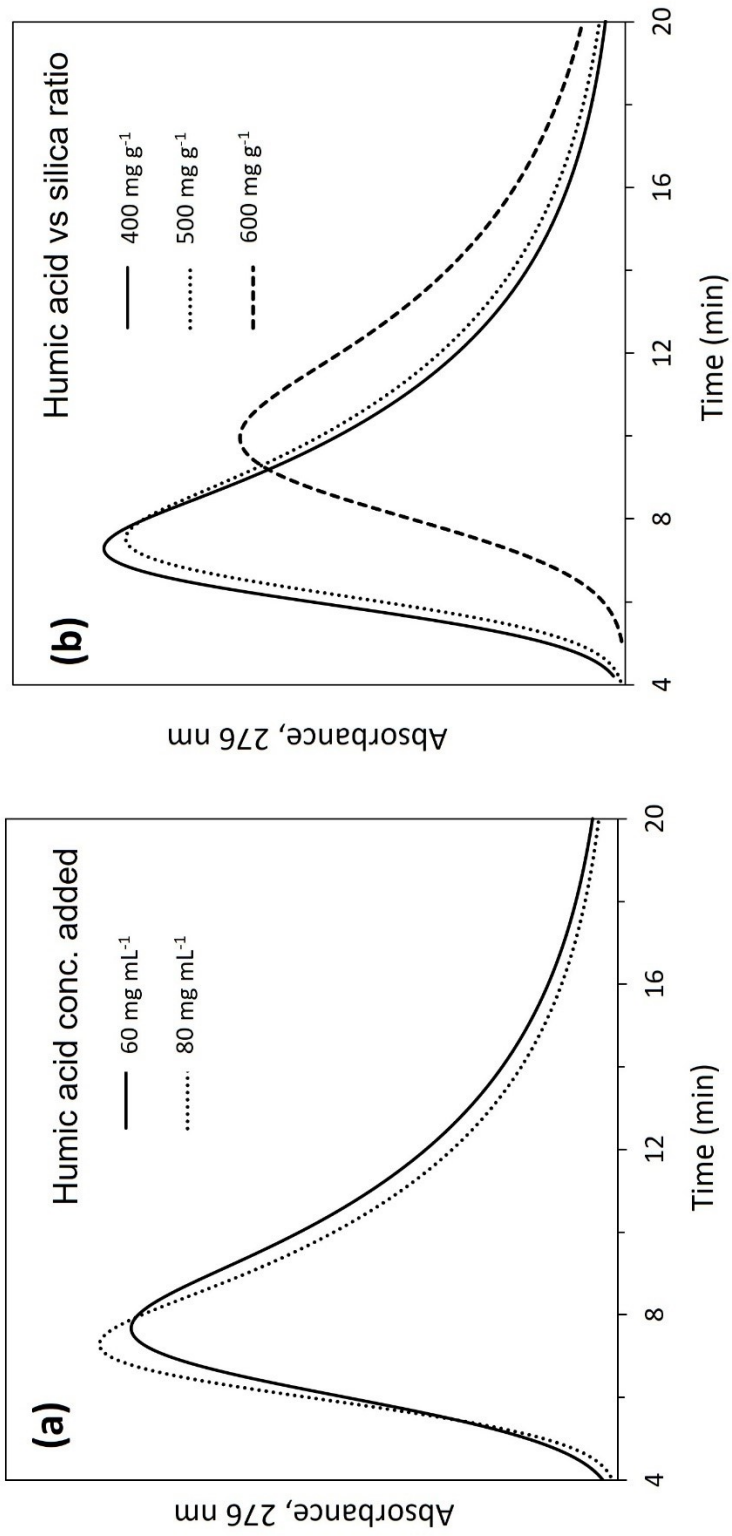
(a)
Humic acid conc. added
60 mg mL-1
80 mg mL-1
Absorbance, 276 nm
Time (min)
4
8
12
16
20
(b)
Humic acid vs silica ratio
400 mg g-1
500 mg g-1
600 mg g-1
Absorbance, 276 nm
Time (min)
4
8
12
16
20

Figure 5.

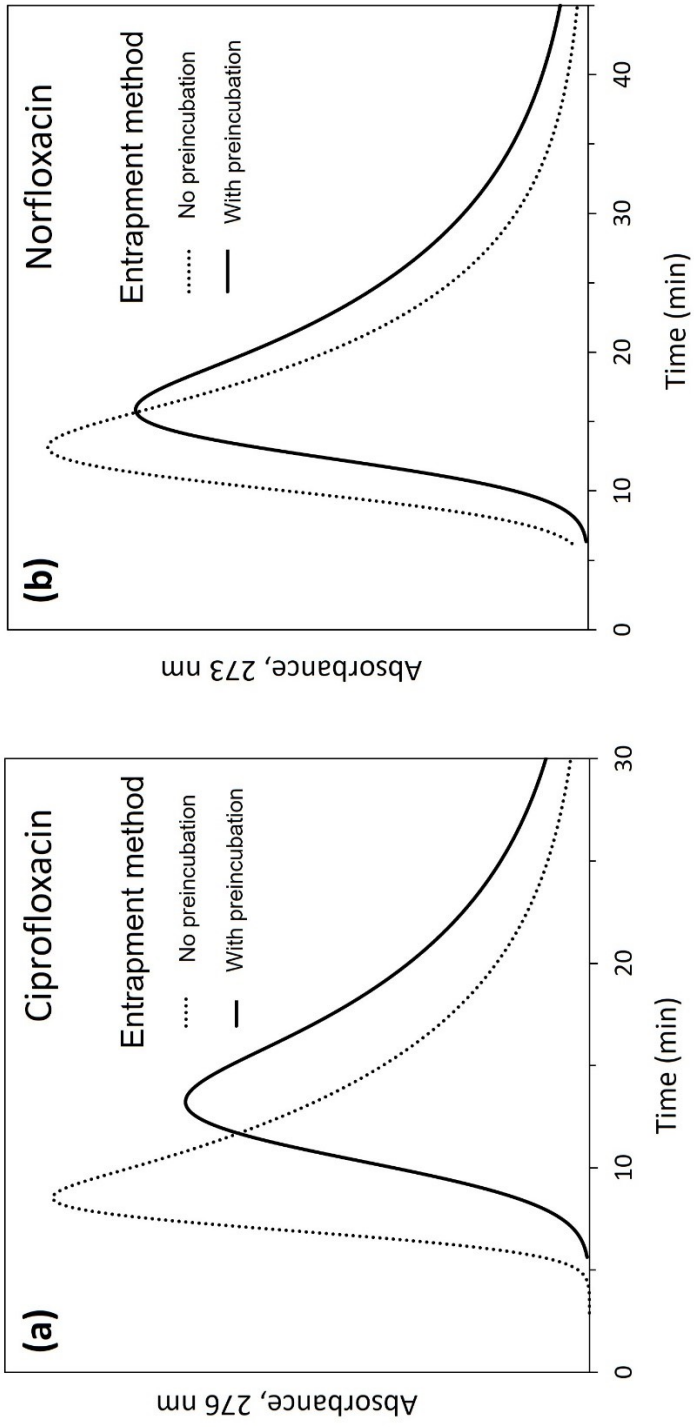
(a)
Ciprofloxacin
Entrapment method
No preincubation
With preincubation
Absorbance, 276 nm
Time (min)
0
10
20
30
(b)
Norfloxacin
Entrapment method
No preincubation
With preincubation
Absorbance, 273 nm
Time (min)
0
10
20
30
40

Figure 6.

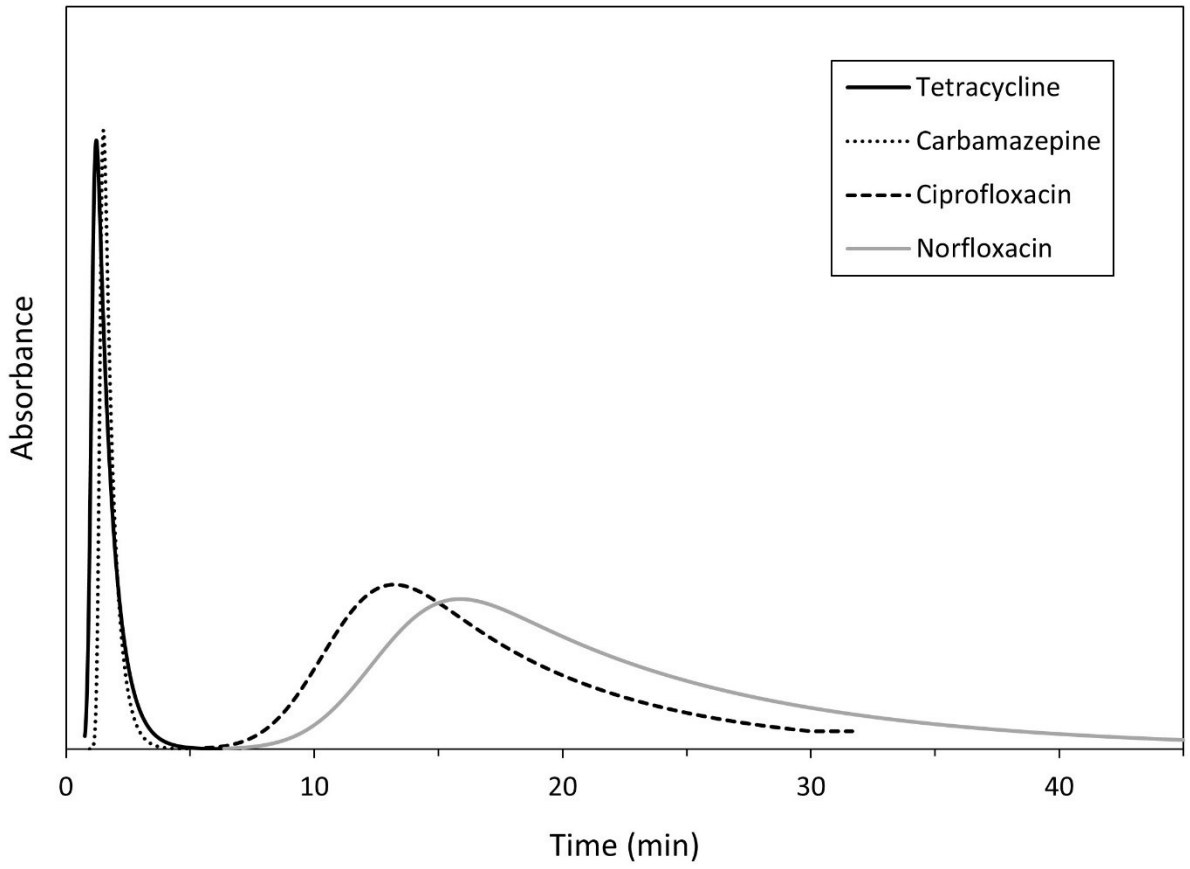
Tetracycline
Carbamazepine
Ciprofloxacin
Norfloxacin
Absorbance
0
10
20
30
40
Time (min)

Figure 7.

Table 1. Specific retention factors measured for several drugs on a humic acid microcolumn prepared by entrapment[a]

Flow Rate (mL min^{-1})	$k'_{Tetracycline}$[b,c]	$k'_{Carbamazepine}$[b,c]	$k'_{Ciprofloxacin}$[b,c]	$k'_{Norfloxacin}$[b,c]
0.10	27.8 (± 0.4)	13.6 (± 0.1)	333 (± 4)	455 (± 2)
0.25	24.6 (± 0.2)	13.6 (± 0.1)	310 (± 2)	414 (± 1)
0.50	21.2 (± 0.4)	13.0 (± 0.1)	300 (± 2)	418 (± 2)
Average k'[d]	24.6 (± 0.6)	13.4 (± 0.2)	314 (± 5)	424 (± 3)

[a]The numbers in the parentheses represent a range of ± 1 S.D. for four sample injections. The specific retention factors were measured at pH 7.4 and 25 (± 0.1) °C. The microcolumn was made by entrapment using the final optimized conditions described in Section 3.2.3.

[b]The following sample concentrations were used in this study: 10 µM tetracycline; 10 µM carbamazepine; 20 µM ciprofloxacin; and 20 µM norfloxacin.

[c]The relative contributions of non-specific binding to the total retention of the listed drugs on the humic acid microcolumn were as follows: tetracycline, 4.5-5.8% (average, 5.0%); carbamazepine, 41.6-48.0% (average, 43.8%); ciprofloxacin, 0.43-0.50% (average, 0.45%); and norfloxacin, 0.36-0.41% (average, 0.38%). The specific retention factors in this table have been corrected for this non-specific binding.

[d]This *k'* value is the average of the retention factors measured for a drug at 0.10, 0.25, and 0.5 mL min^{-1}.

Table 2. Equilibrium constants for the interaction of drugs with humic acid at 25 °C[a]

Drug	K_D[b] (L kg^{-1})	nK'_a[c] (L mol^{-1})	Literature value(s) (Type of Humic Acid, pH) [Ref.][d]
Carbamazepine	0.99 (± 0.01) × 10^3	3.49 (± 0.05) × 10^4	3.8 (± 0.5) × 10^4 M^{-1} (Amherst, pH 7) [18][e]
Tetracycline	1.84 (± 0.05) × 10^3	6.41 (± 0.16) × 10^4	1.8 × 10^3 L kg^{-1} (Aldrich, pH 8) [6][f]
Ciprofloxacin	2.34 (± 0.04) × 10^4	8.19 (± 0.13) × 10^5	1.719 (± 0.069) × 10^5 L kg^{-1} (Pahokee Peat, pH 7) [8]
Norfloxacin	3.15 (± 0.03) × 10^4	1.11 (± 0.01) × 10^6	7.50 (± 2.56) × 10^4 L kg^{-1} (Pahokee Peat, pH 7) [8]

[a]The numbers in the parentheses represent a range of ± 1 S.D. for four sample injections, as based on the error propagation using measured precision of the retention factors and the estimated precision of the TGA results used to provide the mass of humic acid per gram support. The values for nK'_a and K_D were determined at pH 7.4 and 25.0 (± 0.1) °C. The humic acid microcolumn used for this work was made by entrapment under the final optimized conditions described in Section 3.2.3. Terms: nK'_a, global affinity constant; K_D, distribution equilibrium constant.

[b]The value of K_D was calculated by using the average k' value for each drug over multiple flow rates, as provided in Table 1, along with the measured humic acid content of the support (37.28 mg per g silica) and the known packing density of this support (0.45 mg mL^{-1})

[c]The value of nK'_a for each drug was found by combining the calculated value of K_D, as provided above, along with an estimated average molar mass for Aldrich humic acid of 35,000 g mol^{-1} (based on information from the supplier; typical range, 20,000 to 50,000 g mol^{-1}).

Supplementary Material

Characterization of Supports by FTIR

FTIR spectra of humic acid, a support containing entrapped humic acid, and a control support that was made by entrapment but with no humic acid added were compared in Figure S1. The successful entrapment of the humic acid in the support was confirmed by the increase in absorbance (and decrease in %transmittance) that was seen for the broad peak centered around ~3300 cm^{-1}, which was due to O-H plus N-H stretching modes and/or appearance of additional peaks at approximately 1610 and 1651 cm^{-1}, as expected from the C=C stretch of aromatic groups and the C=O stretch from carboxylic acid groups in humic acid, respectively [1,2].

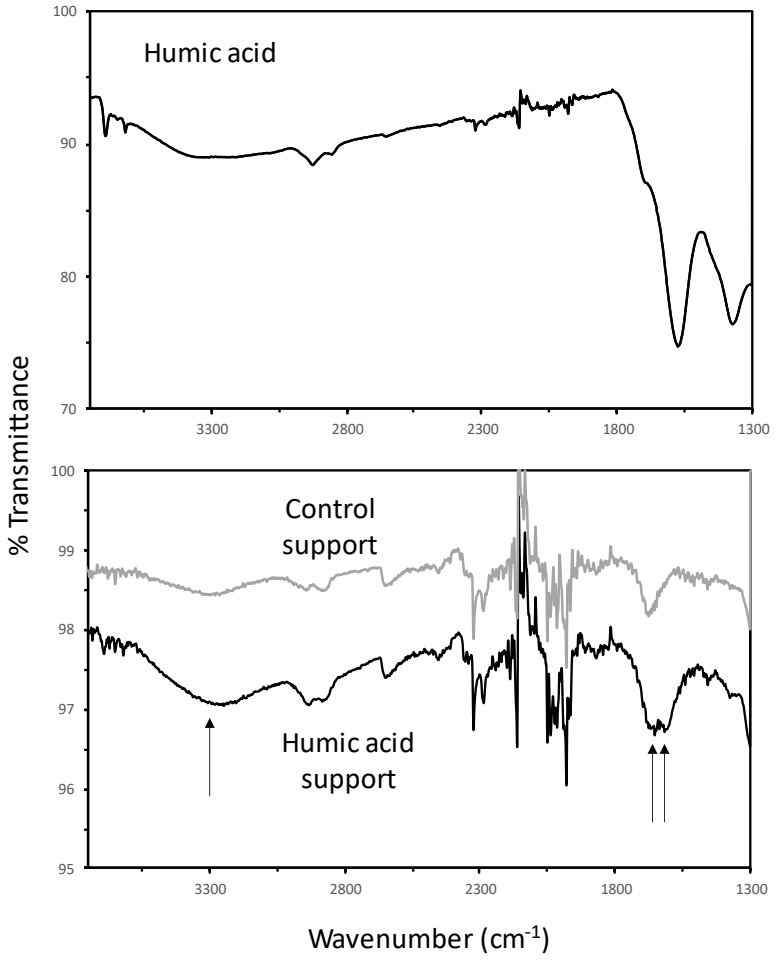

Figure S1. Typical results obtained by attenuated total reflectance FTIR for humic acid (top), a humic acid support that was prepared by entrapment (bottom, black line), and a control support that was prepared by entrapment but with no humic acid present (bottom, gray line). The humic acid support used in this example was prepared with no preincubation of the humic acid with the hydrazide-activated silica and using an initial humic acid concentration of 80 mg mL^{-1} along with a mass ratio of 500 mg humic acid per g silica.

Characterization of Supports by SEM

Figure S2 shows some SEM images that were acquired for 1) hydrazide-activated silica, 2) a control support that was prepared from the hydrazide-activated silica by reacting this material with oxidized glycogen, but with no humic acid present, and 3) a humic acid support prepared in the same manner as the control support and with humic acid being present during the entrapment process. These images indicate that no significant cross-linking or aggregation occurred between the support particles upon capping the hydrazide-activated silica support with oxidized glycogen or upon entrapment of humic acid within the support.

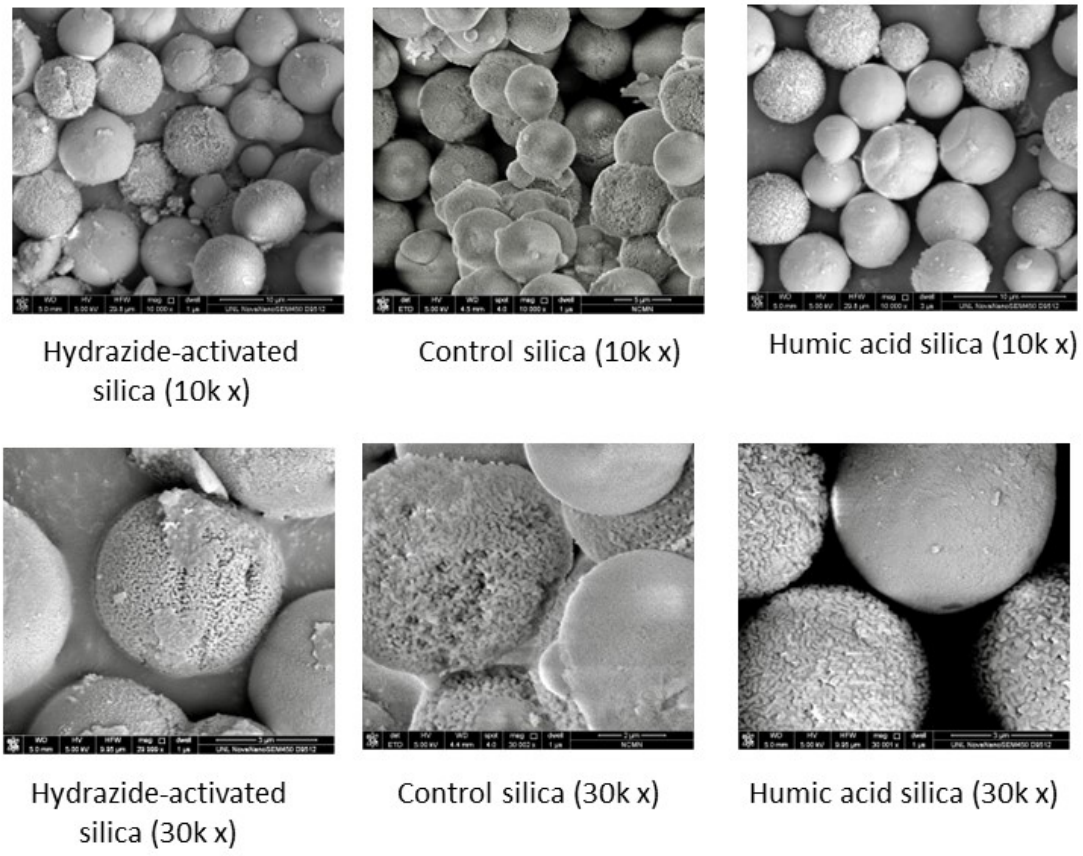

Figure S2. SEM images obtained (from left-to-right) for hydrazide-activated silica, control silica made by reacting the hydrazide-activated silica with oxidized glycogen, and humic acid silica that was prepared in the same manner as the control silica but with humic acid being present during the entrapment process. These supports were prepared using the final conditions described in the text under Section 3.2.3. These images are shown at (top) ×10,000 (10k) magnification and (bottom) × 30,000 (30k) magnification.

Energy-Dispersive X-Ray Analysis of Humic Acid Silica and Related Materials

Energy-dispersive X-ray spectroscopy (EDS) was used to examine the control silica and humic acid silica that were prepared under the final entrapment conditions discussed in Section 3.2.3. The results are summarized in Table S1. The carbon and oxygen content increased by 3.0- or 3.3-fold, respectively, for the humic acid silica versus the control silica. The presence of the various aliphatic chains and aromatic rings in humic acid probably contributed to the observed increase in carbon content for the humic acid silica vs the control support. The increase in oxygen content for the humic acid silica vs control silica can be attributed to the carboxylate, quinone, and phenol groups that are found in humic acid. The nitrogen content was found to increase by 2-fold when comparing the humic acid silica to the control support, due to the presence of peptides and other amines in humic acid [3]. These increases in the carbon, oxygen, and nitrogen content of the humic acid silica meant that its relative content of silicon had a corresponding decrease vs the control silica.

Table S1. Chemical composition, as determined by energy-dispersive X-ray spectroscopy (EDS) of the control silica, and humic acid silica that were prepared under final entrapment conditions used in this study.

Element	*Control support (Atomic %)*	*Humic acid silica (Atomic %)*
C	1.83	5.5
N	0.22	0.44
O	17.94	59.27
Si	80.02	34.8

Characterization of Long-Term Stability of Humic Acid Silica

The stability of affinity microcolumns containing entrapped humic acid was evaluated by making ~100 injections of 20 μL samples containing 10 μM carbamazepine onto a 10 mm × 2.1 mm i.d. humic acid microcolumn. The microcolumn used in Figure S3 was prepared using a starting humic acid concentration of 40 mg mL^{-1} and a humic acid vs silica ratio of 400 mg per g silica. Data that were obtained at 0.50 mL min^{-1} are shown in Figure S3. In this experiment, the humic acid microcolumn was found to be quite stable, with a maximum variation of only 16% occurring in the retention factor for carbamazepine during the application of 380 mL of mobile phase (~1.1×10^4 column volumes). Similar results were obtained for microcolumns that were prepared using an initial humic acid concentration of 80 mg mL^{-1} and a humic acid vs silica ratio of 600 mg per g silica. The backpressure across such a microcolumn during this type of study ranged from 2.7-4.0 MPa (390-580 psi), which was well below the maximum pressure that could be used (i.e., ~28 MPa or 4000 psi, the pressure at which this microcolumn was packed).

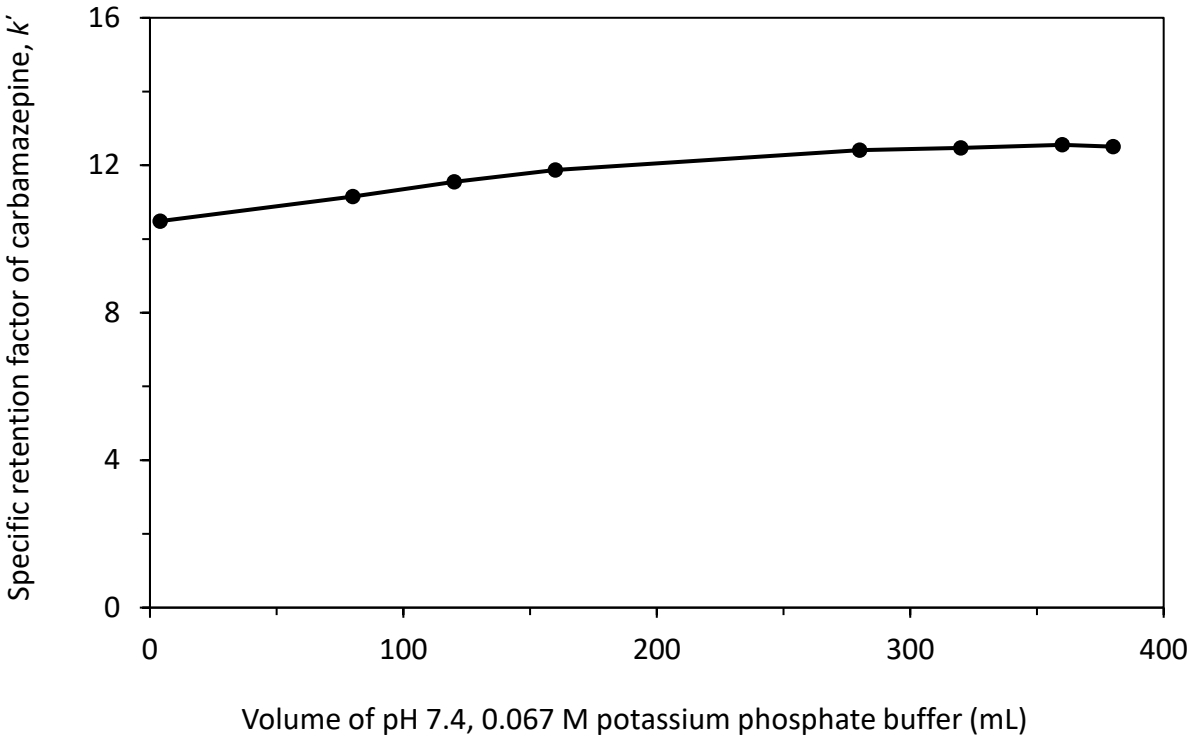

Figure S3. Effect of increasing the volume of applied mobile phase (pH 7.4, 0.067 M potassium phosphate buffer) on the specific retention factors measured at 0.50 mL min^{-1} for 10 µM carbamazepine on a 10 mm × 2.1 mm I.D. humic acid microcolumn prepared using an initial humic acid concentration of 40 mg mL^{-1} and a humic acid vs silica ratio of 400 mg g^{-1}. The error bars represent a range of ± 1 S.D. for four sequential injections and were comparable to the size of the data symbols. The specific retention factors were measured at 25.0 (± 0.1) ℃.

Measurement of Entrapped Humic Acid by TGA

The humic acid content of the humic acid silica was estimated by comparing the relative change in mass obtained by TGA for samples of the humic acid silica and a control support (i.e., with no humic acid present). This was done by using the following eq. (S1),

$$\text{Entrapped material from humic acid, } F_{HAo} = \left(\frac{\%W_{h110} - \%W_{h650}}{\%W_{h110}}\right) - \left(\frac{\%W_{c110} - \%W_{c650}}{\%W_{c110}}\right) \quad \text{(S1)}$$

where ($\%W_{h110}$ - $\%W_{h650}$) is the difference in percent weight seen for the humic acid silica at 110 °C vs 650 °C, and ($\%W_{c110}$ - $\%W_{c650}$) is the difference in weight (%) seen for the control support under the same temperature conditions. Inorganic material made up 20% of the humic acid preparation, based on information provided by the supplier. To correct for the mass due to the inorganic component, the value of F_{HAo} was multiplied by 0.80 to obtain F_{HA}, the total fraction (w/w) of humic acid in the humic acid silica. Multiplying the value of F_{HA} by 1000 then gave a relative weight in units of parts-per-thousand, or the equivalent to expressing the humic acid content in units of mg humic acid per g support.

References (Supplemental Material)

[1] T. Tanaka, Functional groups and reactivity of size-fractionated Aldrich humic acid, Thermochim. Acta 532 (2012) 60–64.

[2] S. Yan, N. Zhang, J. Li, Y. Wang, Y. Liu, M. Cao, Q. Yan, Characterization of humic acids from original coal and its oxidization production, Sci. Rep. 11 (2021) 1–10.

[3] B.A.G. De Melo, F.L. Motta, M.H.A. Santana, Humic acids: structural properties and multiple functionalities for novel technological developments, Mater. Sci. Eng. C. 62 (2016) 967–974.