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Analysis of Interactions between Pharmaceuticals and Humic Acid: Characterization using Entrapment and High-Performance Affinity Microcolumns

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Abstract

The presence of pharmaceuticals as microcontaminants in the environment has become a particular concern given the growing increase in water reuse and recycling to promote global sustainability of this resource. Pharmaceuticals can often undergo reversible interactions with soluble dissolved organic material such as humic acid, which may be an important factor in determining the bioavailability and effects of these compounds in the environment. In this study, high-performance affinity microcolumns containing non-covalently entrapped and immobilized humic acid are used to examine the binding strength and interactions of this agent for tetracycline, carbamazepine, ciprofloxacin, and norfloxacin, all common pharmaceutical microcontaminants known to bind humic acid. The binding constants, as measured with Aldrich humic acid, have good agreement with values reported in the literature. In addition, the effects of temperature, ionic strength, and pH on these interactions are examined with the humic acid microcolumns. This technique made it possible to determine the relative importance of electrostatic interactions vs non-polar interactions or hydrogen bonding on these binding processes. This study illustrates how affinity microcolumns can be used to screen and uniformly quantify binding by pharmaceuticals with humic acid, as well as to study the mechanisms of these interactions, with this information often being acquired in minutes and with small amounts of binding agent (~0.3 mg per microcolumn, which could be used over 200-300 experiments). Use of entrapment and affinity microcolumns can support similar research for a wide range of other microcontaminants with humic acid or alternative binding agents found in water and the environment.

Key words: Humic acid; Pharmaceuticals; Interaction studies; Affinity microcolumn; High-performance affinity chromatography

1. Introduction

Pharmaceuticals are a diverse class of compounds differing in their physicochemical and biological properties and mechanisms of action [1]. Pharmaceuticals are commonly used to not only treat humans and animals for diseases and care but also as part of animal feed and aquaculture [1-4]. However, the overuse of pharmaceuticals has led to their discharge into the environment, leading to their presence as microcontaminants in surface waters, groundwater, sediments, and soil [1,5,6]. This occurrence is of concern given the limited knowledge on the fate and behavior of pharmaceuticals in wastewater and environment, along with the possible effects such compounds may have on the aquatic ecosystem, antibiotic resistance, and potential health effects in humans [7-9].

In the environment, pharmaceuticals can undergo steps such as adsorption, migration, degradation, and transformation [10-12], which are influenced by the presence of complex binding agents such as dissolved organic matter [7,13]. One such binding agent is humic acid. Humic acid is an operationally-defined mixture of recalcitrant organic compounds, soluble at pH > 2 in water and produced from the breakdown of plant and animal matter [14-17]. This mixture is comprised of a large, heterogeneous set of organic polymers with molar masses that may range from 2 to 1300 kDa. As shown in Figure 1, humic acid can contain multiple functional groups, such as hydroxyl, carboxylic, phenolic, or enolic functional groups and quinones [15,17]. Depending on the source of the humic acid, this material may also contain peptides and sugars in its structure [15,17]. The carboxyl and hydroxyl groups in humic acid give this material an overall negative charge over the pH range seen in environmental water [18]. In addition, the size and shape of humic acid in solution is influenced by both pH and the presence of natural salts [19]. In a neutral or slightly alkaline medium, humic acid is present in an expanded state due to mutual repulsion of its negatively-charged groups. In a more acidic medium, contraction occurs due to charge

reduction and the humic acid becomes more spherical in shape. If the solution is quite acidic (e.g., pH 2.5 or less), aggregates of humic acid can also form [18].

Humic acid occurs widely in surface and groundwater and is known to bind many pharmaceuticals; this agent is even employed as an additive in a variety of drug formulations and related products [20-22]. The complex structure and variety of functional groups that may be present in humic acid make it difficult to predict, in advance of experimental studies, the nature of binding by this material with specific pharmaceuticals [23,24]. However, these interactions do each involve reversible, non-covalent interactions that may span from non-polar binding to dipole related interactions or electrostatic interactions, depending on the structure of the given pharmaceutical and type of humic acid that is present [25-29]. This has resulted in great interest in determining the interactions of pharmaceuticals with humic acid, as these interactions may affect the bioavailability, transportation, and solubilities of these microcontaminants in the environment [16,25-30].

Several methods have been used previously to examine humic acid binding to pharmaceuticals and related agents. These methods have included equilibrium dialysis, UV-Vis spectroscopy, fluorescence spectroscopy, nuclear magnetic resonance spectroscopy, and liquid chromatography-mass spectrometry [27-29,31]. However, many of these methods require relatively large sample volumes or amounts of material and may have long equilibration or extraction times [27,28]. An alternative approach that has recently been developed and validated for this work is the use of high-performance affinity chromatography (HPAC) and affinity microcolumns (i.e., columns with volumes in the low-to-mid microliter range) that contain humic acid [32]. As shown in Figure 1, the humic acid is non-covalently entrapped by this approach in a soluble form, providing a system that should be a good model for the humic acid found in

environmental water and microcolumns in which only a small amount of this material is required for many binding and interaction studies [32-35]. Other advantages of using these microcolumns with HPAC include the ability to conduct interaction studies in an automated system and to acquire precise data binding in short periods of time (i.e., minutes per sample injection) [36-38].

The objective of this research was to see how affinity columns with entrapped humic acid could be used for a detailed examination of the interactions of this binding agent with representative pharmaceuticals found in the environment. The effects of changes in conditions such as the temperature, pH, and ionic strength of surrounding medium were considered. The microcolumns were prepared with Aldrich humic acid, which has often been used as a reference material and model system in examining drug binding by humic acid [27,31,32]. The pharmaceuticals that were examined included tetracycline, carbamazepine, ciprofloxacin, and norfloxacin (see Figure 2). These compounds were employed because they are often found as microcontaminants in the environment; they are also known to undergo reversible, non-covalent interactions with humic acid [26-30,32]. These interactions were studied by HPAC and affinity microcolumns to aid in characterizing how humic acid binds to such pharmaceuticals as microcontaminants. The results were then compared to prior observations from the literature and used to provide fundamental information on the strength and expected extent of these interactions in the environment.

2. Materials and methods

2.1 Materials

Aldrich humic acid (~20% inorganic residue, product 53680, lot BCB7247), periodic acid (99% pure), oxalic dihydrazide (98%), sodium borohydride (98%), glycogen (bovine liver, type IX, total glucose ≥ 85%, dry basis), sodium chloride (≥ 99%), tetracycline (≥ 98%), ciprofloxacin

(≥ 98%), and norfloxacin (≥ 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Carbamazepine (≥ 99%) was acquired from Tocris Bioscience (Minneapolis, MN, USA). The silica (Nucleosil Si-300, pore size 300 Å, particle diameter 7 μm) was from Macherey-Nagel (Duren, Germany). Amicon ultra centrifugal filters (30 kDa cutoff; Millipore Sigma, Burlington, MA, USA) were employed for purification of the oxidized glycogen. All aqueous solutions and buffers were prepared using water purified with a Milli-Q system (Barnstead, Dubuque, IA, USA). GNWP nylon membrane filters (0.22 μm, Fischer Scientific, Pittsburgh, PA, USA) were used to filter the buffers. All other reagents were of the purest grades available.

2.2 Instrumentation

Thermogravimetric analysis (TGA) was carried out using a TGA 550 system (Waters, New Castle, DE, USA) equipped with nitrogen flow to maintain the inert analysis conditions and controlled by TRIOS v5.1.1.46572 software from Waters. The zeta potentials were measured using a Zetasizer Nano ZS90 system, and these data were analyzed using Zetasizer v8.02 software (Malvern Panalytical, Worcestershire, UK).

The affinity microcolumns containing humic acid and the control microcolumns were packed using a Prep 24 pump from ChromTech (Apple Valley, MN, USA). The HPLC system utilized for chromatographic studies consisted of a PU-2080 pump, a DG-2080-54 degasser, an AS-2057 autosampler, a UV-2075 detector, a CO-2067 column oven, a HV-2080-01 column selection unit to regulate buffer and sample flow through the column, and an LCNet control unit from Jasco (Easton, MD, USA). Data acquisition from the HPLC system was carried out using ChromNAV v1.18.04 software (Jasco, Easton, MD, USA). The chromatographic peaks were processed using the progressive, linear, and exponentially modified Gaussian (EMG) functions of

PeakFit v4.12 software (Jandel Scientific, San Rafael, CA, USA). Additional data analyses were carried out using Excel (Microsoft Office 36, Redmond, WA, USA).

2.3 Preparation of microcolumns

The humic acid was non-covalently immobilized within porous HPLC-grade silica via a slurry entrapment method with split mixing, as described previously [32]. Prior to entrapment, commercial HPLC-grade silica was converted to diol-bonded silica [34,35]. As shown in Figure 1, the diol-bonded silica was then placed into an aldehyde-activated form by treating it with periodic acid, followed by conversion of the support into hydrazide-activated silica through combining the oxidized support with oxalic dihydrazide [33-35,39].

Humic acid was first dissolved in 5.0 mL of pH 11.0, 0.10 M potassium phosphate buffer by stirring for 2 h at room temperature to obtain an initial concentration of 80 mg/mL [32,40,41]. The pH of this solution was then slowly adjusted to pH 6.0 by adding pH 2.5, 0.10 M potassium phosphate buffer, giving a final concentration of humic acid of approximately 14.25 mg/mL. This humic acid solution was then combined with 70 mg of hydrazide-activated silica, giving a slurry that contained 600 mg humic acid per g hydrazide-activated silica. The slurry was degassed for 10 min to remove any trapped air within the support and then mixed on a wrist action shaker for 3.5 h at room temperature.

Mildly oxidized glycogen was prepared as described previously to give a final working solution that contained about 4.25 mg/mL glycogen in pH 6.0, 0.10 M potassium phosphate buffer [32,42]. The oxidized glycogen solution was then added to a slurry containing hydrazide-activated silica and humic acid (see Figure 1). The oxidized glycogen was present in this mixture at a level of 18 mg glycogen per g silica [32,42]. This mixture was adjusted to a volume of 3.0 mL by adding pH 6.0, 0.10 M potassium phosphate buffer and then shaken for 18 h at room temperature using a

wrist action shaker. After the entrapment step, any unreacted aldehyde groups on the oxidized glycogen or on the support were removed by adding to the slurry 50 μL of 1 mg/mL oxalic dihydrazide in pH 6.0, 0.10 M potassium phosphate buffer, with this new mixture then being shaken for another 2 h at room temperature [42]. The support containing humic acid was washed with pH 7.0, 0.1 M potassium phosphate buffer. A control support was prepared in the same manner where pH 6.0, 0.10 M potassium phosphate buffer was used in place of the humic acid solution in the incubation and entrapment step.

The entrapped humic acid support and control support were placed into separate stainless-steel columns with lengths of 10 mm and an inner diameter (i.d.) of 2.1 mm. The packing solution was pH 7.4, 0.067 M potassium phosphate buffer. All the microcolumns were downward slurry packed at 4000 psi (28 MPa). When not in use, all supports and microcolumns were stored at 4 °C in pH 7.4, 0.067 M potassium phosphate buffer. Supports and microcolumns that were used and stored under these conditions were typically stable over up to a year [32] and several hundred injection cycles (e.g., less than 12% change in retention for carbamazepine over 400 injections). This stability was noted over the full range of temperature and mobile phase ionic strengths that were employed. Similar stability was seen when a humic microcolumn was used at a fixed pH; however, going from a neutral to more acidic pH (e.g., pH 7.0 to 3.0) did result in an increase in microcolumn back pressure, which was presumably due to humic acid aggregation [18]. Thus, in the pH studies, separate microcolumns were used at each pH, while in the temperature and ionic strength studies the same microcolumn were employed over many experimental conditions.

2.4 Characterization of supports and humic acid

The humic acid content of the supports was determined by thermogravimetric analysis (TGA). In this analysis, the samples were heated from room temperature to 110 °C at 5 °C/min

and then held at this temperature for 20 min to remove any moisture that was originally present. Next, the temperature was increased from 110 °C to 650 °C at a rate of 20 °C/min [32,43,44]. During this analysis, a stream of nitrogen applied at a flow rate of 20 mL/min was used to purge oxygen from the system.

The weight fraction of entrapped humic acid (F_{HA}, in *w/w*) was estimated from the relative change in the mass of the humic acid support and control support (i.e., a support prepared in the absence of humic acid) by examining the mass change in these materials over 110 °C to 650 °C. This weight fraction was obtained by using the following equation,

$$\textit{Weight fraction of the entrapped humic acid}, F_{HA} = \frac{\%W_{h110} - \%W_{h650}}{\%W_{h110}} - \frac{\%W_{c110} - \%W_{c650}}{\%W_{c110}} \qquad (1)$$

where $\%W_{h110}$ and $\%W_{h650}$ are the percent of the initial weights of the humic acid silica at 100 °C and 650 °C; and $\%W_{c110}$ and $\%W_{c650}$ are the percent of the initial weights of the control silica at 100 °C and 650 °C. Because this equation only considered the combustible organic content of the humic acid, a correction was also made for the known inorganic content of the humic acid (i.e., 26.8%, as provided by the supplier for the lot used in this work) to obtain the overall final amount of humic acid (F_{HA_o}) in each support [32]. To make this latter correction, the value of F_{HA} from eq. (1) was multiplied by (1 – 0.268), or 0.732, to obtain F_{HA_o}. The value of F_{HA_o} was then multiplied by 1000 to give a result in parts-per-thousands, or the equivalent to expressing the humic acid content in units of mg humic acid per g support [32].

Zeta potential measurements were made under various pH conditions by preparing samples that contained 0.10 mg/mL Aldrich humic acid in 0.10 M potassium phosphate solutions with adjusted pH values that ranged from pH 2.0 to pH 8.0. These solutions were placed into disposable

capillary cells (product DTS 1070, Malvern Instruments, Worchestershire, UK) at 25 °C and were analyzed after a 2 min equilibration time. Each sample was measured using ten replicates.

2.5 Chromatographic binding studies

In the temperature studies, the mobile phase was pH 7.0, 0.10 M potassium phosphate. The same buffer with 0.10-0.40 M sodium chloride added was used in the work with solutions at various ionic strengths. The effect of pH was determined using mobile phases that consisted of 0.10 M potassium phosphate solutions with pH values adjusted from 3.0 to 8.0 in 1.0 pH unit intervals. The chromatographic system and column were maintained at 25 °C in all experiments except the temperature studies, in which several values in the range of 10-45 °C were utilized.

Retention times were determined by injecting sample solutions of each pharmaceutical in replicate (n = 4). These samples contained 10 μM carbamazepine or 20 μM tetracycline, ciprofloxacin, or norfloxacin prepared in the same mobile phase that was used in the given study. These pharmaceutical concentrations were found to represent linear elution conditions, in which no significant change in retention was seen with a further decrease in sample concentration [32,36]. All the working sample solutions were stored at 4 °C when not in use and were used within one week of preparation. The peaks for the pharmaceuticals were detected by monitoring the absorbance of the column eluent at 276 nm for tetracycline, 286 nm for carbamazepine, 276 nm for ciprofloxacin, and 273 nm for norfloxacin. The void volumes of the HPLC system and microcolumns were determined by injecting 20 μM sodium nitrate as a non-retained marker, as monitored at 205 nm [32,35]. A flow rate of 0.50 mL/min was used as the default condition in these studies, with equivalent retention factors also being obtained at lower flow rates (i.e., 0.10-0.25 mL/min). This latter behavior confirmed that a local equilibrium was present at the true

centers of these peaks under such conditions, as is ideally needed when using retention factors in binding and thermodynamic studies [32,36].

The retention time of each pharmaceutical or solute was determined by using the central moment of its chromatographic peak [36,38]. The retention factor (*k*) of the solute was then found by using the following relationship,

$$k = \frac{t_R - t_M}{t_M - t_0} \quad (2)$$

where t_R is the solute's retention time, t_M is the microcolumn's void time (e.g., as measured using sodium nitrate as a non-retained marker), and t_0 is the void time of the chromatographic system with no microcolumn present [35,36,38]. The difference in retention of a pharmaceutical on the humic acid microcolumn vs the control microcolumn provided the specific retention factor (*k'*) due to the interaction of the injected pharmaceutical with the immobilized binding agent (i.e., humic acid), as shown in eq. (3).

$$\textit{Specific retention factor } (k') = k_{humic\ acid\ column} - k_{control\ column} \quad (3)$$

This same approach has been used in the prior work with affinity microcolumns containing humic acid, serum proteins, or other binding agents to provide the specific retention factors of these agents for applied solutes [32,35]. Although the focus of this study was on retention and binding studies, information on the efficiencies of the affinity microcolumns is also provided in the Supplementary Material.

3 Results and discussion

3.1 Initial characterization of humic acid microcolumns

The amount of entrapped humic acid, as determined by TGA, was 20.7 (± 4.0) mg humic acid per gram of silica. This value was consistent with an entrapment level obtained in a previous report under similar conditions [32]. Prior characterization by scanning electron microscopy has

also shown that the entrapment of humic acid by the approach used in this study gives a uniform support preparation with no aggregation or significant cross-linking between the modified particles [32]. A 10 mm and 2.1 mm i.d. microcolumn packed with this material contained approximately 0.3 mg humic acid and was typically used over the course of 200-300 experiments. This meant the equivalent of only 1.1-1.6 μg humic acid was required per experiment. Furthermore, each microcolumn and entrapped preparation of humic acid could be used with multiple pharmaceuticals and under various mobile phase and temperature conditions. This combination of reusable microcolumns with HPAC gave an analytical platform with reproducible conditions and that could quickly provide precise, robust results for the types of binding and mechanistic studies that are described in this report.

Typical chromatograms that were obtained with the humic acid microcolumns and for the pharmaceuticals examined in this study are provided in the Supplementary Material (Figure 1S). When a 10 mm and 2.1 mm i.d. humic acid microcolumn was used at 0.50 mL/min and 25 ℃ in the presence of pH 7.0, 0.10 M potassium phosphate buffer, tetracycline and carbamazepine gave peaks with retention times of 0.9-1.2 min and that eluted within 2-3 min. Ciprofloxacin and norfloxacin had stronger retention under these same conditions, with retention times of about 13-17 min and overall elution times between 50-60 min. The order of elution for these compounds and the general range of retention was consistent with results that have previously been reported at pH 7.4 and 25 ℃ during the initial development of the entrapped humic acid supports and microcolumns for such studies [32]. The back pressure across the microcolumns ranged from 3.5-5.5 MPa (508-798 psi), which was fully compatible with a standard HPLC system.

The level of humic acid-specific vs. non-specific binding by the microcolumns for these pharmaceuticals was evaluated by comparing the measured retention times on both a microcolumn

containing entrapped humic acid and a control microcolumn that was prepared in the same manner but with no humic acid being added during the entrapment process (Supplementary Material, Table 1S). It was determined through this process that non-specific binding to the support made up 11.6% of the total retention measured for tetracycline, 55.3% for carbamazepine, 0.22% for ciprofloxacin, and 0.38% for norfloxacin. These results, including the higher level of non-specific binding noted for carbamazepine compared to the other pharmaceuticals, were also consistent with those noted in prior work with these pharmaceuticals using related supports [32,45]. The specific retention factor (i.e., the binding of a solute to only the entrapped agent) was found by using eq. (3) to take the difference between the retention factors measured on a humic acid microcolumn and control microcolumn for a given pharmaceutical. The humic acid-specific retention factors that were obtained at 25 °C and in pH 7.0, 0.10 M potassium phosphate ranged from values that reflected moderate-to-high retention. Moderate retention and binding strengths were seen for tetracycline, with a *k'* of 7.83 (± 0.39), and carbamazepine, with a *k'* of 7.83 (± 1.09). High retention and much stronger binding were observed for ciprofloxacin and norfloxacin, with *k'* values of 450 (± 28) and 523 (± 30), respectively. The typical precision of these specific retention factors under these conditions ranged from ± 5.0-13.1% (mean, ± 7.5%). Similar precisions were seen in the temperature, pH, and ionic strength studies described later in this report.

3.2 Determination of equilibrium constants for humic acid-pharmaceutical interactions

The following relationship shows how the specific retention factor (*k'*) for a pharmaceutical or injected solute is related to the strength of its interactions with an immobilized or entrapped binding agent such as humic acid [36,42].

$$k' = (nK_a') \frac{m_L}{V_M} \quad (4)$$

In this equation, $nK_a{}'$ is the overall global affinity constant (in units of L/mol) for the pharmaceutical as it distributes between its surrounding solution and the binding agent. Other terms in this equation include m_L, the total moles of the active and specific binding sites for the pharmaceutical in the microcolumn, and V_M, the void volume of the microcolumn. This equation assumes a local equilibrium for injected solute between the mobile phase and binding agent occurs at the true center of the pharmaceutical's peak and that linear elution conditions are present during the experiment (i.e., the amount of injected pharmaceutical is small compared to the amount of immobilized binding agent) [36]. Eq. (4) is a general expression that describes the overall interactions of a solute with an immobilized agent that has *n*-independent binding sites for the solute (i.e., as may occur for a heterogeneous binding agent) and that can be applied to either a homogeneous or heterogeneous binding agent and stationary phase [32,33,36]. If only a single binding site is present, the term of $nK_a{}'$can be replaced by the association equilibrium constant for this site (K_a) [36,42].

It can be seen from eq. (4) that the specific retention factor is directly proportional to nK_a' (or K_a). This means *k'* can be used directly to compare the relative binding strength of a series of solutes under the same conditions to an immobilized binding agent. In addition, if the value of *k'* is used with an independent estimate of m_L, it is possible to also determine the equilibrium constant for the interaction of the injected solute with the immobilized agent [36]. An alternative form of eq. (4) may also be used if the mass but not the moles of the immobilized binding agent in the system are known [32].

$$k' = \frac{(K_D)\, m_g}{V_M} \tag{5}$$

In eq. (5), the value of k' is now related to the mass-per-volume amount of binding agent in the column (i.e., $\frac{m_g}{V_M}$, in units such as kg/L) and the distribution equilibrium constant or partition

coefficient (K_D, in units such as L/kg) for the pharmaceutical or solute with the binding agent [26,27,32].

If the average molecular weight (M_w) for the binding agent is known, it is possible to convert between the value of nK_a' (or K_a) and the distribution equilibrium constant K_{D} by using the relationship $nK_a' = K_D M_w$ (or $K_a = K_D M_w$ for a system with a single type of binding site) [32]. Prior work with humic acid and related forms of dissolved organic matter have reported binding constants for pharmaceuticals and other solutes by using either nK_a' or K_a and K_{D} [26,27,32]. Thus, both approaches will also be used to describe pharmaceutical-humic acid interactions in this study.

3.3 Effects of temperature on pharmaceutical-humic acid interactions

The binding and retention by the selected pharmaceuticals with entrapped Aldrich humic acid increased with temperature over the range of 10 to 45 °C and in the presence of pH 7.0, 0.10 M potassium phosphate buffer as the mobile phase. Figure 3 shows some typical chromatograms for carbamazepine during these experiments. Similar behavior was seen for the other pharmaceuticals that were examined, with these variations in retention reflecting the relative changes in solute interactions with both the mobile phase and stationary phase (i.e., humic acid) as the temperature was varied. The specific retention factors obtained at various temperatures are provided in the Supplementary Material (Table 1S); the corresponding binding constants determined from these data for the pharmaceuticals with the humic acid are given in Table 1. For tetracycline and carbamazepine, the specific retention factor due to humic acid decreased by up to 19% or 36%, respectively, between 10 and 40-45 °C. Ciprofloxacin and norfloxacin decreased in their specific retention factors for humic acid by 21% and 39% under the same conditions.

Table 1 gives the values of K_D and nK_a' that were calculated from the observed specific retention factors for these pharmaceuticals. For instance, K_D was calculated from k' by using eq.

(5) along with the measured humic acid content for the support (20.7 mg humic acid/g silica) and the known packing density of this material (0.45 g/mL, as supplied by the manufacturer of the original silica). In a similar manner, nK_a' was obtained from K_D by using an average molar mass for Aldrich humic acid of ~35 kDa [32]. The precision of these two types of binding constants over the temperature studies ranged from ± 2.6% to ± 18% for all the tested pharmaceuticals, as determined from precisions of the retention factors used in this analysis. Because K_D and nK_a' were both directly proportional to the specific retention factor, as shown in eqs. (4-5), the values for these calculated binding constants in Table 1 decreased by the same amount as noted earlier for *k'* when increasing the temperature from 10 to 40-45 °C (i.e., a change of up to 18-38%). Tetracycline and carbamazepine had similar, low-to-moderate binding constants at each of temperatures that were examined (i.e., K_D values that ranged from 0.70-1.07 × 10^3 L/kg and nK'_a values of 2.42-3.75 × 10^4 M^{-1}). Ciprofloxacin and norfloxacin had much higher binding constants (i.e., K_D of 4.42-7.19 × 10^4 L/kg and nK'_a of 1.55-2.52 × 10^6 M^{-1}).

The K_D and nK'_a values that were determined in this report at pH 7.0 and 25 °C were compared with results of prior methods under similar conditions [26-28,30,32]. For instance, the K_D obtained for tetracycline with Aldrich humic acid at pH 7.0 and 25 °C, a value of 0.84 ($\pm$ 0.04) × 10^3 L/kg, showed good agreement with estimates of around 1.8 × 10^3 L/kg that have been obtained in two other studies for the same pharmaceutical, binding agent, and temperature at a slightly higher pH of 7.4-8 [27,32]. Similar agreement was noted for the nK'_a of 2.95 ($\pm$ 0.41) × 10^4 M^{-1} that was measured here for carbamazepine and Aldrich humic acid at pH 7.0 and 25 °C with prior values of 3.8 ($\pm$ 0.5) × 10^4 and 3.49 ($\pm$ 0.05) × 10^4 L/mol that have been determined at the same temperature and pH 7.0-7.4 [26,32]. In addition, the K_D of 0.84 ($\pm$ 0.12) × 10^3 L/kg noted at 25 °C and pH 7.0 for carbamazepine with Aldrich humic acid was the same order of

magnitude as values in the range of 10^2-10^3 L/kg that have been reported at 25 °C and pH 7.4 for this system [30,32]. The K_D values for ciprofloxacin and norfloxacin of 4.84 ($\pm$ 0.30) × 10^4 and 5.63 ($\pm$ 0.32) × 10^4 L/kg with Aldrich humic acid at pH 7.0 and 25 °C were consistent with K_D values of 10^4-10^5 L/kg that have been reported for these pharmaceuticals 25 °C with Pahokee peat at pH 7.0 and Aldrich humic acid at pH 7.4 [28,32].

A comparison was next made of the specific retention factors and binding constants measured here in pH 7.0, 0.10 M potassium phosphate buffer for Aldrich humic acid with these pharmaceuticals with the base-10 logarithm of their *n*-octanol-water partition coefficients, or log(K_{ow}). The log(K_{ow}) values for these compounds, which can be used as a general index of polarity, were provided in Figure 2 [1,23,24]. It was found that the general ranking of the retention and binding strength for the pharmaceuticals did not correlate with the order of their log(K_{ow}) values. For example, the order of retention was carbamazepine ≈ tetracycline << ciprofloxacin and norfloxacin, while the order of polarity (from low-to-high log(K_{ow}), or most polar-to-least polar) was tetracycline << ciprofloxacin < norfloxacin << carbamazepine. This result agreed with a prior obervation that, unlike many natural organic pollutants, the mobility of pharmaceuticals in the environment appears to not be directly related to their polarity and log(K_{ow}) values [7]. Instead, it has been suggested that the many non-polar, polar, and even ionizable groups in these pharmaceuticals may lead to a variety of other interactions with binding agents like humic acid [7]. In the following sections, the humic acid microcolumns were used to provide further information on the types of interactions that were present between Aldrich humic acid and the model pharmaceuticals in this study.

3.3.1 Changes in total free energy for pharmaceutical-humic acid interactions

The global affinity constants or association equilibrium constants (nK'_a or K_a) that were obtained in this report by zonal elution at various temperatures and in pH 7.0, 0.10 M potassium phosphate buffer were next used to find the thermodynamic parameters for binding by Aldrich humic acid to the given pharmaceuticals. The mechanisms and forces involved in this binding were more closely studied by determining the changes in free energy that occurred during the interactions of these compounds with the mobile phase vs humic acid. The changes in total free anergy for such interactions were obtained as follows [46,47].

$$\Delta G = \text{-RT ln } (nK'_a) \quad (6)$$

In this equation, ΔG was the total change in free energy, nK'_a was the global affinity constant (or the association equilibrium constant K_a for a system with a single binding site) that measured at absolute temperature (T), and R was the ideal gas constant.

The values that were obtained for ΔG at 25 °C and by using eq. (6) are provided in Table 2. These values were all negative, representing spontaneous reactions [48], with ΔG at 25 °C being around -25.5 kJ mol^{-1} for tetracycline or carbamazepine and -35.6 to -35.9 kJ mol^{-1} for ciprofloxacin and norfloxacin. The more negative ΔG values exhibited for the latter two pharmaceuticals reflected the higher affinities measured for these compounds with humic acid under the given solution conditions. The value of ΔG was similarly estimated at the other temperatures with the affinity microcolumns and gave precisions ranging from ± 0.25 to ± 1.4%, as based on error propagation. As the temperature was increased from 10 to 45 °C, ΔG became more negative, indicating that binding became more thermodynamically favorable for each of the model pharmaceuticals. The ΔG for tetracycline decreased from -24.5 to -27.4 kJ/mol when the temperature was increased from 10 to 45 °C, and carbamazepine had a ΔG that went from -24.8 to

-26.7 kJ/mol at these temperatures. Over the same temperature range, ΔG for ciprofloxacin went from -34.1 to -37.7 kJ/mol, and the value for norfloxacin decreased from -34.7 to -37.7 kJ/mol.

The general range of ΔG values obtained for these pharmaceuticals with Aldrich humic acid in pH 7.0, 0.10 M potassium phosphate buffer were consistent with prior work for the same solutes with other types of humic acid. For instance, the interactions of tetracycline with humic substances extracted from peat have been reported to have ΔG values of -23.33 to -23.40 kJ mol^{-1} at pH 5.0 and temperatures of 22 to 30 °C (Nascimento et al., 2024). A ΔG of -26.1 kJ mol^{-1} at pH 7.0 and 25 °C was calculated for carbamazepine with Amherst humic acid [26]. The binding of ciprofloxacin with humic acid was found to have a ΔG of -18.1 kJ mol^{-1} at 25 °C [49]. Also, ΔG values of -31.32 to -32.54 kJ mol^{-1} at pH 5.0 and 15 to 35 °C have been reported for norfloxacin with humic acid extracted from weathered coal [48].

For each pharmaceutical, the absolute value of ΔG was below 40 kJ mol^{-1} over the temperature range of 10 to 45 °C. This result indicated that the binding of these pharmaceuticals with humic acid and at the given pH and mobile phase conditions was reversible in nature, rather than involving irreversible adsorption or bond formation [48,50]. This model agrees with the observation made in this study that these pharmaceuticals could be applied, reversibly bound, and then eluted over this set of temperatures and under the mobile phase conditions that were employed with the affinity microcolumns containing entrapped humic acid.

3.3.2 Changes in enthalpy and entropy for pharmaceutical-humic acid interactions

It was further possible from the retention and binding studies made at several temperatures to determine the changes in enthalpy (ΔH) and entropy (ΔS) that were present for each pharmaceutical with the entrapped samples of humic acid and in the presence of pH 7.0, 0.10 M

potassium phosphate buffer. For instance, ΔG for a reaction such solute-ligand binding can be related to ΔH and ΔS by using the van't Hoff equation [28,46-48].

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

In addition, eqs. (6) and (7) can be combined to obtain the following relationship for a system with reversible solute binding that is now expressed in terms of nK'_a (or K_a) instead of ΔG [46,47].

$$\ln(nK'_a) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (8)$$

Eq. (8) predicts that such a system should give a linear relationship for a plot of $\ln(nK'_a)$ vs $\frac{1}{T}$. Furthermore, this plot should have a slope equal to $-\frac{\Delta H}{R}$ and an intercept of $\frac{\Delta S}{R}$, which can be used to provide the values of ΔH and ΔS [46].

Figure 4 shows plots made according to eq. (8) for retention data that were acquired on the humic acid microcolumns in the presence of pH 7.0, 0.10 M potassium phosphate buffer and between 10 and 45 °C. All the tested pharmaceuticals gave good linearity for these plots, with correlation coefficients that ranged from 0.9774 to 0.9949 (n = 6-7). This linearity indicated that eq. (8) and a reversible binding model described by nK'_a (or K_a) gave a reasonable description for the interactions of these solutes with Aldrich humic acid over this temperature range and set of solvent conditions [46,51]. Similar behavior based on this model has been seen in the binding of pharmaceuticals and other small solutes with serum transport proteins with comparable binding affinities to those measured here for humic acid [46,52]. This type of behavior has further been noted when using relationships equivalent to eq. (8) to examine the binding by norfloxacin and ciprofloxacin with Pakohee peat humic acid [28] or the binding by tetracycline with humic substances extracted from peat [53].

The values obtained for ΔH and ΔS from these plots are provided in Table 2. The change in enthalpy, ΔH, for Aldrich humic acid with all the model pharmaceuticals was negative and

ranged from -5.0 to -10.3 kJ mol^{-1}. These negative values for ΔH indicated that the overall energy gain by bond formation vs bond breakage (e.g., disruption of some solvent interactions with the pharmaceuticals or humic acid) was energetically favored. The change in entropy, ΔS, spanned from 53.6 to 102.6 J mol^{-1} K^{-1} for the same interactions. These positive values for ΔS meant the value for $-T\Delta S$ in eq. (7) was negative, or that the overall binding process led to an increase in entropy for the system. Similar increases in entropy have been observed for the binding of pharmaceuticals and solutes with serum proteins or humic acid [46,48,49,52,53]. Such an increase in entropy is typically related to the release of solvent molecules from the solute and/or binding agent as these two components interact to form a complex [46,54]. When the contributions of ΔH and $-T\Delta S$ were compared in eq. (7), the entropy term ($-T\Delta S$) was found to make up the largest contribution to ΔG at 25 °C. This entropy term contributed 79% to the overall negative value of ΔG at this temperature for tetracycline, 62% for carbamazepine, 85% for ciprofloxacin, and 70% for norfloxacin.

There are many types of interactions that could occur between a solute and a complex binding agent such as humic acid. Depending on the polarity, acid-base properties, and charge of the solute, binding of this agent to humic acid could involve non-polar or electrostatic forces, hydrogen bonding, and dipole-dipole interactions, among others [47,48]. It has been reported previously that some information on the relative contributions of these forces can be obtained from the values for ΔH and ΔS [47,55]. For example, in the case of protein-ligand and protein-protein binding, a situation where $\Delta H > 0$ and $\Delta S > 0$ indicates that hydrophobic association (i.e., due to partial withdrawal of non-polar groups from water) probably dominates the interaction [55]. If $\Delta H < 0$ and $\Delta S < 0$, van der Waals interactions and hydrogen bonds may be the most important forces in binding, while a system where $\Delta H < 0$ and $\Delta S > 0$ probably has electrostatic interactions playing

a major role [55]. In this study, all the model pharmaceuticals had $\Delta H < 0$ and $\Delta S > 0$ for their binding to Aldrich humic acid. If the same general trends in thermodynamics could be applied here as for protein-ligand binding [55], these results indicate that electrostatic forces likely played a significant role in binding for many of the model pharmaceuticals with the entrapped humic acid. The importance of such forces compared to other possible interactions was examined further in the next two sections.

3.4 Effect of ionic strength on pharmaceutical-humic acid interactions

Further information on the forces that were present between the model pharmaceuticals and entrapped humic acid was obtained by altering the ionic strength and salt content of the mobile phase. In these experiments, the ionic strength of the mobile phase was varied by adding various concentrations of sodium chloride (NaCl), an inert and fully dissociated salt, while the pH was held at pH 7.0 and the temperature was kept at 25 °C. The total ionic strength of the mobile phase, including the ionized components of 0.10 M potassium phosphate buffer at pH 7.0, ranged from roughly 0.19 M to 0.59 M as the added NaCl was raised from 0.00 M to 0.40 M. Figure 5 shows how the change in ionic strength affected the observed binding by each pharmaceutical with the humic acid microcolumns. Further details on the retention data that were used to create this figure are provided in the Supplementary Material (Table 2S).

As shown in Figure 5, all the pharmaceuticals had a decrease in their specific retention factors and overall binding for Aldrich humic acid as the ionic strength of the mobile phase was increased. For tetracycline and carbamazepine, the specific retention factors decreased by 33% and 53%, respectively, as the ionic strength was increased from 0.19 M to 0.59 M at pH 7.0 and 25 °C. Under the same conditions, the specific retention factors for ciprofloxacin and norfloxacin decreased by 72% and 69%. This type of decrease in binding strength as the ionic strength was

increased could have been produced by the shielding of electrostatic and dipole-dipole interactions [56,57]. Thus, the behavior in Figure 5 was consistent with the observation made in Section 3.3 that electrostatic interations were probably important in the binding by humic acid with many of the tested pharmaceuticals. Another possible effect of the increase in ionic strength may have been a change in electrostatic interations between ionized groups within humic acid, thus affecting the structure of this agent and also leading to a possible alteration in its binding strength for solutes [58].

3.5 Effect of pH on pharmaceutical-humic acid interactions

Additional information on the interactions between the tested pharmaceuticals and Aldrich humic acid was obtained by altering the pH of the mobile phase that was passed through the humic acid microcolumns. This made it important to also consider how the pH affected the net charges and/or acid-base forms of both the humic acid and pharmaceuticals during such experiments.

3.5.1 Effect of pH on humic acid

A variation in pH is known to alter the overall charge and ionization of acidic or basic groups within humic acid [58]. In addition, it is known a change in pH can affect the conformation of humic acid [29,59-61]. For instance, as the carboxylic groups on humic acid become protonated (i.e., as the pH falls below the pK_a of these groups at ~pH 5.0), this can facilitate intra- and intermolecular hydrogen bonding and lead to the aggregation of humic acid [58]. Non-polar compounds can be more attracted to this aggregated form of humic acid due to hydrophobic interactions [29,59,60]; however, such interactions may have negligible contributions when the aggregated form of humic acid binds with the polar and ionizable compounds [29,62].

The effect of pH on the ionization of Aldrich humic acid was examined by measuring the zeta potential, or the effective surface charge, for this binding agent [63]. This was done at 25 °C and using solutions with a pH of 2.0 to 8.0 that contained 0.10 mg/mL Aldrich humic acid and 0.10 M potassium phosphate (Note: The ionic strengths of these solutions were 0.10 M to 0.24 M based on the potassium phosphate concentration and the acid-base species for phosphate that were present at each pH). The zeta potential measured for these solutions is given in the Supplementary Material (Table 4S). The zeta potential went from -15.5 mV at pH 2.0 to -35.9 mV at pH 8.0, with each of these values having relative precisions of ± 3.9-7.8% (mean, ± 5.8%). The presence of negative zeta potentials for a commercial preparation of humic acid from Sigma-Aldrich and over this pH range has been noted previously [64]. In addition, the zeta potential of -20.8 (± 1.0) mV that was measured here for Aldrich humic acid at pH 3.0 was equivalent to a reported value of -21.0 mV for Suwannee River humic acid at the same pH and 25 °C [65].

Even at a low pH, it has been found that humic acid can have a negative charge due to some dissociation of its acidic functional groups [22,66]. In addition, the increase in negative potential seen for Aldrich humic acid as the pH was raised was expected, as the acidic functional groups would become deprotonated as the pH was increased above the pK_a values for these regions [67]. For instance, in the Supplementary Material (Figure 2S), the decrease in zeta potential seen for Aldrich humic acid in going from pH 2.0 to around pH 5.0-6.0 probably corresponded to the ionization and dissociation of carboxylate groups (pK_a range, 3.1-4.5); the further decrease in zeta potential seen above pH 5.0-6.0 could similarly reflect the ionization of phenolic acid groups (pK_a range, 6.0-10) [56]. The zeta potential for Aldrich humic acid reached about -36 mV at pH 7.0 and remained at this approximate value up through pH 8.0, which was the highest pH examined in this study. Besides changing the surface charge of humic acid with pH, the dissociation of acidic groups

may have further resulted in breaking of hydrogen bonds within humic acid and to an expansion in its configuration, thus exposing more potential binding sites to solutes [68].

3.5.2 Effect of pH on pharmaceuticals and humic acid interactions

The effect of a change in pH on the charges and acid-base forms of the model pharmaceuticals was next examined. This was done by using the known pK_a values for these compounds (see Figure 2) to determine which acid-base species were present over the pH range that was used in this study, as illustrated in Figure 6 [23,24]. The net charge of each pharmaceutical as function of pH was also calculated from these results (see Supplementary Material for equations used to calculate these values and the fraction of each acid-base species, as well as a summary of the acid-base forms of the pharmaceuticals that were examined in this report) [69].

The combined effect of pH on the binding of each pharmaceutical with humic acid was also investigated by determining the specific retention factors of these pharmaceuticals with entrapped Aldrich humic acid as the pH of the mobile phase was varied. The results are summarized in Figure 7. The specific retention factors that were used to generate these plots are provided in the Supplementary Material (Table 3S). The pK_a values that were present over the tested pH range of 3.0-8.0 are provided for reference in these plots. These conditions corresponded to the pH range of stability for the silica support used in the microcolumns and covered the pH range seen in most environmental water samples [18]. The net charge of each tested compound over this pH range is also provided, as determined by using the equations given in the Supplementary Material.

For tetracycline, the retention factors shown in Figure 7(a) had a strong dependence on pH over the tested pH range. At pH 3.0, the specific retention factor for tetracycline was around 42 and increased to a maximum of 54 at pH 4.0. This value then had a large decrease by pH 5.0,

leveled off between pH 6.0-7.0, and showed a more modest decrease from pH 7.0 to 8.0. This behavior can be explained by comparing Figure 7(a) with the expected acid-base forms for tetracycline in Figure 6(a) [70,71]. At a pH between 3.0 and 4.0, both the cationic (TET^{+}) and zwitterionic ($TET^{\pm}$) forms of tetracycline should have been present at significant levels (i.e., with TET^{+} being dominant at pH 3.0 and $TET^{\pm}$ at pH 4.0). This meant that electrostatic interactions, such as between the cation TET^{+} and negative charge on humic acid, and hydrogen bonding (e.g., between $TET^{\pm}$ and humic acid) could have led to the high retention seen in Figure 7(a) for tetracycline at these pH values and the increase in retention when going from pH 3.0 to 4.0. As the pH increased from 4.0 to 5.0-7.0, tetracycline was mainly present in its neutral zwitterionic form, so electrostatic interactions would have been greatly reduced; this would also now have made other forces such as non-polar binding, hydrogen bonding, or dipole-related interactions more important in binding [71]. As the pH increased from pH 7.0 to 8.0, the observed decrease in retention likely occurred as the singly-charged anion of tetracycline (TET^{-}) became a more important species and led to electrostatic repulsion with the negative charges on humic acid [71].

In the case of carbamazepine, Figure 7(b) shows that the specific retention factor for carbamazepine reached a maximum value of around 6.7-7.0 at a pH 5.0-6.0 and decreased as the pH was either further increased or decreased. Throughout this pH range, the neutral form of carbamazepine was dominant, and the net charge of this compound was at or close to zero, as shown in Figure 6(b) [72]. Thus, electrostatic interactions between this pharmaceutical and humic acid should not have been important in determining binding under these pH conditions [73]. Instead, non-polar interactions, hydrogen bonding, or other effects were more likely to be the main forces leading to binding between carbamazepine and humic acid [29,59,60,73]. At a lower pH of 3.0-4.0, the specific retention factor of carbamazepine for Aldrich humic acid was only about 0.4-

1.4. This low retention could have been due to the aggregation of humic acid in this pH range and subsequent decrease in accessible binding regions for carbamazepine [26,68]. The decrease in specific retention factors between pH 5.0-6.0 and pH 7.0-8.0 may further have reflected changes in non-polar interactions within humic acid as more acidic groups within this agent dissociated, thereby affecting hydrogen bonding within humic acid, along with its overall charge and structure [26].

Figures 7(c-d) indicate that similar retention was seen as the pH was varied for the two fluoroquinolones, ciprofloxacin and norfloxacin. In each case, the specific retention factor for Aldrich humic acid increased from pH 3.0 to a maximum around 6.0 and then decreased as the pH was further raised to 8.0. As shown in Figure 6(c-d), the main form for these compounds at a pH up to 6.0 was their cationic form (CIP^+/NOR^+) [49,74]. The negative charge on Aldrich humic acid also increased over this range, as shown in the Supplementary Material (Figure 2S). This meant an increase in electrostatic interactions could have produced the increase in retention seen for these two pharmaceuticals between pH 3.0 and 6.0; a change in hydrogen bonding with the increasing amount of the zwitterionic form over this pH range may also have been a factor. As the pH increased from pH 6.0 to 8.0, the zwitterionic form of these compounds ($CIP^{\pm}$/$NOR^{\pm}$) became dominant and some of the anionic form (CIP^-/NOR^-) also began to increase in its abundance [75,76]. The latter form would have led to some electrostatic repulsion with humic acid and a decrease in binding, although some changes over this range may have also occurred in hydrogen bonding, as is known to take place between these compounds and humic acid [23,24,49,74].

4. Conclusions

In this study, affinity microcolumns that had prepared by non-covalent immobilization of humic acid with porous silica were used to study the interactions of Aldrich humic acid with some

common pharmaceutical microcontaminants that are found in the environment. Each microcolumn contained only 0.3 mg humic acid, which was used with multiple compounds, temperatures, and mobile phase conditions over 200-300 experiments (i.e., the equivalent of 1.1-1.6 μg humic acid per analysis). These studies were also quick to perform, occurring on the minute time scale, and provided robust retention and binding data with high precision. In addition, the binding constants measured by this approach gave good agreement with the literature.

This approach was first used to determine the binding constants of the pharmaceuticals with Aldrich humic acid at pH 7.0 and temperatures ranging from 10 to 45 °C. Tetracycline and carbamazepine had similar, low-to-moderate binding constants at each of the temperatures that were examined, with K_D values in the general range of 0.70-1.07 × 10^3 L/kg and nK'_a values of ~2.42-3.75 × 10^4 M^{-1}. Ciprofloxacin and norfloxacin had much stronger binding, with K_D values of around 4.42-7.19 × 10^4 L/kg and nK'_a values of ~1.55-2.52 × 10^6 M^{-1}. The general order of these binding strengths did not correlate with the order of the log(K_{ow}) values for these pharmaceuticals, agreeing with a prior observation that the mobility of pharmaceuticals in the environment does not appear not be directly related to their polarity [7]. The effects of changing temperature, ionic strength, and pH on this binding were examined further to determine the types of interactions that were occurring between these pharmaceuticals and humic acid. It was found that 62-85% of the total change in free energy (ΔG) at 25 °C for this binding was due to the change in entropy. It was further found that electrostatic interactions were important for the binding of several of these pharmaceuticals with humic acid and over a pH range seen in environmental water. Other forces that were present during this binding may have included non-polar or polar interactions and hydrogen bonding.

Improved understanding of binding by these and other pharmaceuticals with humic acid is essential for understanding the transport, bioavailability, and behavior of these microcontaminants in water and the risks they pose in the environment [7,24]. This work demonstrates how affinity microcolumns can be used to rapidly screen and rank the binding by pharmaceuticals and other microcontaminants with humic acid, as well as to provide detailed mechanistic studies of these interactions. The ability to work with a small quantity of binding agent and to use the same agent across many experiments is a key advantage in allowing uniform conditions to be provided in such work. The speed, precision, and ease of automation of HPAC that can be combined with these microcolumns are also attractive features for their use in binding and mechanistic studies. The same approach and techniques can be applied in the future to study binding by other microcontaminants with humic acid or other agents present in water. This data, in turn, can be used to examine the role of this binding in affecting transport, bioavailability, and overall behavior or potential risks of such compounds in the environment.

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Figure Legends

Figure 1. General structure of humic acid, including ionizable acid-base groups that may occur in this structure [15,17], and scheme used for the entrapment and non-covalent immobilization of humic acid within a silica-based support. In this process, HPLC-grade silica was first converted to a diol-bonded form and then treated with periodic acid to give aldehyde-activated silica. This support was then combined with oxalic dihydrazide to give hydrazide-activated silica. The hydrazide-activated silica was combined in a slurry with humic acid and mildly oxidized glycogen. The hydrazone bonds that formed between the hydrazide groups on the silica and aldehyde groups on glycogen acted to entrap the humic acid within the pores or at the surface of the support. However, the final support still allowed small molecules such as many pharmaceuticals to access the entrapped agent for use in chromatographic studies and measurements of binding constants.

Figure 2. Structures of the pharmaceuticals examined in this study for their binding to humic acid. The literature values for the -log of the acid dissociation constants (pK_a) and the log of the *n*-octanol-water partition coefficients (log K_{ow}) for these compounds are also provided [1,23,24]. Tetracycline is an antibiotic containing a tricarbonylamide group (pK_a, 3.32), a phenolic diketone group (pK_a, 7.78), and a dimethylamino group (pK_a, 9.58) [71]. Carbamazepine is a dibenzoazepine-class anticonvulsant drug that contains a carboxamide group ($RCONH_2$); protonation and deprotonation of this group corresponds to pK_a values of 1.0 and 13.9, respectively [72]. Ciprofloxacin and norfloxacin are fluoroquinolone antibacterial agents that contain fluorine, a carboxylic acid (pK_a, 6.2-6.3), two tertiary arylamines, and a

secondary alkylamine (pK_a, 8.8) [23,24]. All these compounds are often found as contaminants in the environment and are known to undergo reversible, non-covalent interactions with humic acid [26-30,32].

Figure 3. Typical chromatograms and overall retention of peaks for carbamazepine on a 10 mm × 2.1 mm i.d. humic acid microcolumn at 0.50 mL/min and in the presence of pH 7.0, 0.10 M potassium phosphate buffer at 10, 20, 30, or 40 °C.

Figure 4. Plots prepared according to eq. (8) for injections of (a) tetracycline (●) or carbamazepine (■) and (b) ciprofloxacin (◆) or norfloxacin (▲) onto a 10 mm × 2.1 mm i.d. microcolumn containing entrapped humic acid that was used at 0.50 mL/min and temperatures of 10 °C to 45 °C. Other experimental conditions are given in the text. The equation for the best-fit lines were as follows: tetracycline, y = [6.6 (± 0.7) × 10^2] x + [8.1 (± 0.4)], with a correlation coefficient of 0.9773; carbamazepine, y = [11.5 (± 0.9) × 10^2] x + [6.4 (± 0.3)], with a correlation coefficient of 0.9853; ciprofloxacin, y = [6.1 (± 0.5) × 10^2] x + [12.3 (± 0.2)], with a correlation coefficient of 0.9838; and norfloxacin, y = [12.4 (± 0.6) × 10^2] x + [10.4 (± 0.2)], with a correlation coefficient of 0.9949 (n = 6-7 for all plots). The error bars represent a range of ± 1 S.D. The relative precisions of the y-values ranged from ± 0.7-6.9%.

Figure 5. Effect of ionic strength on the specific retention factors for (a) tetracycline (●) or carbamazepine (■) and (b) ciprofloxacin (◆) or norfloxacin (▲) on 10 mm × 2.1 mm i.d. microcolumns containing Aldrich humic acid. These results were acquired by using mobile phases in which 0.00, 0.10, 0.20, 0.30, or 0.40 M sodium chloride

was added to pH 7.0, 0.10 M potassium phosphate buffer. These results were obtained at 25 °C and 0.50 mL/min. The error bars represent a range of ± 1 S.D.

Figure 6. Calculated fractions (%) of the individual acid-base forms present as a function of pH for (a) tetracycline, (b) carbamazepine, (c) ciprofloxacin, and (d) norfloxacin. The pK_a values of these compounds, as provided in Figure 2, are represented by the vertical dashed lines. The net charge for each compound as a function of pH is also provided (see Supplemental Material for more details on the equations used for these calculations).

Figure 7. Effect of pH on the specific retention factors for the binding of entrapped Aldrich humic acid with (a) tetracycline (●), (b) carbamazepine (■); (c) ciprofloxacin (◆), and (d) norfloxacin (▲). These results were acquired by adjusting the pH of the mobile phase from 3.0 to 8.0 in a 0.10 M potassium phosphate solution and by injecting the pharmaceuticals at 25 °C onto 10 mm × 2.1 mm i.d. microcolumns at 0.50 mL/min. The error bars represent a range of ± 1 S.D. The vertical dashed lines show the pK_a values for each pharmaceutical over the sampled range of pH (i.e., as given in Figure 2). The net charge for each compound over the given pH range is also provided for reference (see Supplementary Material for more details on the equations used for these calculations).

Table 1. Distribution equilibrium constant (K_D) and global affinity constant (nK'_a) for Aldrich humic acid with several model pharmaceutical microcontaminants at pH 7.0[a]

Temperature (°C)	Tetracycline		Carbamazepine		Ciprofloxacin		Norfloxacin	
	K_D (× 10³ L/kg)[b]	nK'_a (×10⁴ L/mol)[c]	K_D (× 10³ L/kg)[b]	nK'_a (× 10⁴ L/mol)[c]	K_D (× 10⁴ L/kg)[b]	nK'_a (× 10⁶ L/mol)[c]	K_D (× 10⁴ L/kg)[b]	nK'_a (× 10⁶ L/mol)[c]
10	0.96 (± 0.02)	3.37 (± 0.09)	1.07 (± 0.06)	3.75 (± 0.22)	5.60 (± 0.34)	1.96 (± 0.12)	7.19 (± 0.35)	2.52 (± 0.12)
20	0.92 (± 0.06)	3.20 (± 0.21)	0.92 (± 0.12)	3.21 (± 0.42)	5.18 (± 0.30)	1.81 (± 0.11)	6.31 (± 0.37)	2.21 (± 0.13)
25	0.84 (± 0.04)	2.95 (± 0.14)	0.84 (± 0.12)	2.95 (± 0.41)	4.84 (± 0.30)	1.70 (± 0.10)	5.63 (± 0.32)	1.97 (± 0.11)
30	0.81 (± 0.06)	2.84 (± 0.21)	0.76 (± 0.09)	2.66 (± 0.31)	4.89 (± 0.90)	1.71 (± 0.31)	5.27 (± 0.91)	1.84 (± 0.32)
37	0.79 (± 0.05)	2.76 (± 0.16)	0.74 (± 0.10)	2.58 (± 0.34)	4.56 (± 0.27)	1.60 (± 0.09)	4.92 (± 0.29)	1.72 (± 0.10)
40	0.78 (± 0.02)	2.74 (± 0.07)	0.71 (± 0.05)	2.48 (± 0.19)	4.54 (± 0.21)	1.59 (± 0.07)	4.81 (± 0.21)	1.68 (± 0.07)
45	0.90 (± 0.04)	3.14 (± 0.13)	0.70 (± 0.07)	2.42 (± 0.25)	4.43 (± 0.28)	1.55 (± 0.10)	4.42 (± 0.28)	1.55 (± 0.10)

[a]The numbers in the parentheses represent a range of ± 1 S.D. for four sample injections, as based on the error propagation using the precision of the specific retention factors and the TGA results that were used to provide the mass of humic acid per gram support.

[b]The value of K_D was calculated by using the average k' for each drug, as provided in the Supplementary Material (Table 1S), along with the measured humic acid content of the support (20.7 mg/g silica) and the known packing density of the support (0.45 mg/mL).

[c]The value of nK'_a was found by combining the calculated value of K_D with an estimated average molar mass for Aldrich humic acid of 35,000 g/mol, as based on information from supplier (typical molar mass range, 20,000-50,000 g/mol).

Table 2. Change in Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) for several model pharmaceutical microcontaminants with Aldrich humic acid at pH 7.0[a]

Drug	ΔG (kJ mol^{-1}) at 25°C[b]	ΔH (kJ mol^{-1})[b]	ΔS (J mol^{-1}K^{-1})[b]
Tetracycline	-25.5 ($\pm$ 0.1)	-5.5 ($\pm$ 0.6)	67.4 ($\pm$ 3.3)
Carbamazepine	-25.5 ($\pm$ 0.3)	-9.6 ($\pm$ 0.7)	53.6 ($\pm$ 2.5)
Ciprofloxacin	-35.6 ($\pm$ 0.2)	-5.0 ($\pm$ 0.4)	102.6 ($\pm$ 1.2)
Norfloxacin	-35.9 ($\pm$ 0.1)	-10.3 ($\pm$ 0.5)	86.2 ($\pm$ 1.5)

[a]The thermodynamic parameters were measured with a 10 mm × 2.1 mm i.d. microcolumn containing entrapped humic acid. These results are for data collected with tetracycline, carbamazepine, ciprofloxacin, and norfloxacin at 0.50 mL/min. The numbers in 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.

[b]The values of ΔG were calculated by using eq. (6). The values of ΔH and ΔS were obtained from the plots by using eq. (8).

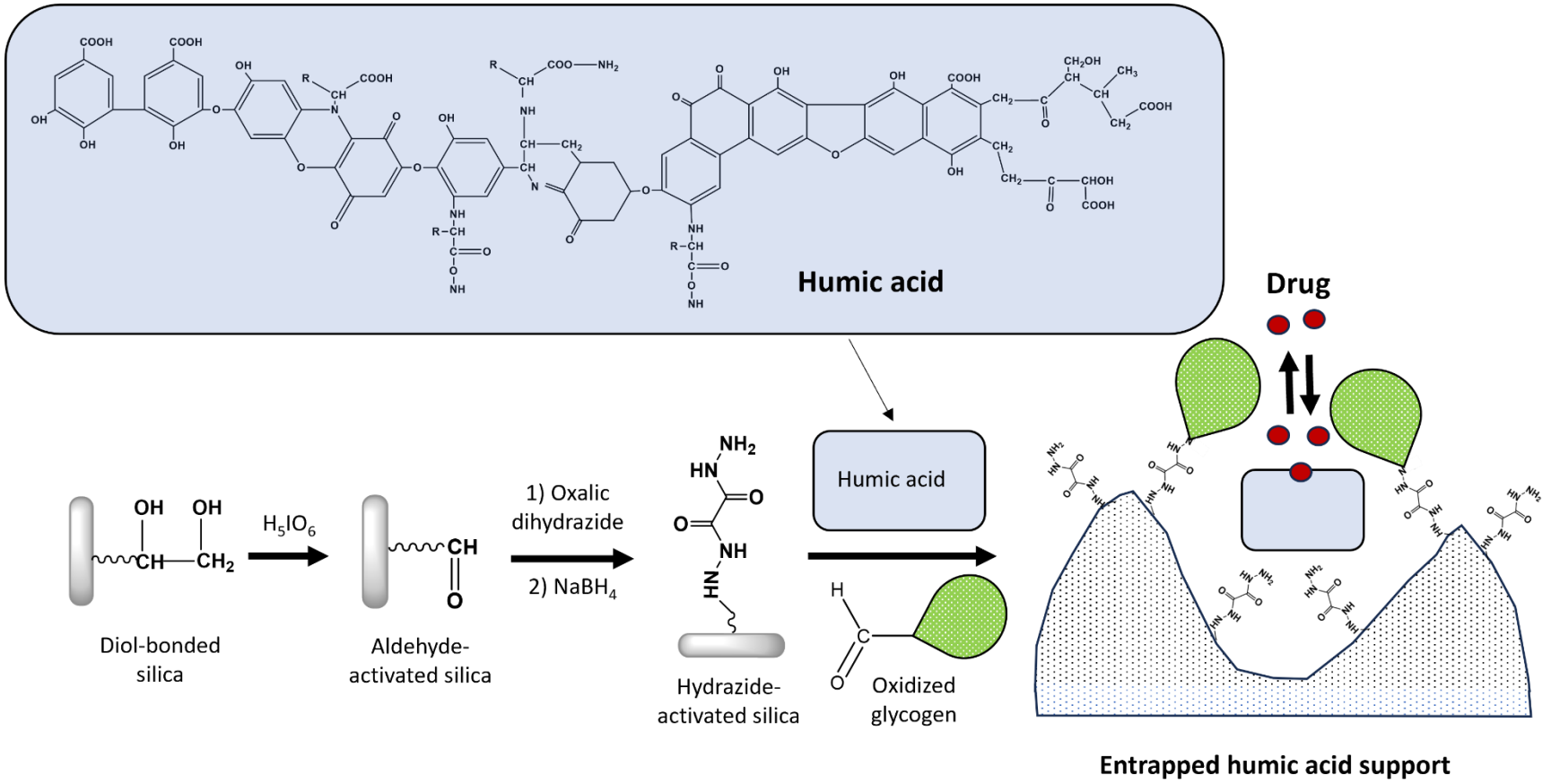
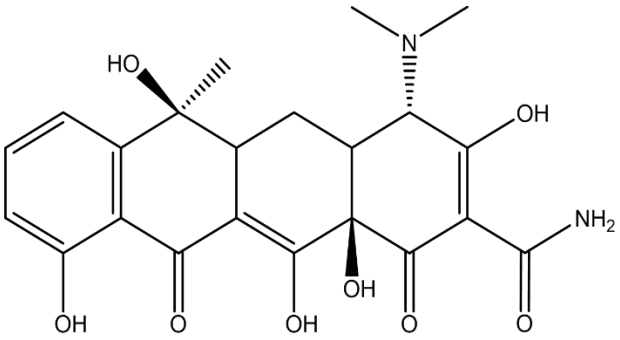
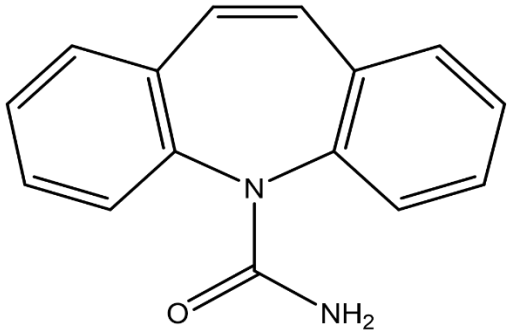

Humic acid
Drug
Diol-bonded silica
H_5IO_6
Aldehyde-activated silica
1) Oxalic dihydrazide
2) $NaBH_4$
Hydrazide-activated silica
Humic acid
Oxidized glycogen
Entrapped humic acid support

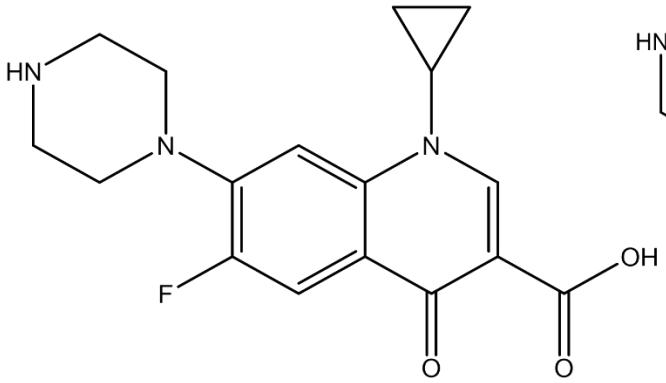
Figure 1



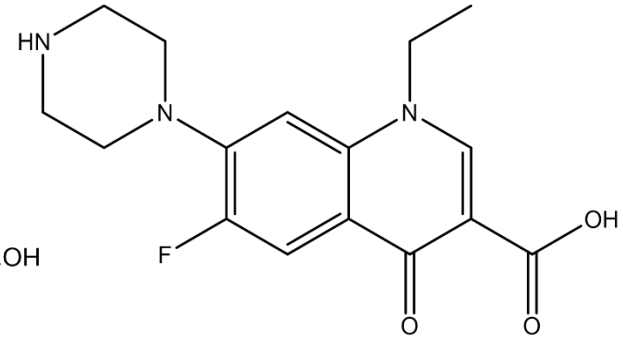
Tetracycline (TET)



Carbamazepine (CBZ)



Ciprofloxacin (CIP)



Norfloxacin (NOR)

Compound	pK_a	log(K_{ow})
Tetracycline	3.32, 7.78, 9.58	-1.37
Carbamazepine	~1, 13.9	2.45
Ciprofloxacin	6.18, 8.76	0.28
Norfloxacin	6.30, 8.76	0.46

Figure 2

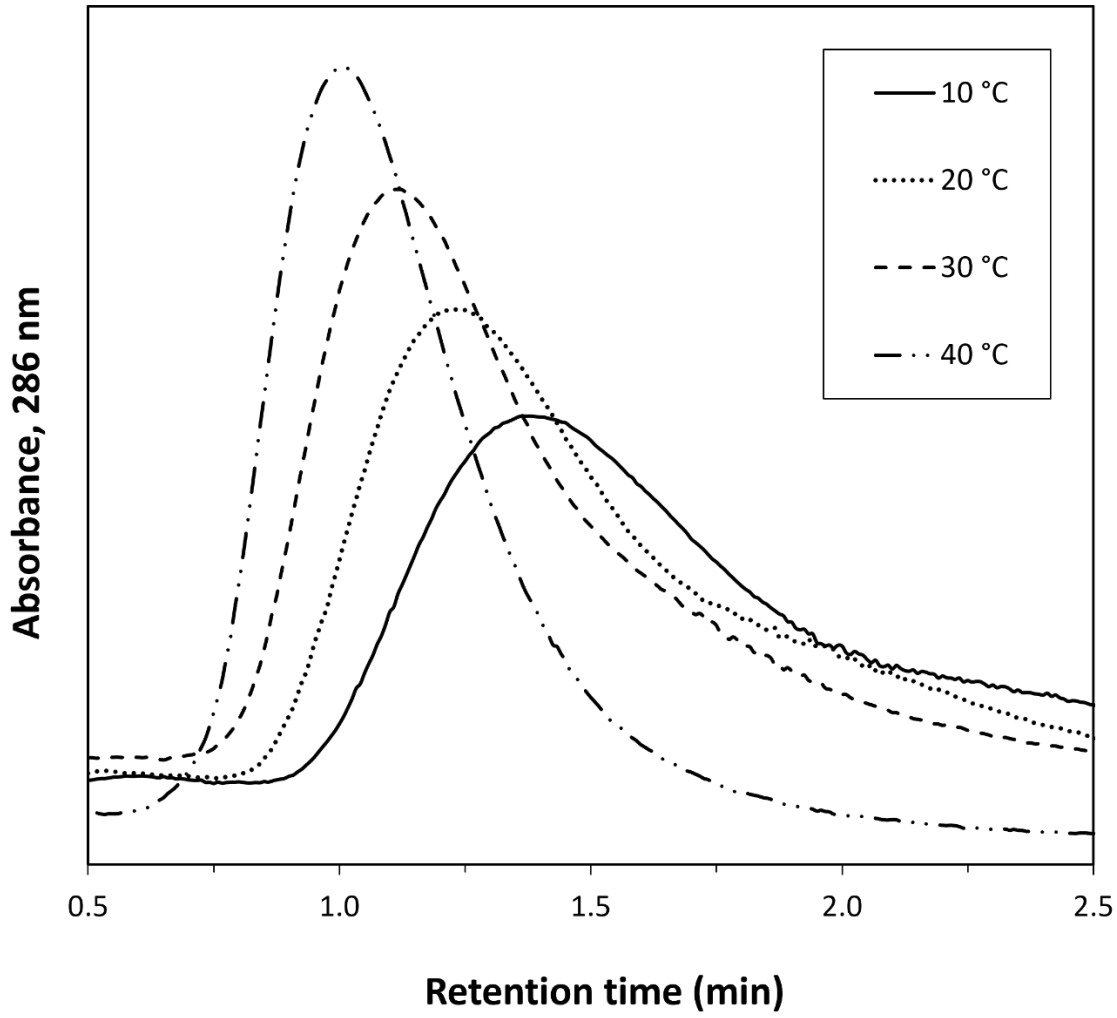

10 °C
20 °C
30 °C
40 °C
Absorbance, 286 nm
0.5
1.0
1.5
2.0
2.5
Retention time (min)

Figure 3

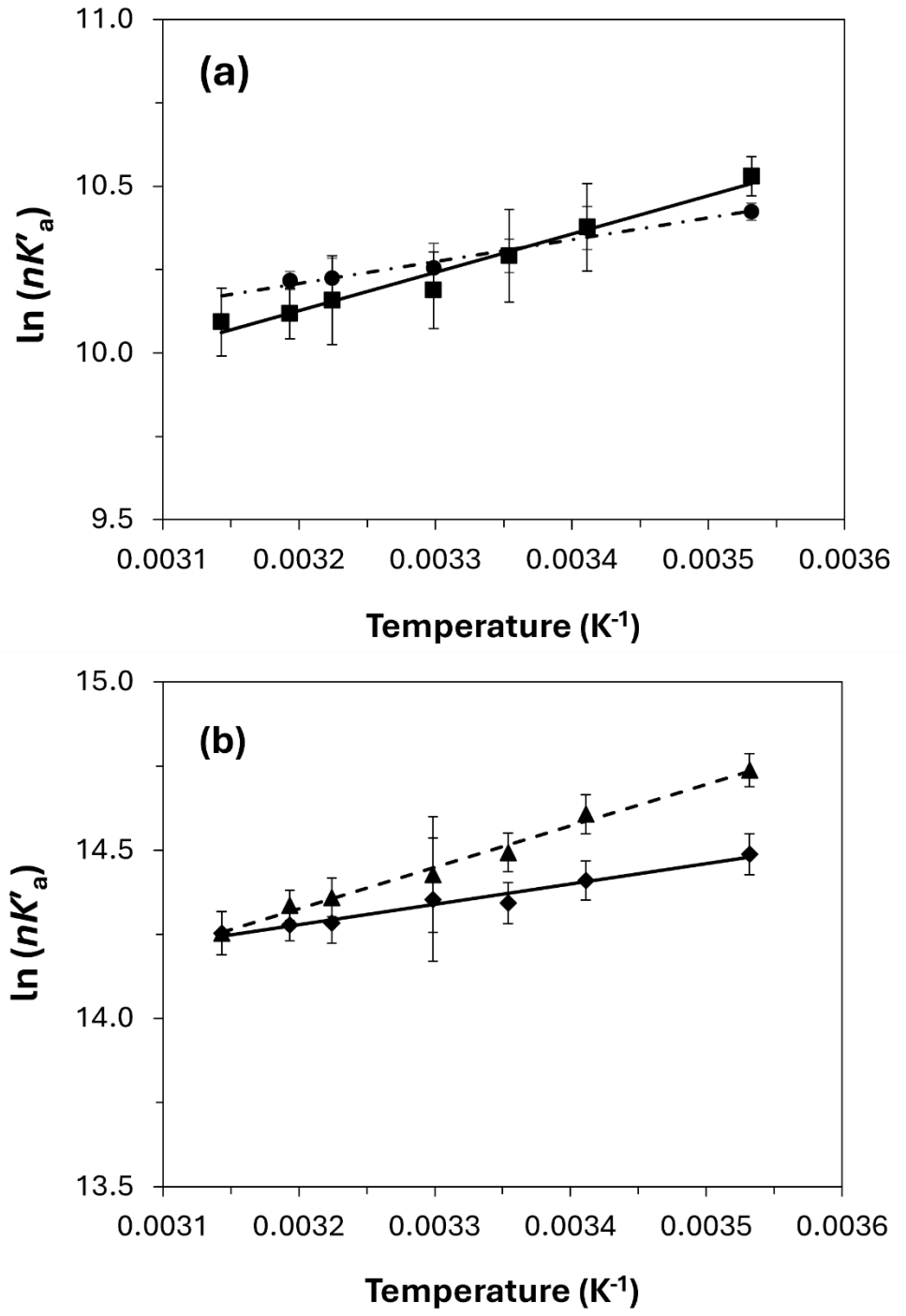

(a)
11.0
10.5
10.0
9.5
ln (nK'a)
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
Temperature (K-1)
(b)
15.0
14.5
14.0
13.5
ln (nK'a)
0.0031
0.0032
0.0033
0.0034
0.0035
0.0036
Temperature (K-1)

Figure 4

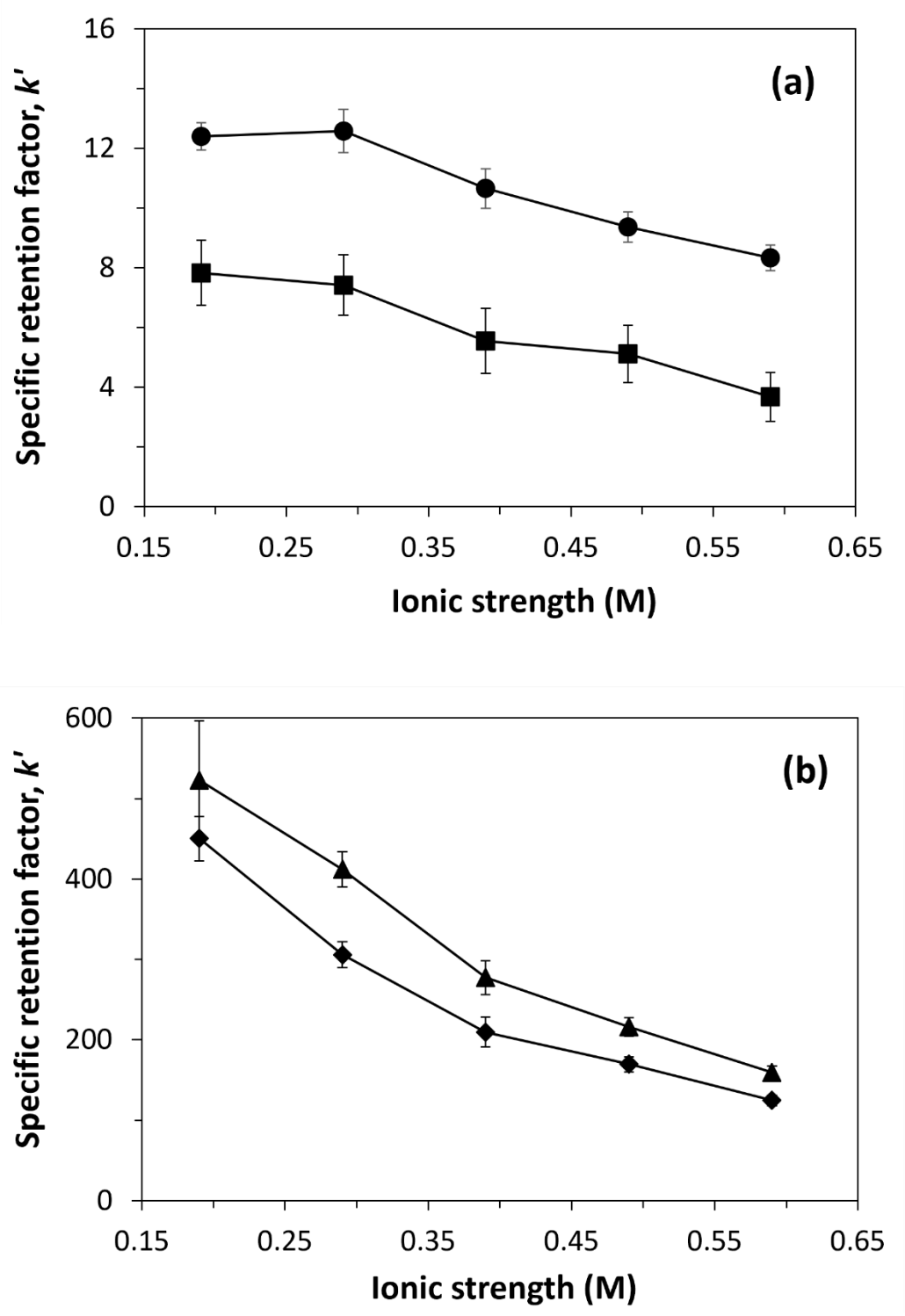

(a)
Specific retention factor, k'
16
12
8
4
0
0.15
0.25
0.35
0.45
0.55
0.65
Ionic strength (M)
(b)
Specific retention factor, k'
600
400
200
0
0.15
0.25
0.35
0.45
0.55
0.65
Ionic strength (M)

Figure 5

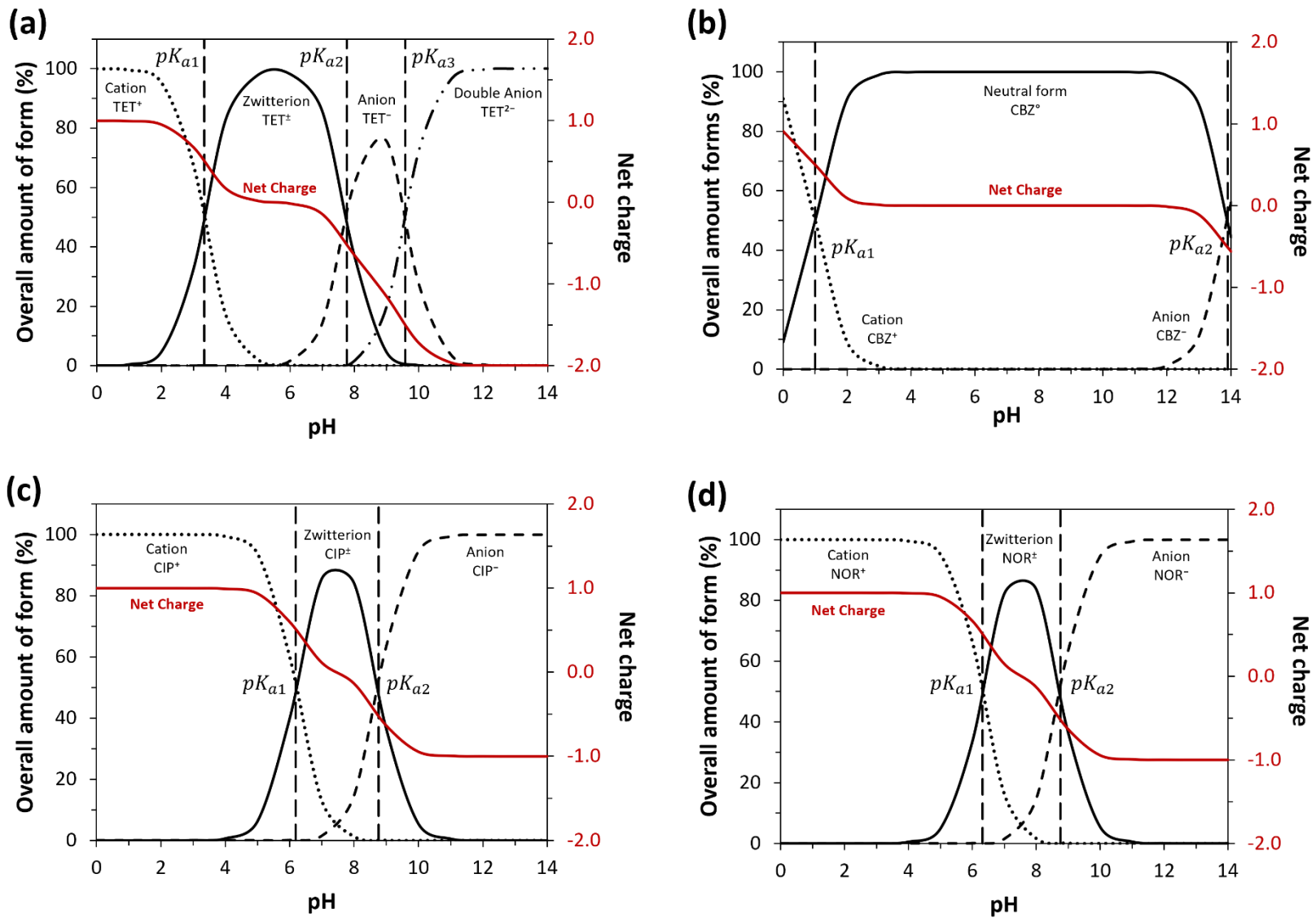

(a)
Overall amount of form (%)
Net charge
pH
pK_{a1}
pK_{a2}
pK_{a3}
Cation TET⁺
Zwitterion TET±
Anion TET⁻
Double Anion TET²⁻
Net Charge
(b)
Overall amount forms (%)
Net charge
pH
Neutral form CBZ°
Net Charge
pK_{a1}
pK_{a2}
Cation CBZ⁺
Anion CBZ⁻
(c)
Overall amount of form (%)
Net charge
pH
Cation CIP⁺
Zwitterion CIP±
Anion CIP⁻
Net Charge
pK_{a1}
pK_{a2}
(d)
Overall amount of form (%)
Net charge
pH
Cation NOR⁺
Zwitterion NOR±
Anion NOR⁻
Net Charge
pK_{a1}
pK_{a2}

Figure 6

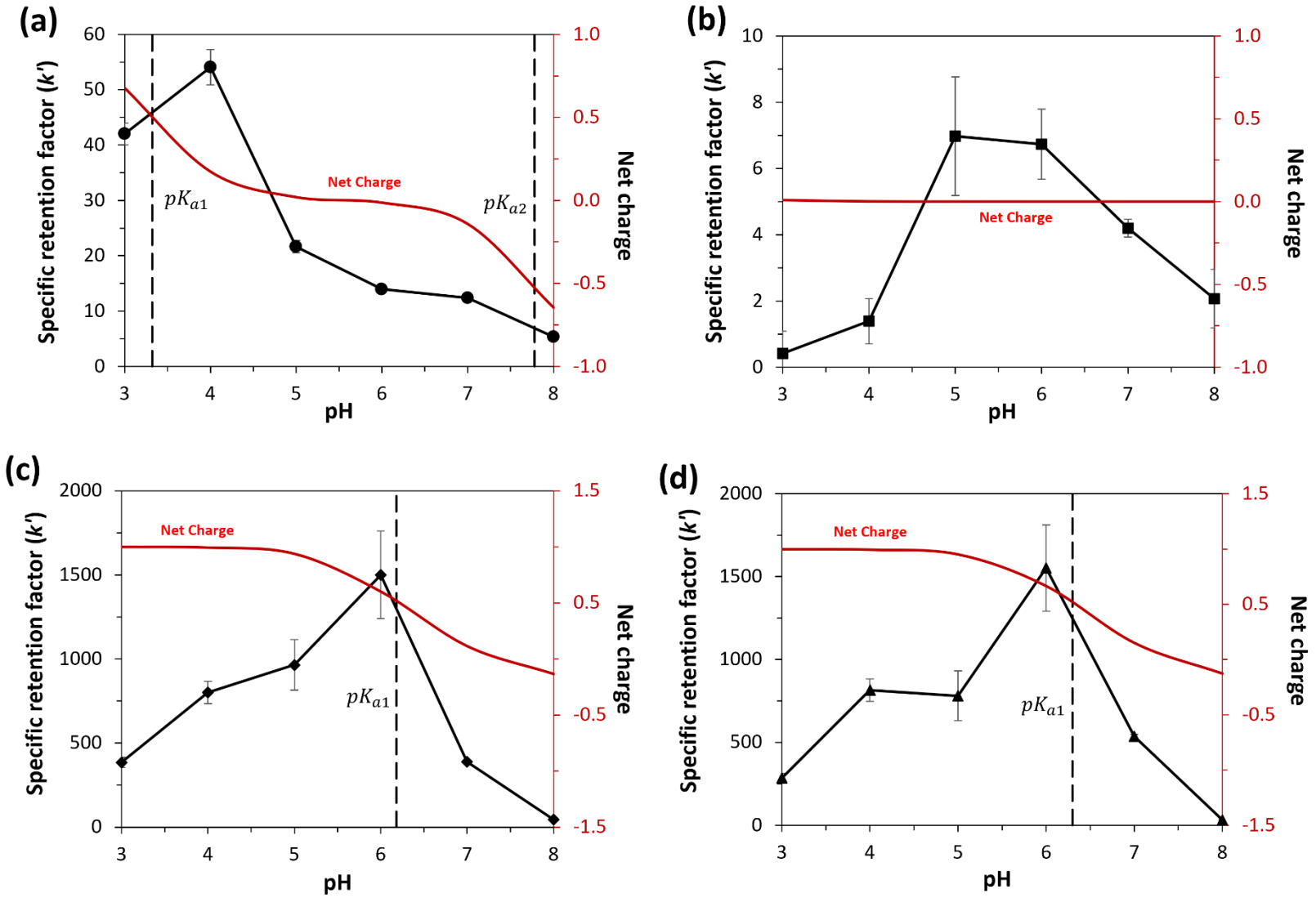

(a)
Specific retention factor (k')
Net charge
pH
Net Charge
pK_{a1}
pK_{a2}
(b)
Specific retention factor (k')
Net charge
pH
Net Charge
(c)
Specific retention factor (k')
Net charge
pH
Net Charge
pK_{a1}
(d)
Specific retention factor (k')
Net charge
pH
Net Charge
pK_{a1}

Figure 7