A Hydrodynamic Method For Measuring Aqueous Nanoparticle Surface Interactions

Kellen John Sorauf
University of Denver

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A HYDRODYNAMIC METHOD FOR MEASURING AQUEOUS NANOPARTICLE SURFACE INTERACTIONS

A Dissertation
Presented to
the Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Kellen J. Sorauf
June 2012
Advisor: Keith E. Miller
ABSTRACT

The objectives of this research dissertation were to develop and present novel analytical methods for the quantification of surface binding interactions between aqueous nanoparticles and water-soluble organic solutes. Quantification of nanoparticle surface interactions are presented in this work as association constants where the solutes have interacted with the surface of the nanoparticles. By understanding these nanoparticle-solute interactions, in part through association constants, the scientific community will better understand how organic drugs and nanomaterials interact in the environment, as well as to understand their eventual environmental fate. The biological community, pharmaceutical, and consumer product industries also have vested interests in nanoparticle-drug interactions for nanoparticle toxicity research and in using nanomaterials as drug delivery vesicles. The presented novel analytical methods, applied to nanoparticle surface association chemistry, may prove to be useful in assisting the scientific community to understand the risks, benefits, and opportunities of nanoparticles.

The development of the analytical methods presented uses a model nanoparticle, Laponite-RD (LRD). LRD was the proposed nanoparticle used to model the system and technique because of its size, 25 nm in diameter.

The solutes selected to model for these studies were chosen because they are also environmentally important. Caffeine, oxytetracycline (OTC), and quinine were selected
to use as models because of their environmental importance and chemical properties that can be exploited in the system. All of these chemicals are found in the environment; thus, how they interact with nanoparticles and are transported through the environment is important.

The analytical methods developed utilize and a wide-bore hydrodynamic chromatography to induce a partial hydrodynamic separation between nanoparticles and dissolved solutes. Then, using deconvolution techniques, two separate elution profiles for the nanoparticle and organic solute can be obtained. Followed by a mass balance approach, association constants between LRD, our model nanoparticle, and organic solutes are calculated. These findings are the first of their kind for LRD and nanoclays in dilute dispersions.
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Chapter 1 Introduction

1.1 Research Objectives Nanoparticle

Nanomaterials in general are a burgeoning research field; a *Web of Knowledge* database search in April 2012 indicated that to date there have been over 200,000 published journal articles referencing nanoparticles and over 100,000 published since 2008. These nanoparticles have wide potential uses in the fields of drug delivery (1-3), as well as energy production and storage, cosmetics and paints (4), and material production. As such, new methods to quantify nanoparticle-drug surface associations are needed because of the unique properties of nanoparticles, which are different from bulk material of the same composition and chemical structure (5-9). For a novel method to become accepted as an adequate method for quantitative chemistry, precision, accuracy and reproducibility are of the utmost importance. Then, if the method is fast (minimal sample preparation and experimental runtime), and inexpensive (utilizes common instrumentation and equipment), the method will be more apt to be adopted as a preferred one.

Surface and adsorption processes govern characterizations of chemical interactions of organic solutes with dispersed nanoparticles in the aqueous phase. One parameter that can help describe surface interactions is the magnitude of association constants. The methods developed and presented here obtain these association constants,
rapidly and reproducibly, using wide-bore hydrodynamic chromatography in conjunction with data decomposition methods.

The format of the dissertation follows a brief introduction into nanoparticles in the environment and their potential toxicity, and a brief overview the composition of clay minerals and how they are defined. This is done to give the reader a background on the chemical composition and properties of clay minerals and to build a foundation for the discussion of LRD, a synthetic hectorite clay nanoparticle. Also, Chapter 1 will discuss nanoparticles and their catalytic properties to appreciate the complex chemistry that occurs on nanoparticle surfaces and how that will relate to the work presented with LRD and quinine in Chapter 6. Chapter 2, in addition to a review and discussion of LRD and the model solutes used throughout the performed experiments, will review the theory of traditional chromatography, wide-bore hydrodynamic chromatography and multivariate chromatography methods. Chapter 3 will summarize methods, results, and discussions on work using LRD as a high-performance liquid chromatography (HPLC) mobile phase modifier and present an attempt to calculate effective association constants between caffeine and LRD. Chapters 4-6 present methods, results and discussions of novel experimental work on LRD interactions with caffeine and other xanthine stimulants, a common antibiotic oxytetracycline, and quinine, an anti-malaria drug, respectively. Chapter 7 summarizes the experimental work and comments on the direction of future work.
1.2 Nanoparticles in the Environment and Their Toxicity

Nanoparticles are ubiquitous in the environment and made through natural processes; however, recently humans have been intentionally creating nanomaterials for research and material production that are then being discharged into the environment (5-7, 9-11). Nanoparticles are broadly defined as particles in the nanometer ($10^{-9}$ m) scale, making them larger than molecules and ions on an angstrom ($10^{-10}$ m) scale, and smaller than macromolecules found on a ($10^{-6}$ m) scale. The US Environmental Protection Agency (EPA), the agency that regulates and monitors environmental contaminants, has defined nanoparticles as particles less than 100 nm in at least one dimension (12). The size of nanoparticles is what makes their chemistry and physical properties of interest to scientists. Their small physical size and large relative surface areas leads to variations in the electronic and magnetic properties, physical and chemical properties, reactivity and kinetics relative to macromolecules and bulk material of the same chemical composition (5,6,9,13).

Nanoparticles can be created through natural and anthropogenic processes and both processes can create organic, carbon containing, and inorganic nanoparticles (10). Some examples of naturally produced nanoparticles are humin, viruses, proteins, soot, aerosolized sea salt, as well as many other examples (5,6,10).

With the introduction of anthropogenic nanoparticles into the environment, a critical assessment of their toxicity, transport, and fate in the environment is important to the safety and health of humans and the ecology of Earth. While this can’t happen
immediately, a better understanding will help us make more informed decisions about nanoparticles and nanomaterials in the future. There have been numerous studies and review articles addressing these issues, but many aspects are still unknown and in need of assessment.

Although a comprehensive toxicological assessment of nanoparticles is not available, there have been many studies looking at their effect on humans and the environment (8,14-17). Most toxicity research has been limited to anthropogenic nanoparticles, both as chemical byproducts and engineered nanoparticles (8,15-18). A common source chemical byproduct nanoparticles are from processes involving combustion of hydrocarbons. These nanoparticles are in the form of soot and black carbon, and the toxicity of soot particles greatly increases with a decrease in particle size as they approach the nanoparticle regime. Soot nanoparticles are small enough to migrate deep into lung tissue and may carry toxic polycyclic aromatic hydrocarbons (PAHs) with them (10,19).

Metal oxides that are inert on a macro scale may be more toxic in the nanoparticle scale due to their cellular uptake and their possible catalytic properties (10,17). Inorganic metal oxide nanoparticles such as TiO₂, SiO₂, and ZnO are frequently used in cosmetics for sunscreen applications. These nanoparticles have been shown to display a toxic affect on bacteria but only when the metal oxides were in a nanoparticle size regime, and the toxicity was significantly increased with exposure to UV light (20).
When mice were fed macro copper particles, (25 µm), there was no detectable bioaccumulation of copper in the kidneys and the calculated LD$_{50}$ was $>5000$ mg kg$^{-1}$ body weight for the macro copper particles. When they were fed nanosized copper particles, however, (23.5 nm), the copper was bioaccumulated in their kidneys and had a LD$_{50}$ of 413 mg kg$^{-1}$ body weight. The higher toxicity and bioaccumulation was attributed to the nanoparticle’s ability to pass through cell walls and from cellular uptake (21,22). Carbon nanotubes are a popular area of research and as such have been studied for their cellular uptake and toxicity in bacteria as well. Experiments have shown that carbon nanotubes have been found in the mitochondria of cells and their toxicity is dependent on the purity of the nanoparticles, where the most toxic carbon nanotubes are from impure mixtures of carbon nanotubes (23,24). Silver nanoparticles are being increasingly used in consumer products, such as, deodorizers and antibacterial agents, and as such, their ability to kill bacteria is well documented. How they affect wastewater treatment facilities and the important nitrogen-fixing bacteria in wastewater treatment, however, is not completely understood (25,26).

LRD, the model nanoparticle used in developing this method is an anthropogenic nanoparticle that is being used in consumer products such as cosmetics and household cleaners as well as in industrial applications including building materials, agricultural production, and polymer manufacturing (27). Despite the many applications of LRD and its inevitable introduction into the environment, a full understanding of LRD interaction with the environment is not known. Southern Clay Products, the manufactures of LRD,
list LRD as a nontoxic substance on their brochure and MSDS (material safety data sheet) page. The Occupational Health and Safety Administration (OSHA) has set the permissible exposure limit (PEL) to 5 mg m$^{-3}$ for respiratory fractions and 15 mg m$^{-3}$ for total dust exposure. Furthermore, like other expandable clay minerals, LRD may be capable of transporting pollutants and catalyzing molecules in the environment.

The methods described here for quantifying surface interactions between nanoparticles and organic solutes in the aqueous phase are potentially important in understanding the toxicity of nanoparticles and the molecules that are associated with their surface. Furthermore, these methods may help model how molecules, associated with the surface of nanoparticles are transported through the environment and their eventual environmental fate.

1.3 Soil Composition

When discussing nanoparticles and their transport through the environment, it is first important to understand the chemistry of soils because the chemical composition of soils naturally contains a wide variety of nanoparticles, in the form of metal oxides, humic substances, and clay minerals. In addition to the unique composition of soils, soils are an integral part of life on earth. Biologists and botanists study soils and their effect on vegetation growth, soil scientists study the terrain of land and are interested in soil composition, hydrologists study the formation and transport of water though soil and bedrock, and geologists are interested in the formation and degradation of soil and
bedrock (7,28). The chemical composition of soils is incredibly complex, and contains many different types of organic and inorganic material, consisting of a wide range of chemical composition and size. Inorganic matter in soil consists of metals, metal oxides and clay minerals. There are approximately 4500 classified clay minerals in the environment, and they have a profound effect on the transport of toxic metals and organic contaminants through the environment, as well as the mediation of bacterial growth (13).

Organic materials are also found in soil in biological form as bacteria as well as abiotic forms consisting of humic substances. Humic substances are divided into three classes: humic acids, fulvic acids and humin; each of these have classifications that differ in alkalinity and solubility. Despite the relatively small classes of humic substances, the scientific community does not have an understanding of the chemical structure due to their complexity and heterogeneity (7). Humic substances are the products of organic decomposition and once formed are stable to further degradation. These humic substances play an important role in transport of metals and nutrients for plants. The metal oxides, clay minerals, and humic substances in soil are all found to some extent in the nanoparticle size regime where one dimension is less than 100 nm in diameter.

1.4 Clay Minerals

To understand the unique physical properties of LRD, a synthetic hectorite clay mineral, and why those properties make traditional analytical techniques inadequate
when used to determine the surface interactions at low concentrations, a background on clay minerals is needed.

Classification of clay minerals is dependent on the size of the mineral particle itself. Clay minerals are generally defined as minerals with a Stokes diameter < 2µm (29,30). Naturally occurring clay minerals are formed at the surface of the Earth’s crust over an extended period of time as the crustal materials are subjected to chemical weathering by the atmosphere. There are three types of natural processes that may produce clay minerals. They may be formed from pre-existing parent rock that went through chemical weathering. Secondly, they may be formed through chemical transformations where the parent structure of a rock or mineral is kept intact and chemical changes occur in the interlayer of the rock. Finally, neoformation is a type of clay mineral formation where clay minerals are formed from precipitation and crystallization of a gel or solution (31).

Clay minerals are further classified by their structure and interlayer charge. It is important to note that structure and lattice are different. Lattice is a theoretical structure of a clay mineral without defects and a true homogeneous arrangement of ions in infinite directions. Whereas the structure of a clay mineral is more indicative of a clay mineral found in the environment where it contains defects in the lattice and the clay mineral chemical composition is heterogeneous. The structures of clay minerals, once they are dispersed in solution, are tetrahedral and octahedral sheets separated by cations and water. If the ratio of assembly of a tetrahedral sheet to an octahedral sheet is 1:1, the
mineral is classified as a silicate structure. A 2:1 ratio of tetrahedral sheets to octahedral sheets is a layer silicate. Tetrahedral sheets contain the molecular structure SiO$_4^{4-}$ whereas the octahedral sheets are contain the molecular structure MX$_6^{(m-6b)}$, where M$^{m+}$ is the metal cation and the six anions are X$^{b-}$ (32).

The layer silicates and silicate structure classes of clay minerals are further classified based on the type of cations in the octahedral sheet. If the clay mineral has a cation to anion ratio of 1:2, it contains divalent cations in the octahedral sheet and is classified as a trioctahedral because three divalent cations are needed to balance the hydroxyl charges. If the cation to anion ratio is 1:3, it contains trivalent cations in its octahedral sheet and is classified as a dioctahedral because two trivalent cations are needed to balance the hydroxyl charges (30,33).

Depending on the charge per unit formula of the clay mineral or the interlayer charge, clay minerals can be further classified from the dicotrahedral and trioctahedral layer silicates and silicate structure classifications.
Table 1 Classifications of select clay minerals. Adapted from Moore Reynolds 1997

<table>
<thead>
<tr>
<th>Layer Type</th>
<th>Charge per Unit Formula</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>≈ 0</td>
<td>Kaolins (trioctahedral)</td>
</tr>
<tr>
<td>1:1</td>
<td>≈ 0</td>
<td>Serpentines (dioctahedral)</td>
</tr>
<tr>
<td>2:1</td>
<td>≈ 0.2-0.6</td>
<td>Smectite</td>
</tr>
<tr>
<td>2:1</td>
<td>≈ 0.6-0.9</td>
<td>Vermiculite</td>
</tr>
<tr>
<td>2:1</td>
<td>≈ 1.0</td>
<td>Micas</td>
</tr>
</tbody>
</table>

Table 1 shows the classification scheme of the interlayer charge, the charge between layers, of some select clay minerals. Unless noted in the table, the group is further divided by their dioctahedral and trioctahedral structures into subgroups and finally to the species and name (30). Interlayer charges are a result of chemical defects in the clay mineral lattice, wherein lithium atoms can exchange with magnesium atoms in the octahedral layer creating a negative charge on the clay surface. Due to the interlayer charge of clay minerals when they are dissolved in water there is an electrostatic interaction between the polar water molecules and the clay minerals. Water and cations can enter the interlayer region of the clay between two layer silicate sheets and produce swelling and expansion of the clay. Furthermore, if the clay mineral is in contact with a solution high in ionic strength, there may be a cation exchange where cations from the solution will interchange with the cations in on the clay surface and in interlayer defect locations. Generally cations will replace each other following the trend:
\[
\text{Na}^+ < \text{K}^+ < \text{Ca}^{2+} < \text{Mg}^{2+} < \text{NH}_4^+
\]

where \(\text{K}^+\) is more stable than a \(\text{Na}^+\) cation but less stable than a \(\text{Ca}^{2+}\) cation. If the clay mineral is in an acidic environment \(\text{H}^+\) is a possible interlayer cation. The capacity for these cations to interchange on the surface or in the interlayer of the clay mineral is quantified as the cation exchange capacity (CEC). CEC is typically reported in milliequivalences per 100 grams of clay or (meq). The chemistry of clay minerals varies greatly depending on the surface charge and their size, and is discussed in the next section (24,30).

1.4.1 Clay Mineral Surface Face and Edge Sites

The surface and edge sites of clay minerals are where chemistry of aqueous clay minerals takes place. As the structure of a clay is terminated at the surface and edge, defects, unbalanced charges, and interactions with the solution can occur. It is helpful to think of clay minerals as disks that have a surface face that contains a much larger area than the relatively small edge or height of the clay mineral (34).

Typically the ratio between the edge and the face of a clay mineral disk is 1:100 where the edge or thickness of the disk is 1\% of the disk surface or disk diameter. The surface face of clay minerals is where cation exchange occurs due to the negatively charged terminating tetrahedral silica sheets. If the solution pH of a clay mineral is below or above the point of zero charge (PZC), terminating hydroxyl groups at the edge
of the clay minerals in solution will begin to electrostatically interact with hydronium or hydroxide ions. The edge site interaction with the hydronium and hydroxide ions create an electrostatic repulsion between the clays in solution and will stabilize the clays and keep them dispersed in solution (34).

If the pH of the solution is near the PZC, then the edge of the clay mineral will be in a neutral state and minimal surface repulsions between clay minerals exist. When minimal electrostatic repulsions of the clays are present, the clays will come close enough together in space that van der Waals forces will promote coagulation(32,35,36). Due to the interchangeable cation, edge site pH, particle size, and ionic strength of solutions, the chemistry of clay minerals is complex and difficult to model and classify. This is especially the case with LRD, as will be addressed to a greater extent in later sections. Because of small particle size and large variations in the surface and chemical edge sites in LRD, there are many interactions that are not well understood and defined when LRD in dispersed in solution (35,37-39).

1.4.2 Clay Mineral Secondary Structures

Clay minerals will form secondary structures in solution where the minerals will coagulate. The coagulation is classified in three types of formation. There can be face-face interaction between two parallel clay minerals, an edge-edge interaction between two edges of clay minerals, and finally face-edge interactions. Cartoon pictures of these three types of clay mineral interaction are depicted in Figure 1.
Figure 1 Cartoon illustration of three types of clay mineral interactions.  a. Edge to edge interaction, b. Face to face interaction, c. Edge to edge interaction.

The colloidal secondary structure will spontaneously form depending on the type of clay mineral, the solution pH, ionic strength of the solution, and the concentration of the clay minerals. Colloidal suspensions are considered to be stable, and the clay mineral particles dispersed in solution if the colloid partial size does not increase to the point where gravitational settling will occur. The mechanism of coagulation, aggregation and
eventually settling out of solution is complicated due to the complex nature and heterogeneous surface chemistry of clay minerals. One proposed mechanism that has been proposed is for clay minerals ≤ 1 µm is known as perikinetic flocculation, which is based on Brownian motion and the Stokes-Einstein equation (36). Coagulation is dependent on the diffusion coefficient, D defined through the Stokes-Einstein equation:

\[ \text{Equation 1} \]

\[ D = \frac{k_B T}{6\pi \eta R} \]

where \( k_B \) is the Boltzman constant, \( \eta \) is the shear viscosity of the solution and \( R \) is the radius of the particle and \( T \) is temperature. This equation indicates that the colloid will diffuse faster in solution when the temperature is high, the viscosity of the solution is low and the particle is small. Particles with faster diffusion will have Brownian motion that will overcome the gravitational force that settles particles out of solution. The rate at which two particles will form a dimer is the perikinetic flocculation equation where the second order rate process is calculated as:

\[ \text{Equation 2} \]

\[ k = 2\pi R_{11} D_{11} \]
where $R_{11}$ is the radius of the particle dimer and $D_{11}$ is the diffusion coefficient of the dimer particle. Assuming the dimer has twice the radius and half the diffusion coefficient of the monomer, the two equations can simplify to the rate coefficient of dimer formation as:

**Equation 3**

$$K = \frac{4k_B T}{3\eta}$$

where the rate of dimer formation is only dependent on temperature and solution viscosity (36).

However, this model is oversimplified due to the many causes of an increase in solution viscosity and the charge of the clay minerals themselves. At a specific ion concentration, spontaneous coagulation will occur. This threshold is defined as the critical coagulation concentration (CCC) where rapid coagulation will occur when the smallest amount of electrolyte is needed to reach the CCC threshold. The CCC is determined by the concentration of ions of the opposite charge at the clay mineral surface, and is qualitatively proportional to an inverse power of the valence ions (32,36). In other words, if the surface of the clay mineral is negatively charged, positive multivalent cations will produce coagulation at lower ionic concentrations.

Cations in solution are attracted electrostatically to the negative surface of the clay minerals. Because of this attraction, the ion concentration is not uniform through the
solution. Cations are found in higher concentration near the negative surface of the clay mineral than in the bulk solution, and anions are found in a lower concentration around the surface of the clay. The cations at the surface of the clay are considered to be immobile and do not diffuse through the solution. The immobile area of ions at the surface and the diffuse layer of ions in the bulk solution are known as the electric double layer and illustrated in Figure 2.
Figure 2. Cartoon illustration of the electric double layer near the clay mineral surface called the immobile layer, and the ions in the bulk solution called the diffuse layer.

The electric double layer is an important concept in explaining dispersion and coagulation of particles. As charged particles come into close proximity with one another in solution, van der Waals forces will bind the two particles together into larger secondary structures. With the introduction of ions below the CCC, the surface charge of
the clays extends further into the diffuse layer and two clay particles will not come close enough in proximity for van der Waals forces to coagulate the particles; thus, the particles will stay suspended in solution. However, as the ionic strength increases and approaches the CCC, the diffuse layer of ions becomes smaller and more compact, allowing the surface charge of two clay particles to become close enough in proximity for van der Waals forces to attract the particles together leading to coagulation. Once coagulation begins, Brownian motion will be overcome by gravitational forces and the particles will precipitate out of solution (30).

The electric double layer model provides a conceptual explanation of clay mineral surface and their interaction with ions when dispersed in water. A more robust and mathematical approach to the interaction between clay mineral surfaces and ions is presented as the DVLO theory developed by Derjaguin, Landau, Verway, and Overbeek (40). A robust mathematical interpretation of DVLO theory is unnecessary; however, a qualitative understanding is useful. DVLO theory states that the rapid coagulation of clay particles occurs when the magnitude and range of the repulsive electrostatic forces decrease with an increase in ion concentration (32). The theory sums all the attractive and repulsive forces in solution to predict a theoretical CCC concentration. A summary of factors affecting the stability of clay particles, adapted from Sposito 2008, is shown in Table 2.
To summarize Table 2, as ion concentration increases, the diffuse double layer increases and the repulsive surface charge of the clay surfaces does not extend as far into the solution. Particles then become close enough to one another that van der Waals forces lead to coagulation. If the pH is equal to PZC or the surface charge is zero, the surfaces of the clays are neutral and van der Waals forces are the strongest and coagulation will occur. The chemical properties of clay minerals will change depending on the electrolyte concentration, pH, and how the clay mineral interacts with other particles in solution and in suspension. Understanding the mechanisms and causes of coagulation and diffusion of clay minerals is important when discussing the properties of LRD in later sections.

### Table 2. Factors affecting the stability of soil colloid suspensions. Adapted from Spoito 1984

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effects</th>
<th>Promotes Coagulation</th>
<th>Promotes Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrolyte concentration</td>
<td>Extent of diffuse double layer</td>
<td>When increased</td>
<td>When decreased</td>
</tr>
<tr>
<td>pH</td>
<td>Changes surface and proton surface density</td>
<td>pH ( \approx ) PZC</td>
<td>pH ( \neq ) PZC</td>
</tr>
<tr>
<td>Surface complex with cations</td>
<td>Changes surface charge density</td>
<td>Surface charge ( \approx 0 )</td>
<td>Surface charge ( \neq 0 )</td>
</tr>
</tbody>
</table>

1.5 **Nanoparticles as Catalysts**

When studying nanoparticles and clay minerals it is important to understand the surface chemistry of the nanomaterials and that the surface chemistry of the nanomaterial
may be different than bulk material of the same chemical composition (6). Chemists are beginning to appreciate the uses of metallic nanoparticles as reusable catalysts in a wide variety of chemical applications. Metallic nanoparticle catalysis have been applied to oxidation reactions such as, Fenton-like reactions and hydrocarbon oxidation reactions, carbon-carbon coupling reactions like Suzuki and Heck reactions, hydrogenation reactions of hydrocarbons for petroleum refining, as well as photocatalytic degradation of organic dyes and drugs (41-46).

Clay minerals are well-documented catalysts and have been used for many years in petroleum refining and cracking of hydrocarbons. Organic chemists have used clay minerals as recoverable and reusable catalysis in synthesis reactions as well. The use of clay minerals has been proposed as a possibly environmentally friendly alternative to traditional chemical catalysts that are difficult to recover (47). In addition to catalyzing ‘wet’ chemistry, clay minerals have been used as a substrate for photochemical reactions under dry conditions (48). The catalytic properties of clays arise from electron transfer oxidation or reduction of organic molecules, where the clay minerals can act as a Lewis Acid and Brønsted Base depending on the reaction conditions and the location of interaction (48). Scientists have used the electron accepting/donating properties of clay minerals to catalyze and control polymerization reactions of styrene and methacrylate reactions (49). Electron transfer properties of clay minerals explain the color changes that are observed when certain dyes are adsorbed to the clay surfaces (49,50). Understanding the catalytic properties of clay minerals is important when discussing the
chemistry and results of LRD interactions with quinine and OTC that will be discussed in Chapter 6.
Chapter 2 Materials, Theory, and Data Processing

2.1 Materials

2.1.1 Model Nanoparticle: Laponite-RD

Throughout this research dissertation, LRD is the reoccurring nanoparticle used as a model to represent nanoparticles in the experiments. It has a small size distribution around 25 nm in diameter and 1 nm in height; thus, it falls in the center of the nanoparticle definition of particles between 1-100 nm in diameter. It is synthetic clay mineral manufactured by South Clay Products (Gonzales, Texas). LRD is one of 16 listed Laponite products manufactured by Southern Clay Products; all the Laponite products are manufactured to have different physical properties that affect viscosity and gel formation. Some of the different physical properties are a result of changing the chemical makeup of edge sites of the clay by adding trisodium phosphate or low molecular weight polyethylene glycols (27). Southern Clay Products list a number of physical properties and applications of Laponite and how it is used in industrial and consumer products. The two functional applications of Laponite that are marketed for consumers are in the areas of rheology and coatings. Laponite is used as rheology additive to increase the viscosity and stability of solutions. It is also used as a thin film coating on materials to change the conductivity of the material and act as a barrier coating (27).
The properties of LRD are continually studied and a *Web of Knowledge* database search indicates there are over 1000 published scientific articles on Laponite with the first publication in 1969 (51). These articles mainly focus on the chemical nature of Laponite including the viscosity, swelling and suspension properties of Laponite in an aqueous solution. As with other clay minerals, their aqueous properties, viscosity, and coagulation are dependent on the pH and CCC of the solution. Another area of Laponite research is focused on the adsorption of solutes to Laponite in suspension and the photolysis of the interactions, specifically with degradation of the solute on the Laponite surface (52-55). The remainder of this section will focus on the chemical, colloidal, and viscosity properties of aqueous Laponite as well as adsorption of solutes and photolysis on the Laponite surface. Addressing these research areas is important to understand the current limitations of methods and how our novel technique can be used to measure solute adsorption to Laponite.

LRD is a trioctehedral hectorite clay, which contains two layering sheets of silicate sandwiching an octahedral layer containing magnesium atoms. The silicon and magnesium atoms are charged balanced by hydroxyl and oxide anions. A net negative charge on the clay mineral results from lithium atom defects in the octahedral layer that make the clay mineral surface electron rich. The negative charge is balanced by sodium ions that are electrostatically attracted to the surface of the clay mineral.

The chemical composition of LRD is $\text{Na}^{+}0.7[\text{Si}_{8}\text{Mg}_{5.5}\text{Li}_{0.3}\text{O}_{20}\text{(OH)}_{4}]^{0.7}$, where the sodium ion, (Na$^+$), is attracted to the negative surface charge of the clay mineral by an
electrostatic interaction and is found the interlayer spacing of the clay mineral. LRD has a net negative charge on the surface of the platelet and a variable charge on the edge of the platelets that is pH dependent. The reported surface area of LRD is 360 m²/g with a CEC of 73.3 mEq/100g (35,39,56,57). A 1% solution of LRD is approximately pH=10 and this corresponds to the measured PZC for Laponite (58).

There are a number of literature publications studying Laponite at concentration above 2% (w/w). At these concentrations, Laponite forms gels and aggregates that display thixotropic behavior, where the viscosity of the solution decreases as a sheer force is applied (38). At concentration between 1% and 4% (w/w) Laponite is considered to be an isotropic gel; at concentrations above 4% (w/w) flocculation of Laponite particles begins (38). Once gelation occurs, the viscosity of Laponite increases, but if a shear force is applied to the Laponite gel the viscosity falls to a viscosity of 1 (56). At concentrations below 1% (w/w) Laponite is an isotropic liquid and the clay particles are totally dispersed in solution. The ionic strength where gelation and flocculation concurs has been reported to be 10⁻² M (37). Significant dissolution of Laponite and has been reported in aqueous systems where the pH < 9; hence, the importance of keeping LRD solutions at pH=10 for many of the studies reported here (35).

Adsorption phenomena of aqueous Laponite dispersions are startlingly absent in the literature. This is due in part to the coagulation and gelation of Laponite at high concentrations and ionic strengths. Once gelation and aggregation of Laponite particles occurs, the chemistry of these systems is different than the dispersed Laponite
nanoparticles, and similarities between gel solutions and dispersed suspensions are lost. To date, there are no published results of adsorption or association of solutes with aqueous dispersed Laponite. Dispersed nanoparticles are problematic for measuring adsorption of solutes because the nanoparticles stay dispersed in solution and centrifuging nanoparticles to measure adsorption interactions is difficult (59). The release of pharmaceutical drugs from the interlayer of Laponite 1% (w/w) suspensions has been studied; however, the experimental solution was adjusted to pH = 4, where the clay platelets are not stable and have chemistry which is different than the bulk dispersed Laponite in pH = 10 solution (35,60). Other attempts have been made to study Laponite binding to solutes where the mixtures make a gel or flocculated particles. In these cases, the authors have studied the Laponite-solute particles using dynamic light scattering and viscosity measurements to determine the binding of solutes to Laponite. The flocculated particles were measured between 100-400nm in diameter, outside of the nanoparticle size regime (61). Another study measured the adsorption of pesticides on Laponite. In this study the Laponite concentration was 10% (w/w) and the corresponding precipitate was filtered, and the pesticides adsorbed to the surface were measured using x-ray diffraction analysis (53). All of these examples are measuring the sorption of LRD at high concentrations where the properties of LRD gel will be different than when the LRD concentration is low and dispersed in suspension.
2.1.2 Model Solute: Caffeine and other Xanthines

Caffeine was chosen as a model solute to be used as a molecular probe to study the association of solutes to nanoparticle surfaces because of its chemical properties and its environmental ubiquity (62,63). Caffeine has been proposed as an anthropogenic marker because nearly every society on earth uses caffeine in some form, whether in local tea or coffee or some other edible product (62). Caffeine made a good initial starting molecule to measure an association on the LRD surface since, from previous experimental results, it is reasonable to assume there would be an interaction between caffeine and LRD. It has been shown that caffeine associates and binds to the surface of sodium montmorillonite, a macro clay particle, with a binding constant of 290 ± 2 (L Kg$^{-1}$) (64). The binding of caffeine to marco clay particles indicates clays are a potential environmental sink for caffeine in the environment and may facilitate the transport of caffeine through the environment. It has been proposed that the caffeine is attracted to the negative surface of the clay through the nitrogen on the imidazole ring (64) and shown in Figure 3.
Figure 3 The molecular structure of caffeine. It is thought that caffeine associates with the surface of clays through the nitrogen on the imidazole ring. Caffeine does not have a measurable pKa. The possible interaction location between caffeine and sodium montmorillonite, shown by the arrow.

Although it may be helpful to model sorption to macro clay particles to help understand the potential for association between caffeine and LRD, it may not be accurate, because of the unique properties of nanoparticles and their differences from macro-materials.

Caffeine belongs to a xanthine class of molecules, which are a group of molecules containing a purine ring. Other xanthines that are structurally similar to caffeine are paraxanthine, theobromine, and theophylline shown in Figure 4. Caffeine was also chosen as a model solute to use as a proof of concept because it does not have a pKa that changes over the experimental conditions of the experiments. The pKa for caffeine is reported as being > 14; however, theobromine, theophylline, and paraxanthine have
reported pKa values of 10.05, 8.77, and 8.81 respectively through the protonation of the unsubstituted nitrogen on the purine ring (65).
Figure 4 Molecular structures of other xanthines used to probe the surface interactions of LRD. All three of these xanthine molecules have measurable pKa values from the cyclic ionizable nitrogen.

Due to LRD solutions having a pH = 10, caffeine is the best choice for a proof-of-concept model because it is the only neutral xanthine species during the experiment whereas the other xanthines will have a nontrivial amount of the conjugate base in solution from the high pH of LRD solutions.
2.1.3 Model Solute: Oxytetracycline

Another molecule used as a model in the LRD system is oxytetracycline (OTC), which is a member of a class of molecules called tetracyclines that are common antibiotics (66). Because tetracyclines have been shown to possess antimicrobial activity, they are used in agricultural animal feed to prevent disease and infection. Due to the use of tetracyclines in agricultural practices, tetracyclines are entering the environment in significant quantities. 16 million kg are produced annually, and of that, an estimated 50-80% is excreted without being metabolized (67). As tetracyclines are entering the environment at high concentrations, there have been a number of research papers focused on the accumulation of tetracyclines in soil and on clay minerals (66-68).

OTC has 3 ionization sites corresponding to three pKa values, $pK_{a1} = 3.57$, $pK_{a2} = 7.49$, and $pK_{a3} = 9.88$ as seen in the molecular structure of OTC in Figure 5 (69).
Figure 5 The molecular structure of oxytetracycline. Oxytetracycline has 3 pKa values associated with the nitrogen and hydroxyl structures in the molecule. In natural waters oxytetracycline is a zwitterion.

When the solution pH < pKa₁, OTC is a cation in solution, when the pH is between pKa₁ and pKa₂, OTC is zwitterionic, when the pH is between pKa₂ and pKa₃ OTC has a net negative charge, and finally when the pH > pKa₃, OTC has two negative charges. The mechanism for tetracycline adsorption to clay minerals surfaces is a cationic exchange with the negative surface of the clay mineral surface. There are many publications that report a decrease in tetracycline adsorption with an increase in pH (67,70). This is observed because at low pH where tetracylines are positively charged,
there is an electrostatic attraction followed by a cationic exchange reaction with the counter ion in the clay mineral interlayer. However, when tetracyclines are negatively charged, in basic conditions above their respective pKa$_3$, there is an electrostatic repulsion between the clay and the tetracycline. Due to the charge interactions of OTC and the surface of clays, the adsorption of tetracyclines, is charge dependent through the pKa of the OTC and the pH of solution (70).

When the ionic strength of a solution increases, a slight decrease of tetracyclines absorption is observed. This occurs from increased competition for the interlayer spacing between the cations with a high affinity for the interlayer and the tetracyclines. As expected experiments have shown when Ca$^{2+}$ ions are added in solution of sodium montmorillonite, a decrease in tetracycline adsorption was observed compared to equivalent pH values in lower ionic strength solutions (70).

Although it is helpful to model OTC sorption to LRD after OTC sorption to macro clay particles, however, it is not an analogous comparison. This is because of the unique properties of nanomaterials and their increased surface area compared to otherwise analogous macroparticles. Furthermore, sorption studies of dilute aqueous suspensions of LRD are absent in the literature and they may have different properties than macro clays of similar structure.

It is also important to note that OTC degrades in the presence of light, heat pH and metal redox reactions (71-73). OTC has been shown to be more stable at higher pH values, and as such, a decrease in degradation rate occurs at higher pH (71-73).
2.1.4 Model Solute: Quinine and 6-Methoxyquinoline

Quinine was also used as a molecule used to probe the surface interactions of LRD because the quinuclidine moiety was a probable location for interaction on the surface of the negatively charged nanoparticle. Quinine is also a medically relevant molecule used to treat malaria (74). As such, the interaction between quinine and environmental toxins, such as nanomaterials, are important for understanding the health risks of possible interactions between the two.

The fluorescence chemistry of quinine is well characterized in the literature and is used as a calibration standard for fluorescence lifetime measurements (75). 6-Methoxyquinoline (6MQ) is often used in conjunction with quinine in fluorescence experiments, because 6MQ consists of a quinoline ring and is essentially quinine without the quinuclidine moiety; both are shown in Figure 6.
Figure 6 Molecular structures of quinine and 6-methoxyquinoline. 6-methoxyquinoline was used as a control throughout the LRD experiments because it lacks the quinuclidine moiety. Both molecules have a pKa associated with the nitrogen on the quinoline ring, and quinine has another pKa associated with the nitrogen in the quinuclidine ring.
2.2 Theory

2.2.1 Adsorption Isotherms

The most common method for determining the concentration of analyte adsorbed to the surface of a clay is through batch isotherm experiments. In batch isotherm experiments, a known concentration of analyte is added to a solution with a known mass of clay and brought to a standard volume. After equilibration, the solution is centrifuged to force the clay with adsorbed analyte from the bulk solution. The equilibrium concentration of analyte in the bulk solution is then measured and through mass balance, the amount of analyte adsorbed to the clay surface is calculated. Data from these experiments are plotted as the amount of analyte adsorbed per mass of clay, as a function of the equilibrium concentration of analyte in solution. These isotherm plots usually take three shapes: an L – shaped curve that is fit with a Langmuir curve; and an S – shaped isotherm that is fit with a sigmoidal curve; and a Freundlich isotherm (76, 77). L – shaped isotherms are indicative of a high affinity between the clay and the analyte and typically correspond to strong chemical interactions between the solute and clay. L-shaped isotherms flatten at higher analyte concentrations due to saturation of binding sites (77). S – shaped isotherms are indicative of a weak chemical interaction between the analyte and clay, and there is high analyte concentration at the surface of the clay before adsorption takes place (78). Freundlich isotherms have a small decrease in the
isotherm slope at higher solute concentrations due to heterogeneity of binding sites that are thermodynamically different (77).

Fitting isotherm plots is necessary to calculate the distribution coefficient, $K_D$, a common parameter used in clay chemistry to describe binding constant of analytes to clay surfaces. $K_D$ values are calculated by measuring the equilibrium concentration of the analyte in solution, $C_i$, in units of (mg L$^{-1}$) or (mol L$^{-1}$), and the concentration of analyte on the clay surface, $q_i$, in units of (mg kg$^{-1}$) or (mol kg$^{-1}$). These values represent the respective (x,y) axes of the isotherm plots. $K_D$ is then calculated by finding the slope of the plot.

**Equation 4**

$$K_D = \frac{q_i}{C_i}$$

The resulting $K_D$ units are in L kg$^{-1}$ (70,79). This equation is simplified and is only valid in the linear regions of the isotherm plot. Freundlich isotherms are similar to linear isotherms; however, the $C_i$ term is dependent on an exponent, $n$, that is usually less than one (77).

**Equation 5**

$$K_D = \frac{q_i}{C_i^n}$$
Fitting nonlinear isotherm plots uses the Langmuir isotherm where

**Equation 6**

\[ q_i = \frac{Q b C_i}{1 + bC_i} \]

and \( Q \) is the maximum adsorption capacity of the clay (mol kg\(^{-1}\)) calculated as analyte sorption as \( C_i \) approaches infinity, and \( b \) is the commonly referred to as the Langmuir constant (L mol\(^{-1}\)). Both of these constants are calculated through the intercept and slope through plotting

**Equation 7**

\[ \frac{1}{q_i} = \left( \frac{1}{Q * b} \right) \frac{1}{C_i} + \frac{1}{Q} \]

where \( Q^{-1} \) is the intercept and \( (Q*b)^{-1} \) is the slope (70,76). Although LRD and solute association constants are not calculated through bulk isotherm experiments due the nature of LRD nanoparticles in dilute concentrations to stay suspended in solution, isotherm plots can be created through the use of wide-bore HDC.

### 2.2.2 Chromatography

Chromatography is the separation of analytes dissolved and traveling with a mobile phase, which may be a liquid or a gas, while they are retained on a stationary.
Analytes that are strongly retained on the stationary phase move slowly with the mobile phase, and analytes that are weakly retained on the stationary phase migrate quickly with the mobile phase. The retention of analyte on the stationary phase is often described by the partition coefficient $K_c$ defined as

**Equation 8**

$$K_c = \frac{c_S}{c_M}$$

where $c_S$ is the concentration of the analyte on the stationary phase and $c_M$ is the concentration of the analyte in the mobile phase. The amount of time an analyte is retained in a chromatography separation $t_R$ is the product of the time the analyte spends in the stationary phase $t_S$, and mobile phase $t_M$.

**Equation 9**

$$t_R = t_S + t_M$$

If an analyte is not retained on the column, it elutes at the same time as the mobile phase and this is called the void volume, or the volume it takes for the mobile phase to traverse through the column. It is helpful to think of retention factors for an analyte normalized to the void volume of a column. This is called the retention factor $t'_R$ and is calculated as:
The resolution of two chromatographic peaks $A$ and $B$ is defined as peak resolution $R_S$ and is a function of the retention factors of the two analytes and their respective peak widths $W$:

Equation 10

$$t'_R = \frac{t_R - t_M}{t_M}$$

Equation 11

$$R_S = \frac{2(t_{RB} - t_{RA})}{W_A + W_B}$$

Analyte resolution can also be defined through the theoretical number of plates, $N$, on a column and their height, $H$. Plate number and height are theoretical numbers to describe the resolution power of a given column. An increase in plate numbers will result in an increase in peak resolution, and a decrease in plate height will also increase peak resolution. The, number of plates and plate height, therefore, are inversely proportional to one another; thus, over a given column length the relationship is described by

Equation 12

$$H = \frac{L}{N}$$
where $L$, column length. The number of plates in a column is measured through the peak width and retention time of an analyte; and, therefore, may vary for different analytes on a given column.

**Equation 13**

$$ N = 16\left(\frac{t_R}{W}\right)^2 $$

The factor of 16 arises from measuring the peak width to two standard deviations on either side of the peak maxima. The two standard deviations on either side of the chromatographic peak sums to four and is squared to obtain 16.

The total efficiency of chromatographic columns is expressed as *plate height* through the *van Deemter equation*. The van Deemter equation is a function of $A$, eddy diffusion, $B$, longitudinal diffusion, and the mass transfer of the analyte to the stationary phase and mobile phase, $C_S$ and $C_M$, respectively. The van Deemter equation is also a function of the linear velocity of the mobile phase, $\mu$, and all terms except the eddy diffusion term are dependent on the mobile phase velocity.

**Equation 14**

$$ H = A + \frac{B}{\mu} + C_S\mu + C_M\mu $$
As the linear velocity increases, the longitudinal diffusion term in the equation approaches zero but approaches infinity when the velocity approaches zero. The mass transfer to the stationary phase and mobile phase increase to infinity, with an increase in velocity, but are zero when the linear velocity of the solution approaches zero. The mass transfer term dominates the van Deemter equation and plate height will eventually increase to infinity as the linear velocity of the mobile phase approaches infinity. However, **Equation 14** is a simplified form of the equation and does not show the dependence of the diffusivity of solutes. Where the $B$, longitudinal diffusion is dependent on diffusion coefficient of the analyte, $D_m$, and, $\gamma$, is a constant parameter depending on the column and generally on the order of unity.

**Equation 15**

\[ B = 2\gamma D_m \]

The mass transfer of the analyte to the mobile phase $C_m$ is also dependent on the molecular diffusion of the analyte

**Equation 16**

\[ C_m = \frac{\omega d_p^2}{D_m} \]
where $\omega$ and $d^2_p$ are constants that are dependent on the column and generally on the order of unity. Interpretation of Equation 14 and Equation 15 indicates that the longitudinal diffusion term decreases with a decrease in a diffusion coefficient increasing the separation efficiency; whereas, interpretation of Equation 14 and Equation 16 indicate a decrease in a diffusion coefficient will decrease the separation efficiency. Different diffusion coefficients do not have a major effect on separation efficiency if solutes in the LC separation are on the same order of magnitude in their diffusion coefficients; however, in the separation of nanoparticles this plays a large role in column and separation efficiency (80,81).

2.2.3 Liquid Chromatography

Liquid chromatography (LC), like all partition based chromatography, is the separation of solutes as they partition to a stationary phase while being eluted by a mobile phase. In partitioning based chromatography, the difference in solubility between analytes in the mobile phase and their effective partitioning strength to the stationary phase creates a separation between solutes. Traditional liquid chromatography uses a polar stationary phase and a nonpolar mobile phase to elute analytes. Under these conditions, polar analytes will be retained on the polar stationary phase while nonpolar analytes will elute from the column with the nonpolar mobile phase. Current chromatography in analytical and environmental chemistry favors reversed-phase liquid chromatography (RPLC) where the stationary phase is nonpolar and the mobile phase is a
more polar solvent. RPLC is favored in environmental analytical chemistry because the analytes that are typically of interest are nonpolar and a separation where nonpolar solvents partition to a nonpolar stationary phase creates conditions more favorable for separation. In addition, using water as the polar mobile phase is cheaper and more environmentally friendly because the uses of nonpolar solvents are minimized. To facilitate the elution of nonpolar analytes in RPLC an organic solvent is added as a mobile phase modifier to reduce retention times of solutes on the stationary phase. This is added to the polar mobile phase to increase the solubility of the analytes to the mobile phase. Typically, the mobile phase modifiers are organic molecules with high aqueous solubility, such as methanol or acetonitrile, but there is a desire to have mobile phase modifiers that are easily reusable to cut back on costs to the user.

Advances beginning in the 1960s through column efficiency and decrease separation times have improved the resolution and effectiveness of LC. Column efficiency is improved through a decrease in stationary particle size, and a decrease in separation times was improved through high-pressure pumps to move mobile phase through the stationary column. These advances in LC separation coined the term high-performance liquid chromatography (HPLC) and nearly all LC separation is performed using high-pressure pumps and small particle stationary phase, and as such, LC and HPLC are interchangeable.

There are many different types of stationary phases depending on the separation being performed. Stationary phase columns are optimized for the separation of ions,
proteins, macromolecules, and trace organic solutes to name a few. The two types of stationary phases that are relevant to this research are C₈ columns for the separation of organic molecules and gel permeation columns for separation of macromolecules. C₈ columns contain packed beads of silica with n-octyl (C₈) chains coating the beads. The smaller the bead size, or particle size, the more efficient the separation of solutes. Non-polar molecules partition to the C₈ chains and are retained on the column longer than polar molecules. Non-polar mobile phase modifiers decrease the partitioning of the solute to the C₈ chain and increase its solubility in the mobile phase as the solute moves through the column. With C₈ columns, analytes are separated by their polarity, where the more polar solutes will elute before less polar solutes.

Solvents have a defined polarity index $P'$ used to measure the polarity of solvents. $P'$ varies from 10.2 for water to 0.04 for cyclohexane. The polarity index for a mixture of solvents is

\[
P'_{AB} = \phi_A P'_A + \phi_B P'_B
\]

where $P'_A$ and $P'_B$ are the polarity index for the two solvents and $\phi_A$ and $\phi_B$ are the volume fractions of the two solvents. Adjusting the mobile phase concentration and $P'$ during a separation can increase the resolution between two analytes.

Gel permeation columns contain a permeable silica gel or a permeable organic polymer. These columns are used to separate solutes based on their size. Where small
molecules enter the pores of the gel and have a longer distance to travel through the column, large molecules are too large to enter the pores, are excluded from the gel, and have a relatively smaller distance to travel through the column. The total volume $V_t$ of a gel permeation column is defined as

**Equation 18**

$$V_t = V_g + V_i + V_o$$

where $V_g$ is the volume of the column occupied by the gel, $V_i$ is the volume of solvent held in the pores of the column, and $V_o$ is the volume of solvent outside of the gel. If a molecule is too large to permeate into the gel the volume of solvent, $V_e$, needed to elute the molecule is given as:

**Equation 19**

$$V_e = V_o$$

where $V_e$ is the total exclusion volume of the column. If a solute can permeate into the gel $V_e$ is calculated as

**Equation 20**

$$V_e = V_o + KV_i$$
where $K$ is the distribution constant for the molecule’s ability to permeate into the gel and ranges from 0 for total exclusion to 1 for total inclusion into the gel (80,81).

2.2.4 Wide-bore Hydrodynamic Chromatography

In wide-bore hydrodynamic chromatography (HDC), solutes are separated based on differences in solute diffusivity as solutes travel through the capillary following a laminar flow profile. It has been used in the past to separate submicron particles, and is considered by some to be a technique complimentary to size exclusion chromatography (82-84). In wide-bore HDC as well as most, if not all other separation techniques, solute-solute interactions are purposefully avoided so that the solutes can be separated and quantified. Thus, conditions in wide-bore HDC are designed to minimize intermolecular particle-particle interaction and particle-capillary wall interactions.

The separation of solutes in wide-bore HDC is obtained by relative differences in size and diffusion coefficients. Solutes with a relatively larger cross sectional area will elute from a capillary before solutes with relatively smaller cross-sectional area. If a laminar flow is established in the capillary, the flow can be represented as

\textbf{Equation 21}

$$\mu(r) = \mu_{max} \left(1 - \frac{r^2}{a^2}\right)$$
where $r$ is radial distance from the capillary axis, $a$ is the radius of the capillary, and $\mu(r)$ and $\mu_{\text{max}}$ are the linear velocity of the solvent of the flowing system at $r$ and the maximum linear velocity at the center of the capillary, respectively (85-88). However, in wide-bore HDC, convection and diffusion of solutes diffusing over the cross-sectional area of the capillary impact elution times. Solute diffusion is calculated through the convective-diffusion equation

**Equation 22**

$$D_m \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial r^2} + \frac{1}{r} \frac{\partial C}{\partial r} \right) = \frac{\partial C}{\partial t} + u(r) \frac{\partial C}{\partial x}$$

where $D_m$ is the molecular diffusion coefficient (m$^2$ s$^{-1}$), $C$ is the concentration, and $x$ is the distance along the capillary (87,89,90). Solutes with different diffusion coefficients travel at different speeds through the capillary as governed by their reduced time $\tau$ (unitless),

**Equation 23**

$$\tau = \frac{D_m L}{a^2 \mu_{\text{ave}}}$$
where $L$ is the length of the capillary from injection to detector, and $u_{ave}$ is the average linear velocity of the particle (86-88,91). As $\tau$ approaches 0, the diffusion coefficient of the particle is small and the flow profile exhibits tailing, resulting in an asymmetric flow profile and a relatively faster elution time. As $\tau$ approaches 1, the diffusion coefficient is large and a more symmetrical, and a Gaussian flow profile should be observed and the solute will elute relatively later in time (91).

From Equation 23, the time it takes for an analyte to reach the detector is a function of the diffusion coefficient of the analyte, flow rate, and the length and radius of the capillary. The Stokes-Einstein equation, Equation 1, shows the diffusion coefficient is dependent on the radius of the particle. If there is one order of magnitude difference in size between two solutes, the reduced time in Equation 23 will be sufficiently different to partially separate the two solutes (92). Assuming LRD, the model nanoparticle, is a 25 nm diameter sphere and the experimental temperature is 22°C and with water as the mobile phase with a $\eta=1$, the calculated $D_m$ for LRD following Equation 1 is $1.7 \times 10^{11}$ m² s⁻¹ and a small solute with a radius of 0.5nm, $D_m$ is calculated to be $8.6 \times 10^{10}$ m² s⁻¹. With nearly two orders of magnitude difference in $D_m$, the two solutes can be partially separated (92). In addition, by changing the size and length of the capillary, the reduced time for analytes will change. In this manner, it is possible to obtain Gaussian or asymmetrical peak shapes for the same analyte (92). Thus, flow rate and capillary dimensions can be varied to exploit the differences between molecular diffusion coefficients of solutes. Due to the differences in size and diffusion coefficients a partial
separation of solutes from nanoparticles can be obtained in addition to different flow profiles (82,86,89,91,92).

2.2.5 Detection Methods

Detection during HPLC and wide-bore HDC experiments used ultraviolet (UV) and visible (Vis) molecular spectrometry or UV-Vis spectrometry. UV-Vis spectrometry is based on the ratio of incident light $P_o$, and transmitted light $P$, or transmittance $T$, and as such, the absorbance $A$, of an analyte in solution, is unitless. The absorption of an analyte in solution may also be expressed as a function of the analyte concentration $c$, the path length of the solution $b$, and the analyte’s molar absorptivity $ε$, which is a measured constant based on the efficiency of an analyte to absorb incident radiation. The relationship between concentration, path length and molar absorptivity is commonly referred to as Beer’s Law. Hence the absorbance of an analyte is defined through the following equations:

**Equation 24**

$$A = - \log(T) = \log\left(\frac{P}{P_o}\right) = \varepsilon bc$$

The detectors on the instruments used had either a multi-wavelength detector that was capable of measuring 5 wavelengths simultaneously with a spectral window of approximately 180-800 nm. The other detector used was a photodiode array (PDA)
detector, sometimes referred to as a diode array detector (DAD), which was capable of measuring the entire spectral window of approximately 180-800 nm simultaneously (81).

2.2.6 Fluorescence Experiments

Part of the research performed used fluorescence measurements. Fluorescence, like absorption spectroscopy, arises from the absorption of photons. As a molecule absorbs photons, the molecule is populated to a higher excited state and an electron to a orbital in higher energy. Then, through vibrational relaxation or internal conversion, the molecule relaxes to the first excited state before returning to the ground state. As the molecule relaxes to the ground state, a photon is emitted in lower energy than the incident light, as a fluorescence photon. The lifetimes of fluorescence molecules, the time a molecule stays in the first excited state, is on the order of $<10^{-5}$ seconds. However, if an electron in the orbital with higher energy has a parallel spin with respect to the once paired electron in the vacated orbital in the ground state, a triplet state is formed and phosphorescence occurs. The triplet state is long-lived with respect to the singlet state with a lifetime $>10^{-4}$ seconds and possibly up to several seconds. The lifetime is much longer because the electron spin must be flipped before falling back to the ground state.

Measuring fluorescence intensity, $F$, is proportional to Beer’s law found in the previous section, and is proportional to the intensity of the incident beam and the transmitted intensity. Transmitted intensity is linear to the concentration, $c$, at low
concentrations, of analyte and a factor, $K$, which is dependent on the instrument and quantum yield of the analyte:

**Equation 25**

$$F = Kc$$

Quantum yield, $\Phi$, is a ratio of the total number of photos that are emitted during fluorescence to the total number of photons absorbed. The causes of quantum yield are numerous and vary on the incident light intensity, molecule, and the molecular environment (81,93).

### 2.3 Data Processing

#### 2.3.1 Exponentially Modified Gaussians

Peaks from wide-bore HDC experiments and single wavelength analysis were modeled and fit using an exponentially modified Gaussian (EMG) algorithm. EMGs are used to model chromatographic peaks because the asymmetry that is inherent in chromatography is not modeled well by the purely symmetrical peak shape of a Gaussian function (94-98). The equation for a Gaussian shaped peak $G(t)$, is

**Equation 26**

$$G(t) = H e^{-\frac{(t-t_R)^2}{2\sigma^2}}$$
where \( t \) is the time, and \( H \) and \( t_R \) are the height and time of the peak maximum, respectively and \( \sigma \) is the standard deviation, which is related to the full width at half maximum (FWHM) of the peak (95,99). EGMs are modified from a symmetrical Gaussian peak through a first order exponential decay function (96). The exponential decay modification introduces a tailing and asymmetry that is a better fit of chromatographic peaks (94-98). The asymmetry of peak shape in the EMG equation arises from a \( \tau/\sigma \) ratio, where \( \tau \) is the time constant of the exponential modifier and quantifies the decay time of the modeled system (95,96). The exponential decay function \( f(t) \) is (95):

\[
\text{Equation 27}
\]

\[
f(t) = \frac{1}{\tau} e^{-\frac{t}{\tau}}
\]

Combining the Gaussian function and the exponential decay function gives the equation of EMG and a detailed derivation of the EMG function is out of the scope of this research. Chromatographic peaks were fit using Origin (OriginLab, Northampton, MA) and the built-in peak fitting EMG function. The EMG function in Origin is
Equation 28

\[ f(x) = y_0 + \frac{A}{t_0} e^{\frac{x}{t_0}} \int_{-\infty}^{z} e^{-\frac{y^2}{2}} \, dy \]

where \( A \) is the peak area, \( y_0 \) is the baseline offset, \( t_0 \) is tailing parameter, \( x_c \) is elution time of the peak, and \( w \) is peak width at FWHM (100). When fitting chromatographic peaks, \( x_c \), the elution peak time, was constrained in the EMG equation and the other GaussMod variables were optimized using the Origin algorithm.

Using EMGs to fit chromatographic peaks is only relevant when detection is performed with one wavelength, or univariate detection, as is the case with a single wavelength detector. If a PDA is used and multiple wavelengths are recorded as a function of elution time, another fitting or deviation algorithm could be used to model chromatographic peaks.

2.3.2 Multivariate Curve Resolution

In chromatography, a complete separation of analytes is desired so each analyte can be detected and quantified individually. However, incomplete resolution of analytes and coeluting chromatographic peaks are inherent in chromatography and methods are needed to resolve coeluting, convoluted chromatographic peaks. An entire subsection of chemistry has been devoted to the study of deconvoluting species called chemometrics. Chemometrics was pioneered in part by Bruce Kowlaski at the University of Washington, Seattle, D. L. Massart at the Pharmaceutical Institute of the Vrije Universiteit Brussels,
and Svante Wold at Umeå University in Sweden in the early 1970s (101). In fact, there are a number of peer reviewed journals devoted to chemometrics, such as Journal of Chemometrics, and the Journal of Chemical Information and Modeling, to name a few.

One important chemometric technique is multivariate curve resolution (MCR), a technique used to obtain pure elution and spectral profiles from convoluted chromatographic data (102-105). MCR is a processing technique used in conjunction with DADs where large two-way data matrixes, or arrays, are collected. The data matrix, $D$, of convoluted analytes is collected in rows and elution profiles are collected in columns

**Equation 29**

$$ D = D_A + D_B $$

where $D_A$ and $D_B$ are the pure elution and spectral profiles of the convoluted analytes in the data matrix (106). The data matrix can also be expressed as a function of the spectral profile and elution profile of the two analytes

**Equation 30**

$$ D = c_A s_A^T + c_B s_B^T $$

where $c$ is the elution profile of the analyte and $s'$ is the spectral profile of the respective pure analytes. It is common notation in chemometrics to refer to the spectral profile as
loadings and the elution profile as the scores. Finally, combining the elution and spectral profile of the two analytes the data matrix can be written as:

**Equation 31**

\[ D = CS^T \]

The ultimate objective in MCR is to deconvolute the data matrix, \( D \), into the grouping of the pure spectral and elution profiles without any prior knowledge of the system (106). There are many mathematical approaches to obtain the spectral and elution profiles of the convoluted matrix, which is the purpose of MCR research. The convoluted spectra of the chromatographic data in this research were deconvoluted using MCR using PLS_Toolbox (Eigenvector Research, Inc, Wenatchee, WA) (107).

### 2.3.3 Parallel Factor Analysis

Like MCR, parallel factor analysis (PARAFAC) and parallel factor analysis 2 (PARAFAC2) are other chemometric methods used to obtain pure analyte spectra and elution profiles for convoluted data. PARAFAC was first written by Richard Harshman from UCLA in 1970 (108). Rasmus Bro from the University of Rolighedsvej in Denmark has written many tutorials and reviews of PARAFAC and PARAFAC2 applications to chemometrics (109-113). PARAFAC and PARAFAC2 are superior to MCR and other deconvolution algorithms because they can use multi-way data. Multi-way data have other dimensions added to the data array, usually in the form of variable
concentrations (109). Where MCR is limited to a two-way data matrix, PARAFAC can include multi-way data matrix where multiple experiments with varying concentrations can be deconvoluted at once, and in addition to pure spectral and elution profiles, relative concentrations for the analytes can be determined (110). PARAFAC has the limitation of being used for static measurements, for example absorbance and emission of multiple fluorophores, or at variable pH conditions (110,114). PARAFAC2 is a second generation of PARAFAC and is able to model multi-way data with changes in retention time of eluting compounds (109,112). Some examples of using PARAFAC2 to model chemical chromatography are in the case of gas chromatography mass spectrometry data (GC-MS) where the MS data and GC elution data make a two-way data matrix and multiple samples are stacked and make a multi-way data set (109). PARAFAC2 has also been used to quantify sulfamides in kidney by HPLC-DAD (114). Adding constraints can help MCR and PARAFAC methods deconvolute data (102,104,110,112). The most widely used constraints for spectral data are the use of nonnegativity constraints (110). Using nonnegativity constraints in spectral model is justified because if an analyte is present in solution, there must be a signal and a nonnegative concentration. The convoluted spectra of the chromatographic data in this research were deconvoluted using PARAFAC using PLS_Toolbox (Eigenvector Research, Inc, Wenatchee, WA) (107).
Chapter 3 Laponite-RD as a Mobile Phase Additive

3.1 Introduction

LRD, a nanomaterial, was used as a mobile phase modifier partially from the desire to reduce the use of organic mobile phase modifiers methanol, (MeOH), and acetonitrile, (ACN), and from published research that used organically modified clay surfaces as substrates in chromatography stationary phases (115,116). However, the primary motivation for designing experiments using LRD dispersed in LC mobile phase was to obtain binding constants between the clay nanoparticle and small organic molecules. Both of these processes, mobile phase modifiers to facilitate a separation and binding constants between nanomaterials and solutes, were proposed to be analogous to published research using cyclodextrins in the mobile phase (117-119).

Cyclodextrins, most commonly β-cyclodextrin, have been used as mobile phase modifiers in liquid chromatographic separations. β-cyclodextrin in the mobile phase has been shown to reduce retention times of solutes in reversed-phase liquid chromatography (117,118). The reduction in retention times is attributed to the inclusion of the solutes in the β-cyclodextrin cavity that weaken the solutes interaction with the non-polar stationary phase. Due to this potential explanation, attempts have been made to calculate the binding constant between the solute and β-cyclodextrin in aqueous solutions (117,120). Based on the knowledge that xanthines interacted with clay minerals and early
observations that caffeine mixtures with LRD remained dispersed in solution, an analogous interaction was postulated to exist between LRD and xanthine solutes (64). Thus, using published work in calculating $K_D$ between solutes and cyclodextrin as a model, the binding constant between LRD and xanthines can be estimated. When calculating the binding constant, $K_D$ between a solute and LRD there are assumptions of the equilibria that are established in solution that is shown in **Equation 32**:

**Equation 32**

\[
\begin{align*}
(S)_M + (LRD)_m & \overset{K_D}{\leftrightarrow} (S - LRD)_m \\
(S)_s & \overset{K_1}{\downarrow} \quad (LRD)_s & \overset{K_2}{\uparrow} \quad (S - LRD)_s \\
& \overset{K_3}{\downarrow}
\end{align*}
\]

The subscripts $s$ and $m$ denote the phase of the solute, $S$, and LRD, in the stationary phase and mobile phase, respectively. Under the assumption LRD does not interact with the stationary phase, the two equilibrium constants $K_2$ and $K_3$ are negligible and the dominant equilibrium processes existing in solution are $K_1$ and $K_D$. Using the retention factor of the solute, $k'$, in the presence of LRD, and the retention factor, $k_o'$, of the solute in pure water calculated through **Equation 10**, the binding constant of the solute to LRD can be calculated through (117,118):
Equation 33

\[
\frac{1}{k'} = \frac{1}{k'_o} + \frac{K_d}{k'_o} [LRD]
\]

In addition to finding the binding constant between solutes and LRD, there was an opportunity to use nanomaterials as a way to develop a new method to facilitate separating molecules with a similar retention times in LC systems. The theory of using LRD as a mobile phase modifier to separate analytes with similar retention times is based on the knowledge that molecules with similar partition coefficients \(K_c\), Equation 8, to the stationary phase may have different \(K_D\) values for a nanomaterial. If this is the case, molecules will separate based partially due to their \(K_D\) affinity to the nanomaterial, and partially to their partitioning onto the stationary and mobile phases. Typically, analytes with similar \(K_c\) values will elute at approximately the same time in the presence of a constant concentration of organic mobile phase. Our proposed separation technique occurs when analytes with similar \(K_c\) values but different \(K_D\) values for nanomaterials elute at different times because of a relatively stronger or weaker affinity for the nanomaterial in the mobile phase. Analytes with a higher \(K_D\) value will elute sooner due to their interaction with the nanomaterial, and molecules with a lower \(K_D\) will elute later due to their primary interaction with the stationary phase. This separation technique also has the ability to become a “green” separation because of the reduction or absence of organic mobile phase modifiers. Additionally, using nanomaterials has the possibility to make an easily reusable mobile phase. Reusing mobile phase modifiers keeps down costs.
when running LC analysis on an industrial scale and is a better alternative to disposing of mobile phase waste, especially when only a small fraction of the waste is analyte and the majority is water and an organic solvent (121).

3.2 Experimental

3.2.1 Materials and solutions

Laponite-RD (LRD) was obtained from Southern Clay Product Inc. (Gonzales, TX). LRD stock suspension was prepared by suspending 1.0g of LRD in 90 mL of water. The solution was stirred overnight to allow hydration of the LRD and was diluted to the appropriate concentration. Caffeine, theobromine, theophylline, and paraxanthine were HPLC grade (98% purity or higher) purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents, methanol (MeOH) and acetonitrile (ACN) were purchased from Pharmco (Brookfield, CT), and all solutions were prepared in high-purity water (18MΩ · cm Millipore Milli-Q Water System, Billerica, MA). Xanthine solutions were brought to volume in crimped auto sample vials and were spiked with sodium nitrate to determine void volumes in the chromatographs (81).

3.2.2 Apparatus

LRD mobile phase experiments were performed using an Eldex Laboratories CC series metering pump (Napa, CA). All MeOH and ACN experiments were conducted using an Agilent 1100 series pump (Waldenbronn, Germany). HPLC separations were
performed with a constant flow rate of 1 mL min$^{-1}$. Sample injection volumes were 5µL and injected with an Agilent 1050 pneumatic auto sampler (Waldenbronn, Germany). Samples were detected by ultraviolet (UV) absorbance using an Agilent 1100 series multi-wavelength detector at 210 nm and 271 nm (Waldenbronn, Germany). A Waters Xterra (Milford, MA) C$_8$ column, was used with a 3.5 µm particle size and column dimensions of 3.0x50mm.

3.3 Results and Discussion

Four xanthines (caffeine, theobromine, theophylline, and paraxanthine) were studied for their changes in retention time on a C$_8$ stationary phase column in the presence of varying concentrations of LRD. The changes in the xanthine’s retention times were measured as retention factor and compared with the respective retention factors in volume fractions of typical organic mobile phase solvents MeOH and ACN as well as DI water. Retention factors were calculated following the retention factor equation in section 2.2.2. Each xanthine analyte was injected in triplicate, and a mean retention factor was calculated. Separations of xanthine mixtures were also performed to compare the separation using typical organic mobile phase solvents concentration to LRD. Void volumes were calculated by monitoring 210 nm for the sodium nitrate ion peak to elute. Sodium nitrate is eluted in the void volume because it is a small charged ion and does not interact with the non-polar stationary phase.
A sample chromatograph of a xanthine separation using pure DI water as the mobile phase is shown in Figure 7. All four xanthine analytes are baseline resolved and elute from the column within six minutes.

**Figure 7 Elution profile of xanthines using pure water as the mobile phase.**

When an organic mobile phase solvent is added to the mobile phase, retention times decrease because a larger fraction of the analytes are dissolved in the mobile phase and a smaller fraction is partitioning to the stationary phase. Xanthine separations were performed individually and as a mixture with 30%, 20%, 15%, 10% and 5% (v/v) fractions of MeOH as the mobile phase solvent. A sample chromatograph from the 10%
MeOH separation of the xanthine mixture is shown in Figure 8; as expected, addition of MeOH decreases the elution time for all the analytes. A similar set of experiments were performed with ACN as the organic solvent at 15%, 10%, and 5% (v/v) fractions of ACN, and the 5% ACN separation of the xanthine mixture is shown in Figure 9.

Figure 8 Elution profile of xanthines in 10% MeOH as the mobile phase solvent.
Lower ACN concentrations are needed to decrease retention factors when compared to MeOH because ACN is a stronger solvent of organic molecules than MeOH due to its smaller dielectric constant; as such, less solvent is needed to decrease the analyte partitioning to the stationary phase.

When LRD is the mobile phase modifier in place of the typical organic mobile phase solvents MeOH and ACN, there is also a decrease in retention factors with an increase in LRD concentration suggesting an interaction between LRD and solutes. Three experiments with different LRD concentrations were investigated at 500ppm,
1000ppm, and 2500ppm (m/v) LRD, and mixture chromatographs of each are shown in Figure 10, 11, and 12, respectively.
Figure 10 Separation of xanthines using 500ppm LRD as the mobile phase modifier.
Figure 11 Separation of xanthines using 1000ppm LRD as the mobile phase modifier.
When using LRD as the mobile phase modifier, the caffeine and theobromine peaks are broad and fronting. Usually, broad and fronting chromatographic peaks are indicative of overloading the column with analyte to such an extent where all the stationary phase (nonpolar C₈ chains) are saturated by the analyte. This creates a fraction of analyte that is not retained. However, overloading cannot be occurring here since the same concentrations of xanthines were used throughout the experiments. The broad and earlier eluting peaks are most likely caused by the analyte interactions with LRD. Those xanthines associated with LRD will diffuse with the LRD since LRD has a diffusion coefficient lower than the freely eluting xanthines. Therefore the column efficiency
decreases and plate height increases and the separation has poor resolution. When the diffusion coefficient $D_m$ decreases, the mass transfer to the mobile phase $C_m$ increases as in Equation 16.

**Equation 16**

$$C_m = \frac{\omega d_p^2}{D_m}$$

When the mass transfer from the mobile phase increases the theoretical plate height increases following **Equation 14**.

**Equation 14**

$$H = A + \frac{B}{\mu} + C_8\mu + C_M\mu$$

In addition, the xanthines that are associating the LRD surface are not being retained on the stationary phase and elute earlier in time, whereas, a fraction of xanthines not associated with LRD elute later in time. To summarize, xanthines associated with LRD have smaller diffusion coefficients and have a broad peaks from a decrease in theoretical plate height. Secondly, xanthines associated with LRD are not interacting with the C$_8$ stationary phase and are less retained.

Another feature of the LRD chromatographs is the change in elution order. When performing the traditional separation with: water, MeOH and ACN water mixtures in the
mobile phase, the xanthines eluted from the column with theobromine, paraxanthine, theophylline and caffeine, first to last, respectively. However, when LRD is the mobile phase modifier, the elution order is paraxanthine, theophylline, theobromine, and caffeine, first to last, respectively. The switch in elution order for paraxanthine, theophylline and theobromine is due to change in pH of the solution and the ionized species present. The pKa values for theobromine, theophylline, and paraxanthine are 10.05, 8.77, and 8.81 respectively (65). The LRD mobile phase was at a pH ≈ 10 and was therefore above the pKa of theophylline and paraxanthine. As such, theophylline and paraxanthine would be negatively charged and would minimally interact with the negative surface charge of LRD and have a decreased affinity for the nonpolar stationary phase and elute earlier. Further evidence of minimal interaction between LRD and theophylline and paraxanthine is the absence of fronting peaks on their chromatographs. This is in contrast to theobromine and caffeine solutes, which are not charged and therefore interact with the surface of LRD, and as such they have fronting peaks in the chromatographs shown in Figure 10 and Figure 11.

Plotting retention factors verses LRD concentration for all the xanthines studied further illustrates the interactions of the xanthines with LRD. When LRD is used as the mobile phase modifier, a change in solution pH drops the retention factor for paraxanthine and theophylline, shown in Figure 13 and Figure 14, respectively and does not change with an increase in LRD concentration. Caffeine and theobromine have an
interaction with LRD; thus, their retention factors do decrease with an increase in LRD concentration and are shown in Figure 15 and Figure 16, respectively.

Figure 13 Retention factor for paraxanthine in LRD. With the introduction of LRD as the mobile phase modifier, the retention factor for paraxanthine decreases due to a change in solution pH and not from an interaction with LRD.
Figure 14 Retention factor for theophylline in LRD. With the introduction of LRD as the mobile phase modifier, the retention factor for paraxanthine decreases due to a change in solution pH and not from an interaction with LRD.
Figure 15 Retention factor for caffeine in LRD. With the introduction of LRD as the mobile phase modifier, the retention factor for caffeine decreases due to an interaction with the LRD surface.
Figure 16 Retention factor for theobromine in LRD. With the introduction of LRD as the mobile phase modifier, the retention factor for theobromine decreases due to an interaction with the LRD surface.
The decrease in retention factors observed for caffeine and theobromine with LRD are observed for all xanthines in the presence of MeOH or ACN. A plot of retention factor versus MeOH and LRD concentrations represents the solvent affect that LRD has as an organic mobile phase modifier, and the effective LRD concentration needed to have a similar solvent strength as MeOH when eluting caffeine Figure 17. A 2500 ppm mobile phase solution of LRD has the same retention factor for caffeine as a 30% (v/v) MeOH mobile phase. A similar plot comparing ACN to LRD and their ability to lower the retention factor of caffeine is shown in Figure 18, where a 15% (v/v) solution of ACN gives the same retention factor as a 2500ppm solution of LRD.
Figure 17 A plot of retention factors for caffeine on LRD and MeOH. A 2500ppm mobile phase concentration of LRD (red triangles) has the same retention factor of a 30% (v/v) MeOH mobile phase concentration (black diamonds).
Figure 18 A plot of retention factors for caffeine on LRD and ACN. A 2500ppm mobile phase concentration of LRD (red triangles) has the same retention factor of a 15% (v/v) ACN mobile phase concentration (black circles).

An attempt was made to calculate the binding constant of caffeine with LRD following the methods from cyclodextrin solute binding constants using RP-HPLC separations. A plot of caffeine LRD retention factor data following Equation 33 is shown in Figure 19. Calculating the association constant for caffeine LRD interactions from the slope results in a $K_D = 3000$ L Kg$^{-1}$. 
Figure 19 Plot of reciprocal retention factors for varying LRD concentrations to calculate an effective association constant between caffeine and LRD.

This value is an order of magnitude higher than other methods calculated in later chapters and a reported value for caffeine on a macro clay, sodium montmorillonite. There are two possible reasons for this larger than expected $K_D$ values. First, the relatively large size and small diffusion coefficient of LRD decreases the separation efficiency of LRD. A decrease in the separation efficiency and subsequently poor resolution of chromatographs is a result of the $C_m$ term in Equation 14 and Equation 16, which decreases the theoretical plate height. The decrease in theoretical plate height
causes broad fronting peaks that are not indicative of the true elution time of caffeine associated with LRD, and are evident in Figure 11.

The second possible explanation for the large calculated $K_D$ is that the association of caffeine, and other solutes, to LRD is not constant at variable LRD concentrations. As the LRD concentration increases solutes could be competing with LRD for binding locations on the surface. It is documented that at higher LRD concentrations LRD will begin to form ordered structures and begin to gel (35,37-39). If the amount of caffeine associated with LRD is proportionally higher at lower LRD concentrations than higher LRD concentrations Equation 33 would not accurately predict the $K_D$ of caffeine associate with LRD.

There could be a potential third, but unlikely, possibility for the large $K_D$ is from LRD interacting with the stationary phase. This interaction is not likely because LRD is a charged particle in solution. However, if there is an interaction, the assumption that the $K_2$ equilibrium constant from Equation 32 is occurring is not valid. Is would complicate the assumption used to calculate the $K_D$ value in Equation 33.

3.4 Conclusions

These series of experiments provided valuable insight into the behavior of LRD for a number of reasons. First, it was shown LRD can be used a mobile phase modifier, to a certain degree, in place of organic solvents MeOH and ACN. A 2500ppm LRD mobile phase results in the same retention factor on caffeine as a 30% (v/v) MeOH and a
15% (v/v) ACN solution. The lower retention factors of xanthines with LRD are only observed when there is an interaction between the xanthines and LRD and when the xanthines are uncharged in solution. Despite the reduction of retention factors of caffeine and theobromine with LRD, it is unlikely LRD will be used as a mobile phase modifiers for analytical chemists using nonpolar stationary phase columns because of the fronting and poor resolution of the chromatographs. The broad and fronting peaks will not allow for the separation and resolution needed to separate and quantitate analytes in an analytical lab. Another important conclusion from these experiments is that caffeine does interact with LRD dispersed in solution. However, the attempt to calculate the $K_D$ of caffeine associated with LRD was inconclusive due to the large size and small diffusion coefficient of LRD that skewed the $k'$ values due to the poor resolution of the mixture. These conclusions provided the background to use LRD and caffeine as model nanoparticles and solutes for determining association constants using wide-bore hydrodynamic chromatography, which is detailed in subsequent chapters.
Chapter 4 Measuring Laponite-RD association to Xanthine Simulates Using Hydrodynamic Chromatography

From the research described in Chapter 3 an interaction between caffeine and Laponite-RD was observed. Due to excessive band broadening in the eluting peaks, a quantitative measure of the interaction could not be made. However, based on the differences in diffusion coefficients between caffeine and LRD, it was hypothesized that the interaction could be quantified using another chromatographic technique. The method developed is based on wide-bore hydrodynamic chromatography and is used to obtain the magnitude of interactions between aqueous nanoparticles and small organic solutes. The use of wide-bore hydrodynamic chromatography exploits the differences in diffusion between a nanoparticle and a solute, and if an interaction between the solute and nanoparticle exists, the interaction can be detected and quantified.

4.1 Introduction

The interactions of solutes with clay minerals and other adsorbents are governed by ion-exchange absorption as well as other adsorption processes. These sorption processes are typically characterized, in part, by reporting the magnitude of a binding constant associated with a particular solute-sorbent system. In many cases experimental conditions can be readily developed to determine the binding constants via batch adsorption studies. A common technique is to separate the sorbent from the aqueous
solution via centrifugation or sedimentation; this is followed by analysis of the supernatant to quantify the concentrations of solute that has remained in solution. Some cases, however, prevent this facile separation approach of the sorbent from the free solute in solution. For example, Laponite-RD (LRD), a synthetic hectorite, forms stable dispersions of nanoparticles (25 nm in diameter) in aqueous systems (35). In salt-free suspensions, the Laponite particles are stabilized by electrostatic repulsive forces between the negative charges on faces of the clay. The particles will aggregate or form sols and gels over time, when salts are added to the suspensions or when concentrations of LRD are increased (39,56). These properties make it difficult to determine the magnitude of interaction between the suspended clay particles and a solute via centrifugation, as done in typical adsorption isotherm experiments.

Many synthetic clay particles are on the scale of 100 nm or less, and thus clays can be considered nanoparticles. Indeed, (LRD) has been used as a model nanoparticle to study nanoparticle-enzyme interactions in soils and to study transformation of endosulfans on the surfaces of suspended particles in aqueous solutions. Thus, understanding how these materials behave in the environment is of importance (59,122). As previously stated, nanoparticles and related nanomaterials also have wide potential use in energy production and storage, cosmetics, drug delivery, and many emerging consumer products (1-4). As such, the use of these materials can be expected to become more widespread. However, nanoparticle surface interactions and their ultimate fate in the environment are not widely understood (15,17,21).
A novel approach is presented here that is capable of measuring the binding constants of solute molecules to a model nanoparticle (Laponite-RD) using a wide-bore hydrodynamic chromatography (HDC) apparatus. The apparatus is designed to exploit the principals of wide-bore hydrodynamic chromatography, creating a partial separation of the solute and nanoparticle. Wide-bore HDC has been used to separate submicron particles, and is considered by some to be a technique complimentary to size exclusion chromatography (82-84). In wide-bore HDC as well as most, if not all other separation techniques, solute-solute interactions are purposefully avoided so that the solutes can be separated and quantified. Thus, conditions in wide-bore HDC are designed to minimize intermolecular interaction between particles and between particles and the capillary wall. However, when investigating binding constants between solutes and particles, these intermolecular interactions between the solute and nanoparticle, LRD are the property that is of interest. Therefore, the system designed and reported here, while following the principals of wide-bore HDC, does not achieve a separation of the nanoparticles from the solutes. Rather the wide-bore HDC system and conditions are designed to achieve only a partial separation thereby allowing solute-particle interactions to exist between the solute and LRD.

The design and optimization of the wide-bore HDC apparatus reported uses a model solute system. Using caffeine as a probe solute and the synthetic clay LRD as a model nanoparticle, caffeine association constants with the nanoclay are determined. Caffeine was selected as the probe solute because caffeine has been reported in the
environment, does not have a pKa that will change over the experimental pH conditions, and as been shown to bind to sodium montmorillonite with a binding constant of $290 \pm 2$ (L Kg$^{-1}$) (62,64,65).

### 4.2 Experimental

#### 4.2.1 Materials and solutions

Laponite-RD (LRD) was obtained from Southern Clay Product Inc (Gonzales, TX). Caffeine was HPLC grade (99% purity or higher) purchased from Sigma-Aldrich (St. Louis, MO). Sodium benzoate (USP grade) was purchased from Mallinckrodt Chemical Works (St. Louis, MO). All solutions were prepared in high-purity water (18MΩ⋅cm Millipore Milli-Q Water System, Billerica, MA), and the mobile phase was high-purity water or pH adjusted water with 0.1M NaOH from Rocky Mountain Reagents (Sheridan, CO).

LRD is a synthetic clay with platelets that are approximately 25 nm in diameter and 1 nm in height. The chemical composition of LRD is $\text{Na}^{+}_{0.7}\text{[Si}_{8}\text{Mg}_{5.5}\text{Li}_{0.3}\text{O}_{20}(\text{OH})_{4}]^{0.7}$, where the sodium ion, (Na$^+$), is attracted to the negative surface charge of the LRD by an electrostatic interaction. LRD has a net negative charge on the surface of the platelet, a variable charge on the edge of the LRD platelets that is pH dependent, and has a reported surface area of 360 m$^2$/g (27,35,39,56,57). LRD stock suspension was prepared by dissolving 1.0g of LRD in 90 mL of water. The suspension was stirred overnight to allow hydration of the LRD. The LRD suspension was pH
adjusted to pH = 10.2 using 0.1M NaOH; the solution was then brought to a 100mL volume resulting in a final concentration of 1% (w/w). The pH of the LRD solution was adjusted with NaOH to a pH > 10 to avoid degradation or dissolution of the LRD (35,39,56). The use of NaOH to pH adjust sample solutions was selected to minimize any potential impacts from competing cations since Na is the counter ion in LRD. The pH was measured using a Fisher Scientific Accument Basic AB15 pH Meter (Pittsburg, PA) calibrated with pH=7 and pH=10 buffers prior to use. A series of experiments was also conducted at a pH of 8.5. To minimize any impacts from degradation of the LRD suspensions at these conditions, a 1% (w/w) solution of LRD at a pH=8.5 was prepared as described above, and allowed to equilibrate overnight before the pH=8.5 experiments were conducted.

Mixture solutions for the isotherm experiments were made from stock solutions of the LRD and caffeine. For the experiments reported here, the concentration of the LRD was held constant in all samples at 3000 mg L\(^{-1}\) while varying the caffeine concentration between 25 and 350 mg L\(^{-1}\). Solutions were brought to volume in crimped auto sample injection vials, capped, mixed by shaking and then allowed to equilibrate for 5-30 minutes. Isotherm experiments were conducted the day of sample preparation. Additional isotherm experiments were performed where solutions equilibrated overnight. The same results were obtained from solutions over this time period (5 minutes to overnight); thus the equilibration time used for this study was assumed to be sufficient.
4.2.2 Apparatus and UV measurements

Wide-bore HDC experiments were performed using an Agilent 1100 series pump (Waldenbronn, Germany) with a constant flow rate of 300 μL min⁻¹ unless otherwise noted. Sample injection volumes were 5μL and injected with an Agilent 1050 pneumatic auto sampler (Waldenbronn, Germany). The capillary was a 0.25 mm diameter, 1.5-meter long PEEK tube (Upchurch Scientific Part No. 1581, Oak Harbor, WA). A box diagram of the apparatus is depicted in Figure 20. Samples were detected by ultraviolet (UV) absorbance using an Agilent 1100 series multi-wavelength detector (Waldenbronn, Germany). Caffeine standards were analyzed on the same apparatus, in triplicate, to create a five-point calibration curve.

![Box diagram of the wide-bore HDC apparatus.](image)

Figure 20 Box diagram of the wide-bore HDC apparatus.

Additional UV experiments were conducted to characterize the 200-400 nm spectral range of the mixtures. These UV experiments were performed using a Thermo
Electron Corporation Evolution 300 BB ultraviolet-visible, double-beam spectrophotometer (Loughborough, England). All experiments were performed in matching quartz cuvettes, scanning between 200-400nm with a 4 nm bandwidth.

4.2.3 Modeling of flow profiles

All flow profiles were fit using an exponentially modified Gaussian algorithm (GaussMod) in Lab Pro v8.0 (OriginLab Corporation, Northampton, MA) (100). The peak fitting parameters were: \( A \) = area; \( y_0 \) = baseline offset; \( t_0 \) = tailing parameter; \( x_c \) = elution time; and \( w \) = peak width (full width at half maximum) (100,101). Flow profiles were deconvoluted by fitting two exponentially modified Gaussians, one for the caffeine bound to the LRD surface and other for the free eluting caffeine flow profile. Elution times, \( x_c \), for the pure caffeine flow profiles were averaged over the experimental concentration range in triplicate. Three standard deviations of the elution time averages, \( x_c \), were used to constrain the GaussMod peak of the pure eluting caffeine on the convoluted profile. The other nine GaussMod variables for the convoluted flow profiles were optimized using the OriginLab Pro v8.0 algorithm.

4.3 Theory

In wide-bore hydrodynamic chromatography (HDC), solutes separate based on differences in solute diffusivity as they travel through the capillary under a laminar flow profile. The technique has been used in the past to separate submicron particles, and is
considered by some to be a technique complimentary to size exclusion chromatography (82-84). In wide-bore HDC (as well as most, if not all other separation techniques), solute-solute interactions are purposefully avoided so that the solutes can be separated and quantified. Thus, conditions in wide-bore HDC are designed to minimize intermolecular interaction between particles and between particles and the capillary wall.

The separation of solutes in wide-bore HDC is obtained by relative differences in size and diffusion coefficients. Solutes with a relatively larger cross sectional area will elute from a capillary before solutes with relatively smaller cross sectional area. When a laminar flow is established in a capillary, the flow can be represented as

Equation 19

\[ \mu(r) = \mu_{max} \left(1 - \frac{r^2}{a^2}\right) \]

where \( r \) is radial distance from the capillary axis, \( a \) is the radius of the capillary, and \( \mu(r) \) and \( \mu_{max} \) are the linear velocity of the solvent of the flowing system at \( r \) and the maximum linear velocity at the center of the capillary, respectively (85-88). In wide-bore HDC convection and diffusion of solutes diffusing over the cross sectional area of the capillary impact elution times. Solute diffusion is calculated through the convective-diffusion equation
Equation 20

\[ D_m \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial r^2} + \frac{1}{r} \frac{\partial C}{\partial r} \right) = \frac{\partial C}{\partial t} + u(r) \frac{\partial C}{\partial x} \]

where \( D_m \) is the molecular diffusion coefficient (m\(^2\) s\(^{-1}\)), \( C \) is the concentration, and \( x \) is the distance along the capillary (87,89,90). Solutes with different diffusion coefficients travel at different speeds through the capillary as governed by their reduced time \( \tau \) (unitless),

Equation 21

\[ \tau = \frac{D_m L}{R^2 \mu_{ave}} \]

where \( L \) is the length of the capillary from injection to detector, and \( \mu_{ave} \) is the average linear velocity of the particle (86-88,91). As \( \tau \) approaches 0, the diffusion coefficient of the particle is small and the flow profile exhibits tailing that results in an asymmetric flow profile and a relatively faster elution time. As \( \tau \) approaches 1, the diffusion coefficient is large relative to LRD, and a more symmetrical, (nearly Gaussian) flow profile should be observed and the solute will elute relatively later in time. Thus, due to these differences in reduced time, both a partial separation of solutes from nanoparticles can be obtained in and a difference in flow profile symmetry (82). As expected, from
review of the Equation 22, wide-bore HDC the mechanism of separation is dependent on both the flow rate and radius of the capillary. Thus, flow rate and capillary dimensions can be varied to exploit the differences between molecular diffusion coefficients of nanoparticles and solutes.

In the reported system, experimental parameters are optimized to partially separate the LRD (smaller $D_m$) from the caffeine (larger $D_m$) while simultaneously producing asymmetrical and symmetrical peak shapes, respectively. By inducing two different flow profiles, (an asymmetrical profile for the LRD and a near Gaussian profile for the caffeine), the total concentration profile of the mixture can be modeled with two distinct flow profiles. If an interaction (in the form of adsorption, complexation, and/or ion-exchange) exists between the caffeine and LRD, the fraction of caffeine associated with the LRD surface will assume the asymmetrical flow profile of the LRD particles and elute prior to the free caffeine as depicted in the cartoon in Figure 21. LRD weakly scatters at 271 nm where caffeine has an adsorption maximum, however, apparent absorption contributions from LRD scattering can be corrected, and the fraction of caffeine molecules bound to, or associated with, the LRD surface can be determined. By modeling the LRD flow profile with the fractional area of caffeine that have assumed the LRD flow profile and the fractional flow profile area of the free eluting caffeine, an effective association constant of caffeine with LRD can be determined.
Figure 21 Depiction of solutes separating from larger nanoparticles by HDC. The capillary length ($l$) and radius ($r$) are 1.5m and 0.254 mm respectively. Large shaded circles represent LRD nanoparticles and small shaded circles represent caffeine solutes. (a) Initially caffeine and LRD are injected onto the capillary at the same time before separation begins. (b) If the two particles do not interact, the larger LRD particles will elute before the smaller caffeine solutes. (c) If there is an association between LRD and caffeine, a fraction of caffeine associated with LRD will elute with the LRD before the bulk of unassociated caffeine elutes later in time.

It should be noted that the terminology of an effective association constant, $K_{EFF}$, has been chosen due to the range of surface interactions that can exist in various nanoparticle-solute systems. For discussion purposes, theory that describes adsorption processes is used to describe the system. This adsorption isotherm is based on the
assumption there are only three species in equilibrium during our experiments where \([X_F]\) is the free caffeine concentration, \([X_B]\) is the caffeine concentration bound to the LRD surface, and \([LRD]\) is the freely eluting LRD concentration. Recall from the Introduction that caffeine was selected as a probe since it does not have a pKa value close to the conditions tested; thus, it is safe to assume that it is present in only one form. The equilibrium for all species is described by:

**Equation 34**

\[ [X_F] + [LRD] \rightleftharpoons [X_B] \]

To further ensure that only these three species are in the sample, and minimize the formation of ordered LRD platelet structures (that will possess larger hydrodynamic radii), the pH of most samples were adjusted to a pH value of approximately 10.0 or greater, and the concentration of the LRD in solution was kept below 1% (w/w) (35,39,56).

For quasi-linear adsorption isotherms, adsorption coefficients, binding constants or association constants can be described by

**Equation 35**

\[ K_{\text{EFF}} = [X_B]/[X_F] \]
where $K_{\text{EFF}} \, (\text{L g}^{-1})$ is the association constant at equilibrium for caffeine bound to the surface of LRD and is the slope on the isotherm plots. $[\text{XF}]$ is the free caffeine concentration unbound to the LRD surface (mg L$^{-1}$) and is expressed by

**Equation 36**

$$[\text{XF}] = (A_{\text{F}} / A_{\text{T}}) \, [X_0]$$

where $A_{\text{F}}$ is the peak area of the free caffeine from the deconvoluted flow profile and $[X_0]$ is the initial caffeine concentration (mg L$^{-1}$). $A_{\text{T}}$ is the total area of the flow profile, corresponding to the caffeine peak area at that respective concentration obtained from a caffeine calibration curve. $[\text{XB}]$ is the concentration of the caffeine bound to the surface of the LRD (mg g$^{-1}$), and it is determined by

**Equation 37**

$$[\text{XB}] = (A_{\text{B}} / A_{\text{T}}) \, [X_0] / [\text{LRD}]$$

where $A_{\text{B}}$ is the peak area of the caffeine bound to the LRD surface that can be determined by the difference:

**Equation 38**

$$A_{\text{B}} = A_{\text{T}} - A_{\text{F}}$$
The concentration of the free caffeine is determined using the calculated peak area from the flow profile corresponding to the free caffeine that has been reconstructed from convoluted profile. In this time window, scattering from the LRD is assumed to be negligible. Furthermore, the total peak area of the convoluted profile is not constrained during the fitting algorithm. It is assumed that the contribution from scattering from the LRD as well as the absorption from the fraction of caffeine molecules associated with the LRD is accounted for in the asymmetric flow profile. By using the previous equation, the concentration of the caffeine associated with the LRD, $A_B$, is calculated by mass balance.

4.4 Results and Discussion

4.4.1 Flow profiles

Examples of the different flow profiles obtained under a representative flow regime for both caffeine and LRD based on wide-bore HDC described in the theory section are shown in Figure 22 (a) and (b). Figure 22 (a) is a normalized flow profile, normalized to peak height. It also highlights the partial HDC separation of the larger LRD from the smaller caffeine solute. Figure 22 (b) shows the same flow profile as in Figure 22 (a) without normalization to demonstrate the absorbance/scattering difference between caffeine and LRD. The LRD scattering is between 1-10% of the absorbance of the caffeine depending on the caffeine concentration; a high scattering sample is shown for illustration.
Figure 22 Flow profiles highlighting the hydrodynamic differences in caffeine and LRD. (a) Normalized flow profiles of 50ppm caffeine (solid line), and 3000ppm LRD (dots); flow rate of 300µL/min; detected at 271nm. The caffeine flow profile shows a more symmetrical Gaussian peak shape eluting after the asymmetric peak shape of the LRD. (b) Flow profiles of 50ppm caffeine (solid line), and 3000ppm LRD (dashes). LRD flow profile area is 9.5% of the caffeine area at caffeine’s lowest concentration of 25ppm and 0.7% of caffeine area at caffeine’s highest concentration of 350ppm. Flow rate of 300µL/min; absorbance detected at 271nm. Sample vials were adjusted to pH = 10.2 with NaOH and the mobile phase was adjusted to pH = 8.5 with NaOH.
The Agilent auto sampler was essential in these experiments due to its ability to inject samples onto the column quickly and reproducibly. Initial experiments were performed with a manual sample injector with a 10 µL injection loop. When using the manual sample injector, the results from the wide-bore HDC separation were not reproducible and shown in Figure 23. As such, the pneumatic auto injector was used throughout the experiments.

![Manual Caffeine Injections](image)

**Figure 23** Caffeine and LRD injections using a manual sample injector. Using a manual sample injector is slow to inject sample onto the capillary. As a result, the wide-bore HDC separations are irreproducible.
A representative flow profile demonstrating the solute interaction with the nanoclay particles is shown in Figure 24. The absorbance trace shown depicts the convoluted asymmetric flow profile obtained from a mixture of 3000 ppm LRD and 50 ppm caffeine. A flow profile consisting of an identical concentration of 50 ppm caffeine is overlaid on top. The caffeine flow profile fits over the small shoulder on the mixture flow profile from the unbound caffeine eluting later in time. The asymmetric peak shape in the convoluted flow profile is from a fraction of caffeine bound to the larger and earlier eluting LRD. A flow profile overlay of 3000 ppm LRD is also plotted. The small scattering of LRD alone cannot contribute to the asymmetrical peak shape of the convoluted mixture flow profile, and must be from the bound fraction of caffeine on the LRD surface.
Figure 24 Flow profile showing the interaction between caffeine and LRD. 50ppm caffeine (dots), a mixture of 50ppm caffeine and 3000ppm LRD (solid line) and 3000ppm LRD (dashes); flow rate of 300µL/min; absorbance detected at 271nm. Sample vials were adjusted to pH = 10.2 with NaOH and the mobile phase was adjusted to pH = 8.5 with NaOH.

The control is a mixture of sodium benzoate and LRD. The sodium benzoate and LRD will have minimal interaction due to negative charge repulsions from the two solutes. The pH of the solutions were adjusted to pH = 8.5 with NaOH, well above the pKa=4.2 of benzoic acid (123). The mixture flow profile in Figure 25 demonstrates when there is not an interaction between the solute (benzoate ion) and LRD, and the flow profile of the mixture is nearly identical to the pure sodium benzoate flow profile.
Because sodium benzoate does not bind to the LRD surface, it does not exhibit the flow profile of LRD and is not assuming the flow profile of LRD.

Figure 25 Control flow profile without an interaction. 80ppm sodium benzoate (dots); mixture of 80ppm sodium benzoate and 3000ppm LRD (solid line); 3000 ppm LRD (dashes), flow rate of 300µL/min; detected at 230nm. Sample vials and mobile phase adjusted to pH = 8.5 with NaOH. The two flow profiles are nearly identical because the benzoate ion does not react with the LRD nanoparticles.

When deconvoluting the flow profiles of the caffeine LRD mixtures, the molar absorptivity of the caffeine has the same lambda maximum as the caffeine associated with the LRD in the mixtures. In Figure 26, the UV-Vis spectra of caffeine and a mixture of caffeine and LRD are given. As shown, no detectable shift in the molar
absorptivity of caffeine can be seen with the introduction of LRD. The same molar absorptivity of the sample with and without LRD enables a direct determination of caffeine in solution since there is no spectral shift arising from the association with the LRD surface; thus, the peak area from the calibration curve can be used in a mass balance calculation to find the concentration of caffeine bound to the LRD nanoparticles. The scattering from LRD in the convoluted flow profiles is accounted and corrected for as described in the theory section.
Figure 26 UV-Vis absorption spectra showing that the molar absorptivity does not change with the introduction of LRD. 40ppm caffeine (dashes); 40ppm caffeine with 1500ppm LRD (solid line); 1500ppm LRD (dots).

4.4.2 Apparatus optimization

An optimized flow rate was established by performing caffeine and LRD injections at different flow rates shown in Figure 27. As the flow rate is increased to 700 μL min⁻¹ the HDC separation of caffeine adsorbed to LRD and the free caffeine is not observed. However, as the flow rate decreases to 300 μL min⁻¹ a partial separation begins and a distinct shoulder is observed in the convoluted flow profile. (Pure caffeine was also measured over a range of flow conditions to confirm a Gaussian flow profile for
caffeine was maintained despite a change in flow rate for all flow conditions.) A similar decrease in separation is observed when coiling the capillary, **Figure 28**. Coiling the capillary induces turbulence and mixing in the laminar flow profile and consequently there is a decrease in HDC separation (87). With 2 and 3 coils in the PEEK tubing between the injector to the detector, the HDC separation is less pronounced when compared to a straight capillary. Thus, a straight PEEK capillary at a 300 µL min⁻¹ flow rate was used throughout the isotherm experiments.
Figure 27 Flow profiles at different mobile phase flow rates injecting a mixture of 100ppm caffeine and 3000ppm LRD. 300 μL min\(^{-1}\) (solid line); 100 μL min\(^{-1}\) (short dashes); 500 μL min\(^{-1}\) (dots); 700 μL min\(^{-1}\) (long dashes). Absorbance detected at 271 nm. Sample vials adjusted to pH = 10.2 with NaOH and mobile phase adjusted to pH = 8.5 with NaOH.
Figure 28 Flow profiles showing the affect of coiling the micro capillary of a mixture of 100ppm caffeine and 3000ppm LRD. Straight capillary (solid line); 1 coil (dots); 2 coils (short dashes); 3 coils (long dashes). Flow rate of 300µL/min; absorbance detected at 271nm. Sample vials adjusted to pH = 10.2 with NaOH and mobile phase adjusted to pH = 8.5 with NaOH.

4.4.3 Mixture analysis

Samples of the mixture deconvolutions are shown in Figure 29 (a), (b), and (c), at three different pH conditions. The deconvolution of the mixture illustrates the two distinct flow profiles that make the convoluted flow profile. One reconstructed flow profile is from caffeine adsorbed to the LRD, and the other reconstructed flow profile is from the free eluting caffeine. The sums of the two flow profiles are in good agreement.
with the actual mixture flow profile. Again, the scattering by LRD is assumed to be
negligible or accounted for in the elution profile of LRD. The caffeine flow profile fits
well with the reconstructed free eluting caffeine flow profile Figure 30.
Figure 29 Change in flow profiles with a change in pH. (a) Sample vials and mobile adjusted to pH = 10.2 with NaOH; (b) mobile phase adjusted to 8.5 with NaOH; and (c) sample vials and mobile phase adjusted to pH = 8.5 with NaOH. All flow profiles were a mixture of 300ppm caffeine and 3000ppm LRD (short dashes). All figures include LRD reconstructed flow profiles (long dash); caffeine reconstructed flow profiles (dots); and the reconstructed mixture flow profile (sold line).
Figure 30 Overlay of reconstructed flow profiles of caffeine, caffeine LRD mixtures and freely eluting caffeine.

The calculated isotherms from all concentration trials conducted under the pH conditions described in Figure 29 are shown in Figure 31. The isotherms were constructed by determining the free caffeine concentrations in solution; then by using Eqs. 8 and 9, the concentration of caffeine associated with the LRD nanoparticles was quantified. When pure water was used as the mobile phase, the calculated isotherm for caffeine and LRD is not as strong initially as the higher pH trials, indicating a weaker interaction between the caffeine and LRD at low concentrations. However, at higher concentrations, the isotherms under all conditions (including when the mobile phase is
adjusted to pH = 8.5 with NaOH and the sample vials are pH adjusted with NaOH), exhibit similar behavior. The calculated $K_D$ of caffeine to LRD at pH = 10 is $140 \pm 10$ L Kg$^{-1}$.

Figure 31 Apparent adsorption isotherm for caffeine bound to LRD. Water as the mobile phase (squares); sample vials adjusted to pH = 10.2 with NaOH and mobile phase adjusted to pH = 8.5 with NaOH (diamonds); both sample vials and mobile phase adjusted to pH = 8.5 with NaOH (triangles). LRD concentrations were a constant 3000ppm and caffeine concentrations varied. Flow rate of 300µL/min; detection at 271nm. Standard deviation uncertainties bars are shown from triplicate runs.
4.4.4 Limitations

The apparatus presented does have limitations. First, if the solute-nanoparticle interactions are reversible on the time scale of the measurement, and the desorption reaches equilibrium rapidly (on the time scale of the experiment or faster), then peak broadening will occur and $K_{\text{EFF}}$ measurements will be inaccurate. Second, the experimental apparatus has the limited ability to only calculate a $K_{\text{EFF}}$ value since it is unclear what mechanism (or mechanisms) are responsible for the interactions that are observed. It is hoped these well-characterized systems will provide additional insight into the versatility of the apparatus as well as validate the ability of the apparatus to measure a specific binding constant. Despite this limitation, it is important to note that in many instances, knowing the type of interaction may not be as important as knowing the magnitude of $K_{\text{EFF}}$.

4.5 Conclusion

An apparatus has been described that demonstrates that solute-particle associations between solutes and LRD nanoparticles can be observed and quantified using a form of wide-bore HDC and mass balance equations. The apparatus allows for rapid analysis of solute-nanoparticle systems, on the order of a minute, and is relatively inexpensive to construct if a liquid chromatographic system with UV-Vis detection is available. The technique used does not have the limitation that traditional batch isotherm experiments have when applied to solutes that form stable dispersions and do not
precipitate from solution. Thus, determination of $K_{\text{EFF}}$ associations where interactions are in equilibrium, in solution, or suspension are possible.
Chapter 5 Evaluation of Laponite-RD association to Caffeine and Oxytetracycline using Hydrodynamic Chromatography with Multivariate Deconvolution Methods

5.1 Introduction

Univariate curve resolution of caffeine associated with LRD from wide-bore HDC experiments enabled the determination of freely eluting caffeine in solution. From these results an adsorption isotherm was calculated and an effective association constant, $K_{\text{EFF}}$, between caffeine and LRD was determined. The isotherm and subsequently calculated $K_{\text{EFF}}$ value were based on assumptions of the chemical species present and their concentration profile. While these were appropriate assumptions, additional data evaluation techniques were explored in attempt to determine $K_{\text{EFF}}$ of analytes associated with nanoparticles without using the assumptions made in Chapter 4, (i.e. concentration profile shapes and numbers of species present in solution) (102,105,106). Three types of chemometric methods were applied to caffeine and LRD data, and included multivariate curve resolution, (MCR), parallel factor analysis, (PARAFAC), and parallel factor analysis 2, (PARAFAC2), (109,110,114,124).

In addition to using caffeine, oxytetracycline, (OTC), was selected for evaluation with the wide-bore HDC apparatus to monitor the interaction between OTC and LRD. OTC is a small organic molecule (FW = 460 g mol$^{-1}$), and has been shown to bind to clay
surfaces under certain pH conditions, and thus was predicted to be a molecule that would interact with the clay mineral LRD (67). OTC associated with LRD was modeled using PARAFAC2 methods in wide-bore HDC experiments. While performing the wide-bore HDC experiments, a kinetic effect was observed between OTC and LRD. To verify the observed kinetic effects from the wide-bore HDC experiments, injections of OTC and LRD mixtures were performed on a size exclusion column using a HPLC instrument.

5.2 Theory

In multivariate analysis, complex mixtures are detected in the form of a data matrix, sometimes referred to as a data array. Unlike the univariate approach in Chapter 4, no prior information of the mixtures is needed when deconvoluting the data array using multivariate analyses methods. In MCR, the collected convoluted data matrix is simplified into two simpler matrices that are related to the original data matrix (102,105). When used in the deconvolution of UV-Vis spectral data from chromatographic separation, MCR is primarily used to estimate the pure spectra and elution profiles of the mixture analytes. As such, MCR methods are considered a qualitative method not a quantitative method (102,104-106,125,126). The major limitation that prevents MCR from being quantitative and not quantitative arises from intensity and rotational ambiguity of the data (104,105). (Rotational ambiguity is more correctly referred to as linear transformation ambiguity (127)). Intensity ambiguity arises from the concentration and spectral solutions in curve resolution methods being scaled by some unknown factor.
Knowing the scaling factor is not as important in qualitative analysis because the spectral shape and elution profiles do not change; however, not knowing the scaling factor prevents the method from being quantitative (102,104,105). The limitation of rotational ambiguity occurs when there are two or more linearly independent components, for example coeluting peaks, which do not have a fraction where there is a pure elution. As a result, the estimated spectrum and elution profiles of the linearly independent components will be an unknown linear combination of the true component system (104,105). If measured elution profiles have selectivity, or a fraction of pure elution for one of these components, no rotational ambiguity is present (102,104,105).

The problems of intensity and rotational ambiguity are not present in PARAFAC and PARAFAC2 methods. As such, the true underlying spectra can theoretically be determined if the correct number of components are selected, and the signal-to-noise (S/N) ratio is low (110). In both PARAFAC and MCR methods, constraints can be added to the model if prior knowledge of the system justifies constraints (110,114,128). In deconvoluting UV-Vis chromatography data arrays, constraints can be placed on the concentration, elution and absorbance modes of the data. These constraints are justified because if an analyte is analyzed, a nonnegative concentration and a nonnegative absorbance are required.
5.3 Experimental

5.3.1 Materials

Laponite-RD (LRD) was obtained from Southern Clay Product Inc (Gonzales, TX). LRD solutions were passed through a 0.45 µm cellulose acetate filter supplied by VWR (Radnor, PA). Disposable syringe filters were 0.50 µm Teflon (PTFE) membrane and supplied by Toyo Roshi Kaish (Tokyo, Japan). Sodium hydroxide with a purity >98% and purchased from Fisher Chemicals (Fair Lawn, NJ). Caffeine was HPLC grade (99% purity or higher) and oxytetracycline was (>95%) purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared in high-purity water (18MΩ · cm Millipore Milli-Q Water System, Billerica, MA). The pH of solutions were measured using a Fisher Scientific pH meter (Pittsburg, PA) calibrated with pH=7 and pH=10 buffers prior to use. All experiments were performed at a pH≈9.5. LRD concentrations throughout the experiments were held constant at 1000 mg L⁻¹ while the caffeine and OTC concentrations varied between 10 mg L⁻¹ and 200 mg L⁻¹. OTC concentrations of 200 mg L⁻¹ were the highest usable concentration of OTC on the DAD because at concentrations above 200 mg L⁻¹ the signal became saturated. A concentration of 10 mg L⁻¹ and 20 mg L⁻¹ were the lower limit concentrations for OTC and caffeine respectively. At lower concentrations, the signal-to-noise of the instrument was not sufficiently above the scatter of LRD.
5.3.2 Apparatus and Modeling

Wide-bore HDC experiments were performed using a Shimazu LC-10AT VP pump (Columbia, MD) with a constant flow rate of 300 µL min\(^{-1}\). Sample injections were 5µL in volume and injected with a Rheodyne MX Series II six-port external sample injector (Oak Harbor, WA). The capillary was a straight 0.25 mm diameter, 1.5-meter long PEEK tube (Upchurch Scientific Part No. 1581, Oak Harbor, WA). A diagram of the apparatus is depicted in Figure 20. Detection of the elution of the standards and mixtures was performed using a Shimadzu SPD-M10 AVP diode array detector (Columbia, MD). A representative matrix of the caffeine LRD mixture data collected on the DAD is shown in Figure 32.

Size exclusion chromatography (SEC) experiments were performed using an Agilent 1100 series pump (Waldenbronn, Germany) with a constant flow rate of 500 µL min\(^{-1}\). Sample injection volumes were 10µL and injected with an Agilent 1050 pneumatic auto sampler (Waldenbronn, Germany). Phenomenex, (Torrance, CA), BioSep-SEC-S 2000 300 x 7.80 mm size exclusion column with a 3x10\(^{-5}\) Da exclusion limit was used for particle separation. Samples were detected by ultraviolet (UV) absorbance using an Agilent 1100 series multi-wavelength detector (Waldenbronn, Germany). Computing of the MCR, PARAFAC, and PARAFAC2 models were performed using PLS_Toolbox (Wenatchee, WA) for MATLAB (Natick, MA). All models were constricted to nonnegativity constraints in the concentration and spectral modes.
5.4 Results and Discussion

5.4.1 Caffeine and Laponite-RD using MCR Methods

Caffeine association with LRD was first modeled using MCR with two principal components, one for the caffeine associated with LRD and the other for the freely eluting caffeine. MCR was a first approach in determining the peak shapes of the eluting caffeine and LRD analytes in wide-bore HDC chromatography. In Figure 32 the leading peak from the caffeine and LRD interaction is apparent at approximately 10 seconds into the run. The scattering affect of LRD is observed at about 10 seconds and is apparent over the entire collected wavelength region shown in Figure 33.
Figure 32 Caffeine and LRD mixture injection detected on a DAD.
A series of caffeine and LRD mixtures were injected; where the LRD concentration was held constant at 1000 mg L\(^{-1}\) and the caffeine concentration was varied between 20 mg L\(^{-1}\) and 200 mg L\(^{-1}\). In addition to mixture injections, pure caffeine samples were injected to obtain a 5-point calibration curve. The calibration curve was used to determine the concentration of free caffeine in solution and the concentration of caffeine associated with LRD in the same manner as the univariate approach in Chapter 4. Using the MCR method, pure concentration elution profiles are found with minimal assumptions of the experimental conditions. For the evaluation of these data with MCR
no assumption was made on the spectroscopic absorption of the caffeine-LRD complex. The only assumption when using MCR to deconvolute the spectra was the number of pure components in the mixture that would correspond to the number of principal components selected.

Spectral loadings, sometimes referred to as spectral profiles, of a mixture injection and a pure injection are shown in Figure 34. The loadings contain intensity ambiguity that is inherent in MCR and the magnitudes of their peaks are not useful for qualitative analysis (102,104,125). However, a qualitative explanation of the intensity loadings is important. At lower wavelengths, the mixture spectrum shows higher absorbance than the pure caffeine spectrum. The higher absorbance of the mixture at the lower wavelengths is explained by the scattering of LRD, and as such, the mixture absorbance is higher than the free caffeine at lower wavelengths. There is also a shift in the absorbance maximum around 270 nm between the mixture spectrum and the pure caffeine spectrum. The shift is not explained easily since previous experiments have indicated there is not a spectral absorbance shift between caffeine and LRD mixtures as is shown in Figure 26. Thus, it is likely an artifact of the spectral deconvolution.
A normalized spectrum to maximum peak intensity of pure caffeine from a UV-Vis detector is overlaid as a verification; the loadings accurately represent a pure caffeine spectrum shown in Figure 35. If the spectra were the same it would be an indication the MCR methods are reconstructing the freely eluting caffeine. However, the spectra do not match in shape, and the relative intensity of the reconstructed loadings at 210nm and 270nm do not match the relative intensity of the caffeine spectrum taken from the UV-Vis spectrometer.
Figure 35  Loadings of a caffeine LRD mixture using MCR and caffeine UV-Vis spectra normalized to peak maximum.

Elution scores (elution profile) of a mixture injection are shown in Figure 36. The elution scores show an asymmetrical fronting peak from caffeine associated with LRD and a more symmetrical peak from the free caffeine eluting later in time. Unfortunately, from the reconstructed elution profiles, there is not enough of a HDC separation between the fronting peak and lagging peak for either to be freely eluting. The presence of one analyte eluting by itself would be enough to eliminate the rotational ambiguity that exists with the scores plot, and the actual elution profile could be obtained (102,104,105,125).
One possible explanation for the deconvolution discrepancy might be desorption of caffeine from LRD during the wide-bore HDC separation. In the pure caffeine scores plot, there is a small leading foot at approximately 25 seconds that could be attributed to a portion of the caffeine associated LRD that is desorbed from LRD but has the same profile of pure caffeine. Similarly, in the mixture elution profile there is a trailing peak at approximately 50 seconds that is directly under the peak maximum of the free eluting caffeine peak. Again, this could be attributed to a portion of caffeine associated with the
LRD that is desorbed from the LRD at the time of detection, and as a result it appears as an increase in signal under the caffeine maximum.

Once the scores and loadings are calculated for the mixture run, the data are reconstructed into the scaled flow profile for the mixture. The reconstructed flow profile is shown in Figure 37, and is calculated by multiplying the scores by the loadings, or the elution profile by the spectra. When the scores and loadings are multiplied to create the reconstructed flow profiles, the tailing foot on the mixture flow profile is minimized.

![Reconstructed Flow Profiles Using MCR](image)

**Figure 37** Reconstructed flow profile of caffeine and caffeine LRD mixture.
The elution profile of the mixture tails asymmetrically, which is what one would expect from the known theory of wide-bore HDC of large particles (82,89). The pure caffeine peak still has the leading foot on the flow profile that matches in time with the LRD mixture. While these profiles are similar to the ones obtained with the univariate method, the caffeine flow profile, however, is asymmetric and fronts slightly. This result is unexpected and does not fit the theory of wide-bore HDC and the expected Gaussian peak shape of a small molecule. It also, does not match the elution profile of pure caffeine. Thus, because of inherit intensity and rotational ambiguities in the reconstructed flow profiles the prediction from MCR cannot be taken as a quantitative representation of the true flow profile, but only as a qualitative representation. The qualitative reconstructed flow profiles give important visualization of the two different species in solution. Because the MCR analysis was not quantitative PARAFAC methods were subsequently used evaluate the DAD data.

5.4.2 Modeling Caffeine and Laponite-RD Interactions using PARAFAC Methods

Modeling the caffeine LRD interaction with PARAFAC was thought to be a better method than MCR for one primary reason. In PARAFAC, an additional dimension is added that can be used to include multiple samples of varying concentrations into the data array. This increase from a two-way data array in MCR to a three-way, or multi-way, data array in PARAFAC results in minimizing, if not eliminating the intensity and rotational ubiquity that is inherent in MCR (110,129). In PARAFAC analysis, the
absorbance and flow profiles are appropriately scaled relative to each analyte in the multi-way data array. The data arrays are built with absorbance versus elution time, as in MCR analysis, with multiple runs of the samples and varying concentrations comprising the third dimension.

In the PARAFAC analysis, the third dimension consisted of 20 individual runs of 5 different caffeine concentrations run in multiples of 4. The data in PARAFAC analysis was constrained to 2 principal components as in MCR: one for the mixture of caffeine and LRD and the other for the freely eluting caffeine. A sample of the reconstructed spectra of caffeine and the mixture is shown in Figure 38. From the reconstructed spectra, the similarities of the two species in solution are apparent. As with MCR analysis, the caffeine LRD mixture exhibits scattering at the lower wavelengths, but the spectrum matches in shape with the pure caffeine spectrum and the shift that was observed around 270 nm in the MCR analysis is no longer present. The mixture spectrum also has a higher absorbance than the caffeine spectrum above 300 nm, and this is explained by the scattering of LRD and is observed in the DAD array in Figure 33.
Figure 38 Absorbance spectral loadings of caffeine and caffeine LRD mixtures using PARAFAC methods.

However, when the reconstructed spectra are normalized to their respective peak maximums and compared to a pure caffeine spectrum in Figure 39, their spectra do not match in shape or magnitude. The pure UV-Vis spectrum of caffeine has a larger difference in the relative magnitude of the two maximum peaks, where the peak in the low UV region is much larger relatively than the peak at 271nm. The reconstructed spectra, however, has peaks that are not as different in relative magnitude. The discrepancy could originate from the high scatter of LRD at low wavelengths.
Regardless of the origin, this is indication the model is not reconstructing the freely eluting caffeine accurately.

Figure 39 Normalized, to peak maximum, spectral loadings of a caffeine LRD mixture using PARAFAC and caffeine UV-Vis spectra.

The reconstructed flow profiles of a sample data set are shown in Figure 40. The flow profile of the mixture has the definitive fronting peak from the earlier eluting LRD, but it is not fully deconvoluted and separated from the tailing free caffeine. The free eluting caffeine flow profile still has an asymmetrical peak shape from the fronting of the
flow profile. These results from PARAFAC method are not expected, if a freely eluting peak from caffeine is anticipated, and the true flow profiles of the analytes in solution. PARAFAC deconvolution methods are, however, traditionally used in static systems where elution profiles are univariate, or in other words, the elution time of the analytes do not change (109,112,128). While PARAFAC removed some of the limitations of MCR; the method was not well suited for the system where elution times shift or change. Thus, a modified version of PARAFAC, PARAFAC2, was used to evaluate the caffeine and LRD mixtures.
5.4.3 Caffeine and Laponite-RD using PARAFAC2 Methods

A final attempt to deconvolute the caffeine LRD mixtures was preformed using PARAFAC2 methods. This second generation multivariate analysis method was developed for multi-way data where variations in the elution mode are present, and it has been successfully applied to convoluted GC-MS and HPLC-DAD data (109,112,128). Absorbance spectra of the loadings using PARAFAC2 methods are shown in Figure 41. The spectra show the high scatter of LRD and the peak shape of a pure caffeine spectrum.

Figure 40 Reconstructed flow profile of caffeine and caffeine LRD mixture using PARAFAC methods.
This is an indication the model is deconvoluting LRD and caffeine, and not a mixture of the two.

**Figure 41** Absorbance spectral loadings of caffeine and a mixture of caffeine and LRD using PARAFAC2 methods.

Caffeine loadings have nearly the same general shape as the caffeine spectra taken on a UV-Vis instrument and from a PARAFAC2 reconstructed loadings of a pure caffeine elution. The spectra are normalized to their respective peak maxima and shown in **Figure 42**. The absence of changes in the spectrum is further indication that the mixture and pure caffeine have the same absorbance as the data in **Figure 26** show.
Figure 42 Normalized, to peak maximum, reconstructed PARAFAC2 loadings of pure caffeine and caffeine LRD mixtures. The two loadings are nearly identical in shape indicating there is not a change in the molar absorptivity of the caffeine in mixtures. A normalized UV-Vis spectrum of caffeine is also overlaid to show the similarities in shape.

However, the shape of the normalized loadings and UV-Vis data are much closer in shape and relative magnitudes of the two absorption maxima. This is an indication PARAFAC2 methods are better at reconstructing the data. However, without a shift in absorbance from bound caffeine PARAFAC2 is not able to model and deconvolute the data. If PARAFAC2 were able to model the mixture data, there should be a large
asymmetrical flow profile from caffeine associated to LRD and a more Gaussian flow profile from freely eluting caffeine later in time.

In the mixture spectrum in Figure 41, there is a large absorbance at lower wavelengths where LRD scatters to the greatest extent, and the scattering from LRD is high over all wavelengths. This is consistent with what is observed in DAD array in Figure 33. In addition to LRD causing the scattering and high absorbance, it is possible a change in the refractive index of the sample when the injection is reaching the detector is causing the high absorbance at low wavelengths. If the latter is the case, it is analogous to a change in the refractive index caused by void volumes in HPLC experiments.

The reconstructed flow profiles are shown in Figure 43. The flow profile of caffeine is similar to the caffeine flow profile that is observed before deconvolution and shown in Chapter 4 Figures. There is a large leading shoulder from a fraction of caffeine associated with LRD and a tailing peak from the free caffeine eluting later in time. The mixture or LRD flow profile is similar to the LRD flow profile that is observed when a pure injection of LRD is run. A small early eluting peak appears under the leading shoulder of the caffeine flow profile, and is show in Figure 22 in Chapter 4. The two reconstructed flow profiles have a leading peak from caffeine associated with LRD, an indication of an interaction between LRD and caffeine. However, PARAFAC2 was unable to deconvolute caffeine bound and caffeine free. The most likely reason for the
inability of PARAFAC2 to deconvolute the peaks is due to an absence of a spectral shift between bound and free caffeine.

Figure 43 Reconstructed flow profiles of caffeine and caffeine LRD mixture using PARAFAC2 methods.

It appears that PARAFAC2 method was only capable of deconvoluting pure LRD and caffeine free and associated as the only other species present. The failure of the PARAFAC2 method is attributed to the spectral loadings of the caffeine LRD mixture not changing. If the spectrum of the mixture is the same as pure caffeine, the multivariate analysis methods used for deconvolution will not be able to model two distinct flow
profiles. There are however, two different spectra in the experimental run and PARAFAC2 analysis does model deconvoluted LRD and caffeine as evident in Figure 41 and Figure 43.

In addition to modeling two components, caffeine and LRD mixtures were also analyzed with PARAFAC2 using three principal components. Three principal components were used under the assumption there were flow profiles for free LRD, free caffeine and for caffeine associated with LRD. The spectral loadings for the three component PARAFAC2 deconvolution are shown in Figure 44. In the three component deconvolution, the first principal component is the high scattering LRD as was observed in the two component deconvolution, and the other two loading spectra are similar the pure caffeine. However, the third principal component spectrum goes to zero at about 205nm. Thus, it is most likely an artifact of the model and does not fit with UV-Vis absorption of LRD or caffeine.
Figure 44 Spectral loadings of caffeine and LRD on three principal components using PARAFAC2 methods.

When the reconstructed concentration profiles of the three component deconvolution are interpreted, Figure 45, the results are similar to the two component system, where there is a flow profile of LRD eluting earlier in time under a leading shoulder of the caffeine flow profile. The second and third principal components are nearly identical in shape and a result of the PARAFAC2 model’s and indicates PARAFAC2 is not able to model a three component system.
In summary the lack of a change in the absorbance spectra of caffeine associated with LRD and freely eluting caffeine, and as such, the PARAFAC2 models inability to distinguish freely eluting caffeine from caffeine associated to LRD. However, the model did support the deconvolution of a pure LRD scatter and caffeine interacting with LRD. It was thought the univariate approach from Chapter 4 might be sufficient for determining the association between caffeine and LRD.

Figure 45 Reconstructed flow profiles of caffeine LRD mixtures on three principal components using PARAFAC2 methods.
5.4.4 Univariate Curve Resolution from DAD data.

Due to the short comings of the multivariate analysis methods, where MCR had rotational and intensity ambiguity, PARAFAC methods could not model changes in retention time, and PARAFAC2 methods were unable to model freely eluting and associated caffeine due to an absence of a change in absorption, EMG deconvolution was used to model the data. The univariate methods from Chapter 4 were used to model caffeine’s association with LRD and to compare the differences between the association of caffeine at 1000ppm and 3000ppm (m/m) LRD concentrations. The elution profile from the 271nm channel on the DAD data was extracted for univariate analysis methods. This comparison was performed to assess the differences between 3000ppm LRD samples in Chapter 4 to the 1000ppm LRD samples used on the DAD collected data. One conclusion from Chapter 3 results was that the association between caffeine and LRD may not be uniform over all LRD concentrations, and as a result, the HPLC method was unable to accurately determine the $K_D$ of caffeine associated with LRD.

Comparisons of the isotherms from 3000ppm and 1000ppm (m/m) LRD concentrations are shown in Figure 46. The univariate deconvolution methods from the 1000ppm LRD DAD data have a higher $K_D$, $550 \pm 50 \text{ L Kg}^{-1}$, indicating a stronger association, than the 3000ppm LRD univariate calculated $K_D$, $140 \pm 10 \text{ L Kg}^{-1}$, from Chapter 4. One possible explanation for these observations is that the stronger adsorption is likely due to the smaller concentration of LRD that give more possible binding sites for caffeine. A more concentrated LRD solution would have LRD intraparticle self assembly interactions that
would compete with caffeine for binding sites. This would result in a smaller caffeine LRD $K_D$ value. These intramolecular interactions are assumed to be negligible in the inclusion complex model with cyclodextrins in Chapter 3 that was applied to HPLC data. However, these interactions might not be negligible in the nanoparticle system, giving more validity to why the HPLC methods were inadequate.

![Comparison of the LRD Concentration Effect on Isotherms](image)

Figure 46 Comparison of the affect of changing LRD concentration with the effective isotherm of caffeine.
5.4.5 Oxytetracycline and Laponite-RD using PARAFAC2 Methods

Due to the lack of change in the caffeine LRD mixture absorbance spectra another molecule that would change absorbance when in the presence of LRD was selected to evaluate the deconvolution methods. OTC was selected as a possible molecule that would interact with LRD under certain pH conditions and therefore exhibit a partial HDC separation Figure 47. The OTC LRD mixtures were initially adjusted to a pH ≈ 9.5 to maximize the stability of LRD solutions. At this pH, OTC has two negative charges, and therefore and was not initially thought to interact with the negative surface of the LRD. However it has been shown to interact with sodium montmorillonite at these high pH conditions and therefore was thought to possibly interact with LRD (66). It has also been documented that OTC solutions at high pH are not stable and degrade (66).
Figure 47 OTC and LRD mixture injection detected on a DAD after 4 days of equilibration.

When solutions were initially prepared, mixed and ran on the wide-bore HDC apparatus, a small fronting shoulder from the OTC and LRD was observed. However, after allowing the solutions to equilibrate over 4 days, and subsequently ran on the wide-bore HDC apparatus, the more pronounced fronting shoulder was observed indicating a stronger OTC interaction with LRD Figure 48. These observations implied there was a kinetic effect between OTC and LRD or LRD was interacting with OTC degradation products. This conclusion was reached based on the understanding that OTC is not stable at high pH, pure OTC would have a negative charge like LRD, and the more pronounced
fronting peak from a 4 day equilibrium period. Thus, it was not apparent the observed interaction, (fronting peak on the wide-bore HDC apparatus), was due to LRD interactions and not to OTC degradation products. To test whether a kinetic effect or degradation products of OTC were interacting with LRD a solution of OTC at pH ≈ 9.5 was kept for 4 days before mixing with LRD and subsequently ran on the wide-bore HDC apparatus. When this was performed, the fronting peak from a 4-day old solution of OTC prior to mixing with LRD was not as pronounced as a 4-day old mixture. This implies a kinetic and degradation affect was both contributing to the OTC LRD interaction. A kinetic affect because OTC and LRD had a larger fronting peak when solutions were allowed to equilibrate over 4 days, and a degradation affect because a 4 day old solution of OTC mixed with LRD did not display a pronounced fronting peak.
Figure 48 OTC LRD mixtures and pure OTC before and after a 4 day equilibration period. The fronting peak from LRD OTC mixtures is much more pronounced after a 4 day equilibration period. OTC concentration is 100ppm throughout and LRD concentration is 1000ppm. The flow profile was taken from the 328nm DAD channel.

To ensure the OTC degradation products were not present in the mixture injections on the wide-bore HDC apparatus and contributing to the observed fronting peaks, a sample OTC LRD mixture was passed though a 0.45 μm filter. All of the OTC LRD mixtures were not passed through a filter because OTC was observed on the filter after use. A lose of OTC to the filter would cause a discrepancy in the amount of OTC associated with LRD and OTC free in solution. Instead the solutions were not agitated,
and the mixture supernatant was taken from the top of the vials to ensure there was no precipitate entering the wide-bore HDC apparatus.

Deconvolution of OTC LRD solutions after a 4-day equilibrium period was attempted using two principal components by the PARAFAC2 method. The spectral loadings are shown in Figure 49. The spectral loadings of the mixture are similar in shape to the loadings of OTC; however, there is variance in the absorbance at lower wavelengths. The pure UV-Vis spectra of OTC is overlaid on the spectral loadings plot, and the pure OTC spectrum is different in shape, and the absorption maximum is blue shifted when compared to the spectral loadings of the OTC and OTC LRD mixture. The pure OTC and the free OTC from LRD mixtures cannot be assumed to have the same spectrum because of the degradation that occurs when LRD is at a higher pH and these degradation products could be causing the changes in absorption.
Figure 49 Spectral loadings of OTC LRD mixture, OTC and an overlay of an UV-Vis OTC spectrum. The spectral loadings were found using PARAFAC2 methods.

The reconstructed elution profile of a 4-day OTC LRD mixture is shown in Figure 50. The elution profile of the mixture elutes before the OTC flow profile and it has a distinct asymmetrical shape. Around 45 seconds the mixture flow profile has a slight increase in intensity; however, the increase in intensity does not match with the flow profile maximum of OTC. The reconstructed flow profile of OTC is asymmetrical as well, with a leading shoulder that matches with the mixture flow profile. The two asymmetrical peak shapes from the reconstructed data indicate the PARAFAC2 may not be modeling the data completely. Since there was a possible added complexity of the
mixtures from OTC degradation products a separation of OTC LRD mixtures was performed using size exclusion chromatography.

Figure 50 Reconstructed flow profiles of OTC and an OTC LRD mixture using PARAFAC2 methods.
5.4.6 Oxytetracycline and LRD in Size Exclusion Chromatography

The observations of the association of OTC and LRD were investigated using size exclusion chromatography. A pure solution of OTC, a pure solution of LRD, and a mixture of OTC and LRD were injected on a size exclusion column after a two-week equilibrium period. Overlays of the chromatographs are shown in Figure 51 and separated chromatographs of are shown in Figure 52 and Figure 53. An injection of LRD on to the SEC column is the first to elute at 4 minutes because of the relatively large size of the nanoparticle compared to the solutes. An injection of pure OTC during the first week has two peaks, and the leading peak has a slight leading shoulder Figure 52. When a mixture of OTC and LRD are injected during the first week, a peak larger than pure LRD elutes over the pure LRD peak at 4 minutes Figure 53.
Figure 51 Separations of OTC LRD mixtures on a size exclusion chromatography column. By the second week, the magnitude of the leading OTC peak from an association of LRD grows in size and elutes under the large LRD peak. The pure OTC separation always elutes under the LRD peak.
Figure 52 Separations of OTC LRD mixtures on a size exclusion chromatography column. The interaction of between OTC and LRD are apparent by the larger signal of the leading mixture peak compared to the smaller LRD peak.
Figure 53 The OTC LRD interaction is greater by the second week as evident by the large leading signal of the mixture above the pure LRD signal.

After allowing the solutions to equilibrate for a week, an injection of pure OTC shows the shoulder from the leading peak has grown in size and the tailing peak that eluted at 7 minutes is absent, however, a peak at 4 minutes where LRD elutes is still absent. However, the mixture of OTC LRD at week two has a large peak that elutes at the same time as LRD at 4 minutes. The absence of a pure OTC peak at 4 minutes and the increase of the OTC LRD mixture peak at 4 minutes from week 1 to week 2 indicate there is a kinetic effect between the association between degradation products of OTC and LRD Figure 51. Furthermore, the SEC separation indicates the kinetics of
desorption are slower than time of separation during the SEC experiments. If the kinetics of desorption were faster than the SEC experiments or on the timescale of the SEC experiments, the peak at 4 minutes would have the same peak height as LRD because LRD would separate from the OTC during the SEC separation.

The mixture of OTC and LRD on SEC separations had 4 distinct peaks, one that elutes with LRD at 4 minutes, and an unresolved double peak around 5 minutes and a final OTC peak at 7 minutes. Since the SEC experiments showed 4 distinct peaks, the PARAFAC2 method of deconvolution of the wide-bore HDC separation of OTC and LRD mixtures was performed using 4 principal components, for the 4 peaks observed in the SEC separation.

5.4.7 Oxytetracycline and Laponite-RD using PARAFAC2 Methods and 4 Principal Components

The reconstructed flow profiles of OTC and LRD mixtures with PARAFAC2 methods and 4 principal components in the wide-bore HDC apparatus are shown in Figure 54. The first principal component does elute earlier in time than the other three, but it also has the second tailing peak around 40 seconds that is similar the reconstructed flow profiles on two principal components shown in Figure 50. The second principal component is symmetrical and elutes later in time compared to the first principal component, and it could be the free OTC. The third principal component reconstructed data does elute earlier in time and exhibits an asymmetrical peak shape. The fourth
principal component is similar in shape with the second principal component but elutes slightly earlier in time and has a slightly asymmetrical peak shape. The PARAFAC2 model breaks down because of the complex nature of the OTC mixture, and the degradation products of OTC that are present in solution and evident by the SEC experiments.

Figure 54 Reconstructed flow profiles of OTC LRD mixtures on 4 principal components using PARAFAC2 methods.
5.5 Conclusions

There are obvious limitations using multivariate methods for data deconvolution. First, all modeling techniques in deconvoluting wide-bore HDC experiments are limited because of the qualitative nature of MCR from the intensity and rotational ubiquities. As such, PARAFAC methods, a multi-way technique, are better because of their quantitative results. However, PARAFAC is reserved for static environments where elution profiles do not change from run to run. The best suited multivariate analysis method is PARAFAC2 because it can accommodate changes in elution times from run to run. However, without a change in spectral absorbance between the two eluting analytes, the model cannot deconvolute the data matrix and the method finds LRD and caffeine in the solution mixture and not the associated species. Thus, is believed the univariate methods from Chapter 4 are the best deconvolution methods for data that does not change in absorption. OTC LRD mixtures were difficult for PARAFAC2 methods to model because the kinetics of adsorptions of OTC were not well understood. Furthermore, the SEC experiments indicate there were at least 4 different species in solution.
Chapter 6 Investigating Chemical Interactions between Laponite-RD and Quinoline Drugs

6.1 Introduction

Quinine is a small organic molecule that was predicted to interact with LRD and could be used as an additional drug to evaluate the model development reported in Chapter 4. It was theorized quinine might interact electrostatically with suspended LRD through the positively charged quinoline moiety, shown in Figure 6. Thus, obtaining the magnitude of interaction between the two might be possible. However, when LRD and quinine were injected in the wide-bore HDC apparatus, significant tailing occurred as shown in Figure 55. Thus, the interaction as described for caffeine and LRD could not be quantified. Despite the observed tailing in the chromatographs, an interaction between quinine and LRD was readily apparent. The tailing in the chromatographs most likely resulted from precipitation that occurs between the LRD and quinine when it is a cation in solution. This confounded the attempt to model the mixture in the wide-bore HDC apparatus. When investigating the potential reasons for the observed separation, it was discovered that quinine interacted with the suspended clay mineral in a manner that was unexpected.
Fig. 55 Quinine and LRD in the wide-bore HDC apparatus. The leading peak in the mixture chromatographs shows there is an interaction between the quinine and LRD that can be partially separated in the apparatus. However, the large tailing in the chromatographs makes modeling and quantification difficult.

LRD is used in over-the-counter consumer products such as, paint, cosmetics, and toothpaste, as a rheology additive to increase the viscosity of and stabilize solutions (27). Southern Clay Products, the manufactures of LRD, list LRD as a nontoxic substance on their brochure and MSDS (material safety data sheet) (27,130). The Occupational Health and Safety Administration (OSHA) has set the permissible exposure limit (PEL) to $5 \text{ mg m}^{-3}$ for respiratory fractions and $15 \text{ mg m}^{-3}$ for total dust exposure. These limits reflect the small size of LRD and its potential to infiltrate deep into lung tissue.
Furthermore, like all clay minerals, LRD may capable of transporting pollutants and catalyzing molecules due to its surface interactions with other molecules. The research presented shows a chemical change to quinine, a pharmacological compound, when in the presence of LRD. This chemical interaction highlights the potential for LRD to interact and change the chemical nature of organic molecules in the environment.

6.2 Experimental

6.2.1 Materials

LRD was obtained from Southern Clay Product Inc (Gonzales, TX). Quinine monohydrate had a purity of 90% and was purchased from Aldrich Chemical Company (Milwaukee, WI). 6-Methoxyquinoline (6MQ) was purchased from Aldrich Chemical Company (St. Louis, MO). Sodium hydroxide was HPLC grade with a purity > 98% and purchased from Fisher Chemicals (Fair Lawn, NJ). Ammonium nitrate > 99.9% purity was purchased from Mallinckrodt Baker Inc. (Paris KY). All solutions were prepared in high-purity water (18MΩ ⋅ cm Millipore Milli-Q Water System, Billerica, MA). 6MQ and quinine were kept at a concentration of 10µM throughout the experiments and LRD concentration was 100 ppm (m/m).

6.2.2 Solution Preparation

LRD stock suspensions were prepared by dissolving 1.0 g of LRD in 90 mL of water. The suspension was stirred overnight to allow complete hydration of the LRD
particles. The LRD suspension was pH corrected using 1M NaOH; the suspension was brought to a 100 mL final volume resulting in a final concentration of 1% (w,w). The final pH of the LRD stock suspension was corrected to a pH ≈ 10 to avoid degradation or dissolution of the LRD (35). LRD suspensions that were below a pH = 10 were prepared without the addition of NaOH and those suspensions were used within 5 days of preparation to ensure LRD stability. All LRD suspensions were stored at room temperature in sealed glass bottles. Quinine solutions were 0.1 mM and 6-Methoxyquinoline solutions were 0.15 mM unless otherwise noted, and both were stored at room temperature in dark glass vials to minimize degradation from UV light.

6.6.3 UV-Vis and Fluorescence Experiments

UV-Vis experiments were performed using a Thermo Electron Corporation Evolution 300 BB UV-Vis double beam spectrophotometer (Loughborough, England) scanning from 250-400 nm with a 2 nm bandwidth. Fluorescence experiments were performed using a Varian Cary Eclipse Fluorescence Spectrophotometer (Santa Clara, CA). Samples were excited at 300 nm and 320 nm and fluorescence intensity was recorded from 320-550 nm and 350-550 nm respectively. All experiments were performed in disposable, micro-volume PMA cuvettes supplied by Sigma Aldrich (Saint Louis, MO).
6.3 Results and Discussion

The chemical structures of quinine and 6-methoxyquinoline (6MQ) are shown in Figure 6. The two molecules have the same quinoline ring that is the bioactive moiety of the molecule as it reacts with heme complexes (74,131). Quinine has two acidic protons, one on the quinuclidine moiety with a pKa = 8.43 and the other acidic proton on the quinoline ring where the pKa = 4.34. The quinoline ring on 6MQ has a pKa = 5.13 (132).

When quinine LRD mixtures were injected on the wide-bore HDC apparatus there was significant tailing of the chromatographs as shown in Figure 55. In addition to the tailing of the flow profiles the peak areas were not conserved. The peak area for a 0.1 mM quinine solution shown in Figure 55 was 2130 a.u. sec., however, when the same concentration of quinine was mixed with 100 ppm and 1000 ppm LRD, the peak areas were 2247 and 1761 a.u. sec., respectively. The change in peak area could have been attributed to precipitation of the mixtures or a change in the molar absorptivity of the mixture. Hence, the quinine LRD mixtures were investigated in a UV-Vis instrument to see if a change in the molar absorptivity was responsible for the loss in peak area and the spectra are shown in Figure 56. The two spectra at pH = 10.2 show there is not a change in the molar absorptivity of the mixture, although the solution pH was above the pKa of quinine and an interaction between the two would be minimal. At pH = 7.2 there was a slight decrease in the absorbance from an observed precipitation of the mixture when the quinine cation exchanges onto the surface of LRD. When the spectra are normalized,
there is a decrease in absorbance intensity at the lower wavelengths; however, the shape of the absorbance spectra does not change. When quinine is at pH < 4.34, and quinine is a doubly charged dication, there is a large observable change in the absorbance spectra shown in Figure 57.
Figure 56 UV-Vis absorption spectra of 1.0E-4M quinine and 100ppm LRD mixture at pH=10.5 (a). The normalized spectra (b)
Figure 57 UV-Vis absorption spectra of 1.0E-4M quinine and a 100ppm LRD mixture at pH=7.2 where quinine is a cation and a 1.0E-4M quinine sample at pH=2 where quinine is a dication (a). Normalized spectra of quinine and a LRD mixture at pH=7.2 (b).
Due to the minimal change in the UV-Vis spectra, the mixtures were subsequently investigated by fluorescence spectroscopy to see if a change in the fluorescent emissions were occurring. **Figure 58** shows the emission spectra and normalized emission spectra of quinine and quinine LRD mixtures. Quinine was first measured as a cation at pH = 7.5 where the measured emission wavelength maximum, $\lambda_{\text{max}} \approx 380$ nm. However, a mixture of quinine and LRD at pH = 7.5 had a measured $\lambda_{\text{max}} \approx 450$ nm, this corresponds to a red shift of 70 nm. This large and unexpected fluorescence shift is attributed to the interaction between quinine and LRD. The shift in fluorescence emission to $\lambda_{\text{max}} \approx 450$ nm is the same $\lambda_{\text{max}}$ that is observed when quinine is a dication at pH < 4.34. The pH of the quinine LRD mixture was raised to pH = 8 and pH = 10 where quinine is near pKa$_2$ and a neutral molecule, respectively. At pH = 8, the shift in fluorescence emission to $\lambda_{\text{max}} \approx 480$nm is still observed, but less pronounced, and the fluorescence emission to $\lambda_{\text{max}} \approx 380$nm is observed due to a decrease in the quinine LRD interaction and quinine fluorescing as a cation. At pH = 10, the quinine LRD interaction is lost due to the neutral charge on quinine and the fluorescence emission is observed at $\lambda_{\text{max}} \approx 380$ nm.
Figure 58 The fluorescence spectrum (a) of quinine and LRD shows the emission $\lambda_{\text{max}}$ of quinine changes from $\lambda_{\text{max}} \approx 380$ nm to $\lambda_{\text{max}} \approx 450$ nm indicating a chemical change of quinine; the same shift is observed when quinine is in acid conditions below pH = 3 and in the quinine ammonium nitrate fluorescence experiments. The shift in the emission $\lambda_{\text{max}}$ is further highlighted in the normalized fluorescence spectrum (b).
Thus, the observed shift in the emission spectra can possibly be attributed to the proton rich edge sites of the LRD clay mineral are lower in pH than the bulk solution and this causes the quinine LRD mixture to fluoresce as if quinine is a dication at pH < 4.34. This would be at almost 4 orders of magnitude difference in the local pH of the LRD surface relative to the bulk solution; thus this explanation is unlikely. Another explanation for the shift is fluorescence could be from an excited state pKa shift of quinine and the corresponding excited state lifetime of quinine is long enough to accept a proton from the edge sites of the clay mineral.

A shift in quinine fluorescence has been reported in literature in a sol-gel environment (133). Quinine was mixed in a sol-gel solution (tetraethoxysilane) and the fluorescence of quinine was measured over 14 days. During the sol-gel transformation the fluorescence of quinine gradually shifted from 370 nm to 436 nm over the 14-day period, a similar shift was observed between quinine and LRD. The authors attributed the shift to a conformational change in quinine as the sol-gel began to condense and eventually allowed for intramolecular hydrogen bonding of the quinoline nitrogen (133). In the quinine LRD system, the LRD concentration is too low for a sol-gel to form, and as such, there is not believed there is a hydrogen bond forming between LRD and quinine. A more likely interaction between quinine and LRD is though the positively charged quinuclidine moiety. Thus, further experiments were needed to explain the observed shift in fluorescence.
The interaction between quinine and LRD was thought to occur through the quinuclidine moiety on quinine that is protonated at pH > 8.43. To verify this was the point of interaction, 6MQ was selected as a control. 6MQ does not have the quinuclidine moiety and therefore should not interact with LRD. Fluorescence emissions of 6MQ and 6MQ LRD mixtures are shown in Figure 59. The fluorescence emission maximum of 6MQ when a neutral molecule has a $\lambda_{\text{max}} \approx 430$ nm and a second emission peak a $\lambda \approx 360$nm. When 6MQ is in strongly basic conditions at pH = 12 the emission maximum $\lambda_{\text{max}} \approx 360$ nm and when 6MQ is a cation the emission maximum $\lambda_{\text{max}} \approx 430$ nm. The introduction of LRD into solution does not change the emission maximum of 6MQ and the emission is the same as 6MQ in neutral water. This indicates there is no interaction between LRD and 6MQ and as such gives further evidence that quinine is interacting with LRD through the quinuclidine moiety when it is positively charged. The interaction through the quinuclidine alone would not cause a fluorescence shift of quinine because the shift in fluorescence occurs when the quinoline moiety is protonated. Therefore, another mechanism to describe the shift in fluorescence was needed.
Figure 59 Fluorescence spectrum of 15µM 6MQ in: pure water, pH = 3 adjusted water, pH = 12 adjusted water, and a mixture with 5000ppm LRD. Unlike quinine, LRD does not affect the 6MQ magnitude and the \( \lambda_{\text{max}} \) emission is identical to 6MQ in water.

A literature search revealed ionization constants for molecules in the excited state may differ by several orders of magnitude from the same molecules in the ground state (134). Hence, fluorescent molecules may have a different pKa in their excited state than their ground state. It was thought that an excited state pKa shift could explain the change in the quinine LRD fluorescence emission. If there are proton donating molecules in solution and the fluorescent molecule has an excited state lifetime long enough to accept a proton from the donor, the fluorescing molecule will emit a photon as if it was
protonated in the ground state. This is because the excited state molecule has an effectively different pKa in the excited state than in the ground state. A change in the excited state pKa has been observed with acridine in the presence of ammonium nitrate (93,134,135).

When acridine is a neutral molecule (shown in Figure 60), the emission wavelength is 410 nm, however, when acridine is a cation the emission wavelength is red shifted to 560 nm. Acridine has a ground state pKa = 5.45.

![Figure 60 The molecular structure of acridine. Acridine has a pKa associated with the cyclic nitrogen ring.](image)

When acridine is dissolved in a 2M solution of ammonium nitrate at a pH = 8.3, acridine undergoes an excited state pKa shift and accepts a proton of the ammonium nitrate and fluoresces as a cation at 560 nm, despite a solution pH that is 3 orders of magnitude larger than the pKa of acridine in the ground state (93,134,135). The shift in fluorescence from the acridine experiments was a result of an excited state pKa shift in acridine allowing a proton transfer to occur from the ammonium nitrate to acridine. In aqueous conditions, the excited state lifetime is too short to allow a proton transfer from the water solvent to acridine. However, if ammonium nitrate is added to the solution, the
molecules in their excited state are protonated from the ammonium ions in solutions and a shift in the fluorescence emission is observed. It was thought a similar pKa shift was occurring between quinine and LRD and a set of experiments were performed modeled after the excited state pKa shift observed between acridine and ammonium nitrate.

Published acridine experiments were used as a model to design an experiment demonstrating quinine and 6MQ may undergo a similar excited state protonation due to a change in their excited state pKa. 6MQ in the presence of ammonium nitrate displays a shift in fluorescence from 370 nm to 440 nm despite a solution pH that is higher than the ground state pKa Figure 61.
Figure 61 Fluorescence spectra of 15µM 6MQ in: pure water, pH = 3 adjusted water, a mixture with 1.0M ammonium nitrate pH = 6. The lower pH has a $\lambda_{\text{max}} \approx 440 \text{nm}$, and the neutral solution where 6MQ is deprotonated, has a shoulder at 370nm. The 6MQ and ammonium nitrate mixture with a pH = 6 has a shift in fluorescence $\lambda_{\text{max}} \approx 440 \text{nm}$ similar to 6MQ in an acidic environment and the shoulder at 370nm is absent.

The same type of fluorescence shift was observed with quinine and ammonium nitrate mixtures shown in Figure 62 where emission shifted from 380 nm to 460 nm. Thus, quinine fluoresces like a dication when ammonium nitrate is added into solution, despite the solution pH < 5 where quinine would normally fluoresce as a cation at 380 nm. The ammonium nitrate is not physically associating or binding with quinine or 6MQ; however, a shift in fluorescence is observed. This is opposite to what is observed
with LRD and 6MQ. Mixtures of 6MQ and LRD do not display a shift in fluorescence, indicating mixtures of LRD require an interaction to induce a fluorescence shift. This is also observed with quinine LRD mixtures. When quinine is a cation and associated with LRD, there is a shift in the quinine fluorescence but a shift is not observed when quinine is neutral and not associated with LRD.

![Fluorescence Spectra of Quinine & Ammonium Nitrate](image)

**Figure 62** The fluorescence spectra of quinine shows changes in the $\lambda_{\text{max}}$ emission from $\lambda_{\text{max}} \approx 380$ nm to $\lambda_{\text{max}} \approx 460$ nm with an introduction of ammonium nitrate indicating a chemical change of quinine in the fluorescence experiments. The ammonium nitrate has a quenching affect on the quinine fluorescence.
UV-Vis spectra of 6MQ and quinine mixed with ammonium nitrate were measured as a control (shown in Figure 63 and Figure 64), showing there is not a change in their visible absorbance. A subtraction of the ammonium nitrate absorbance from the 6MQ and quinine ammonium nitrate mixture spectra overlays identically with the pure 6MQ and quinine absorbance spectra, further indicating there is not an interaction with the two species in their ground states (Figure 64). The absence of an emission shift between 6MQ and LRD is an indication that the mechanism for the excited state pKa shift is different between 6MQ and LRD than 6MQ and ammonium nitrate.
Figure 63 UV-Vis absorbance of 10mM quinine and ammonium nitrate. The UV-Vis spectrum of quinine and an ammonium nitrate mixture is identical after a subtraction of the ammonium nitrate absorbance from the mixture absorbance. This indicates there is not a chemical reaction and a physical change in quinine.
The UV-Vis spectrum of 1.5mM 6-methoxyquinoline with and without ammonium nitrate shows an identical absorbance spectrum indicating the 6MQ is not changing chemically with the addition of ammonium nitrate.

To summarize, quinine in quinine LRD mixtures displays a shift in fluorescence from 380 nm to 450 nm, despite a solution pH that is nearly 4 orders of magnitude higher than what is need for a ground state shift. The interaction between quinine and LRD was verified to occur through the quinuclidine moiety using 6MQ LRD mixtures as a control. 6MQ LRD mixtures did not display a shift in fluorescence because 6MQ lacks the quinuclidine moiety with which quinine interacts. Quinine and 6MQ mixtures with ammonium nitrate mixtures displayed a shift in fluorescence that has been reported in
literature to be from an excited state pKa shift. The excited state pKa shift with ammonium nitrate occurs despite a physical reaction between quinine and 6MQ, whereas LRD mixtures require a physical interaction for shift in fluorescence to occur.

6.4 Conclusions

As anthropogenic nanomaterials are entering the environment it is important to understand and model how these engineered nanomaterials interact with pharmaceutical compounds, humans and environmental ecology. The research presented is the first known research on the reaction chemistry of Laponite-RD, a known nanomaterial additive in over the counter consumer products, and its interactions with quinine under environmentally and physiologically relevant pH conditions. The research provides a model for the excited state interaction that might be mediated with suspended nanomaterial in an aqueous environment. It also gives evidence that nanomaterials can change the chemical behavior of molecules in aqueous solutions unexpectedly.
Chapter 7 Conclusion and Future Work

The presented research focused on hydrodynamic chromatography methods for determining association constants of solutes on the surface of nanoparticles. The hydrodynamic chromatography methods were evaluated using multivariate analysis techniques. Finally, quinoline drug interactions on LRD surfaces were investigated.

7.1 Laponite-RD as a Mobile Phase Modifier

Chapter 3 showed the affects of using HPLC systems to determine the association constant between nanomaterials and solutes. Chapter 3 also investigated the use of LRD as a mobile phase modifier in HPLC systems. These were important experiments because they showed there was interaction between xanthine solutes and LRD when the solutes were not negatively charged. However, using HPLC systems for determining the association constant between caffeine and LRD were inconclusive for two specific reasons. First, the diffusion coefficient of LRD is sufficiently small to decrease the theoretical plate height of the separation. This lead to large fronting peaks on the chromatographic separations of xanthine LRD mixtures. Secondly, association constants with LRD may not be constant over variable LRD concentrations. There is evidence this is true from the conclusions in Chapter 5. If association constants of solutes to LRD were not constant over variable LRD concentrations, the plot used to calculate the $K_D$ value in Figure 19 would not be valid because the association constant is not constant.
Future experiments that should be performed along the same experimental conditions are mixtures with smaller increments of LRD concentration. Smaller increments of LRD concentrations will potentially allow a curve to develop on the $K_D$ plot shown in Figure 19. If a curve does begin to develop it would be an indication there is a concentration dependence of LRD and solute association. Another future experiment should attempt to break up the LRD intermolecular interactions that would apparently occur when LRD concentration increases. The addition of a constant organic solvent to the LRD mixtures could keep the LRD particles from coagulating. Other Laponite products that have a higher concentration for coagulation could be used in lieu of LRD.

7.2 Measuring Laponite-RD association to Xanthine Stimulants Using Hydrodynamic Chromatography

Conclusions that are drawn from Chapter 4 are important for proof-of-concept using wide-bore hydrodynamic chromatography to separate large particles from small molecules. The data from Chapter 4 show when a partial separation between a nanoparticle and solute exists, the data can be deconvoluted to obtain an asymmetrical flow profile of the nanoparticle and a more Gaussian flow profile of the solute. If there is an association between the solute and nanoparticle, the solute will assume the flow profile of the nanoparticle, and be modeled differently than the free solute. From these modeled data, binding isotherms and $K_D$ values can be determined.
The limitations of this method are the kinetics of interaction. If the rate of desorption is fast, or on the timescale of the separation, \( \approx 30 \) seconds, then a separation of the once associated solute from the nanoparticle would occur. If this were to happen it could create isotherms that are not indicative of the true \( K_D \) value.

Further experiments with wide-bore HDC chromatography would include expanding the range of solutes and nanoparticles to use in the system. The apparatus has potential to measure binding of drugs and biologically relevant molecules to proteins. Also, the potential for nanomaterials to be used as drug delivery vesicles could be investigated with the wide-bore HDC method.

7.3 Evaluation of Laponite-RD association to Caffeine and Oxytetracycline using Hydrodynamic Chromatography with Multivariate Deconvolution Methods

Evaluation of caffeine and OTC using wide-bore HDC with multivariate deconvolution methods was informative and showed the limitation of multivariate analysis methods. MCR and PARAFAC multivariate methods could not reconstruct the flow profile and spectral data to match experimental data. PARAFAC2 analysis methods were better suited for wide-bore HDC experiments because PARAFAC2 requires multi-way data and can model changes in retention times in chromatographic experiments. The reconstructed caffeine LRD data from PARAFAC2 analysis was not able to model the flow profiles of the mixture because there was not a change in the spectral data of caffeine associated with LRD and freely eluting caffeine. This caused the model to find 2
species in solution, freely eluting LRD and caffeine. The caffeine flow profile showed the leading peak, which is indicative of caffeine associated with LRD, and the larger freely eluting caffeine peak later in time. Without a change in absorbance these two species were modeled as one.

OTC was thought to be better choice for analysis on the wide-bore HDC apparatus; however, the reconstructed data did not match the experimental data. The precipitate of OTC was thought to be the reason for the discrepancies between the reconstructed data and the experimental data. To verify OTC degradation was present in the mixture solutions, and to verify the observed kinetic reaction between LRD and OTC, the mixtures were analyzed using SEC. SEC data validated the observed kinetics and indicated there were at least 4 species present in solution 3 of which were larger than an OTC monomer molecule. The OTC LRD mixtures were too complex for PARAFAC2 deconvolution methods to model the data.

Future work using multivariate methods would use a solute nanomaterial system where there is a change in the absorbance spectrum when the solute is associated with the nanomaterial. This change is spectrum would allow PARAFAC2 methods to deconvolute the data matrix and obtain flow profiles and spectral data for the two species in solution. Using PARAFAC2 methods would facilitate isotherms and $K_D$ values without any chemical assumptions made about the analytes present in solution.
7.4 Investigating Chemical Interactions between Laponite-RD and Quinoline Drugs

Quinoline drugs interactions with LRD were also investigated. They were first investigated with the wide-bore HDC apparatus, but the method failed due to precipitation of the mixture. However, an excited state reaction between quinine and LRD was observed. The observed reaction was from an interaction between quinine and LRD. When excited by UV light, LRD donated a proton to quinine. The proton donation did not occur with 6MQ because it did not associate with LRD. The association between LRD is important for the proton transfer, and this is evident because 6MQ and quinine show the excited state proton transfer in the presence of ammonium nitrate. The experiment was important because it showed there could be interaction between LRD and solutes that are not observable in the visible spectrum.

Future experiments should determine the lifetime of the quinine interaction with LRD; this would enable calculation of the excited state pK$_a$ of quinine. Calculation of the excited state pK$_a$ is an important experiment to determine if the lifetime and excited state pK$_a$ values are different when quinine is associated with LRD compared to quinine in solution with ammonium nitrate.
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