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# A Systematic Investigation of the Effects of Chain Length and Ionic Head Group on Perfluoroalkyl Acid Binding to Human Serum Albumin

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# A Systematic Investigation of the Effects of Chain Length and Ionic Head Group on Perfluoroalkyl Acid Binding to Human Serum Albumin

By

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ABSTRACT

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Perfluoroalkyl acids (PFAAs) are industrial chemicals used in everyday products ranging from non-stick coatings to fire-fighting foam. PFAAs are contaminants of emerging concern (CECs) and are bioaccumulative, persistent and toxic. Unlike other CECs, PFAAs bioaccumulate in areas of high protein concentration, such as the kidneys, liver and blood; therefore, it is vital to study PFAA-protein interactions. Human Serum Albumin (HSA) is the model protein used for PFAA-protein studies because it is the most abundant protein in the human body and it binds and transports endogenous and exogenous ligands. Previously, researchers have investigated PFAA-HSA binding, but most of these studies have focused on medium-chain length PFAAs and lacked a systematic method to quantify PFAA-HSA binding. Additionally, few studies have focused on determining a relationship between PFAA chain length and binding strength. In this study, a systematic method was used to determine relationships between PFAA chain length and ionic head group and protein binding; the binding affinities of short-, medium- and long-chain PFAAs were determined using a systematic equilibrium dialysis method coupled with liquid chromatography-tandem mass spectrometry. The results indicate that the short-chain PFAAs, depending on ionic head group, bind as strongly or only moderately weaker at the high-affinity sites than the medium- and long-chain PFAAs.

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## Introduction

*What are perfluoroalkyl acids?*

Perfluoroalkyl acids (PFAAs) are a family of industrial chemicals that are fluorinated analogs of carboxylic acids. PFAAs are used in a variety of consumer products, such as non-stick coatings, paper plates, microwave popcorn bags and fire fighting foams, due to their unique properties of being both hydrophobic and lipophobic, which causes PFAAs to repel both water and oils. These chemicals have been produced and used in products since the 1940s. However, in recent years PFAAs have been classified as Contaminants of Emerging Concern (CECs) because they have been seen to bioaccumulate in organisms and not degrade in the environment.[1-3] These chemicals accumulate in aquatic environments, sediments and various mammals and organisms; PFAAs have been detected in trout, rats and the Great Lakes, to name a few.[2,4-7]

In addition to being bioaccumulative, PFAAs have also been shown to have adverse health effects. Many of these toxicity studies have been conducted with the PFAAs perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). Studies with PFOA and PFOS in rodents and non-human primates have shown reduced body weight, liver toxicity and development of cellular tumors.[1] Additionally, some epidemiology studies have seen a correlation between prolonged exposure to PFAAs and cancer incidence in humans.[1] Other toxicity tests have shown that PFAAs could cause adverse changes in cell membranes and peroxisome proliferation.[8] Due to PFAAs being bioaccumulative, persistent in the environment and exhibiting toxic effects, they have been designated as PBT (Persistent, Bioaccumulative and Toxic) chemicals by some

groups. Thus it is important to study PFAAs in order to understand more about their fate and transport within the environment and in organisms.

Historically, the main PFAAs used in the manufacturing of products have been perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) and their derivatives. However, the primary U.S. manufacturers of PFOA and PFOS began to phase out the production of these chemicals in 2002 (Figure 1) due to reports that these chemicals bioaccumulate and exhibit toxic effects.[1,2,9] These manufacturers have replaced these longer eight-carbon chemicals with chemicals based on the shorter four-carbon PFAAs, perfluorobutanoic acid (PFBA) and perfluorobutanesulfonic acid (PFBS). Therefore, PFAAs are still currently used in the manufacture of everyday products.

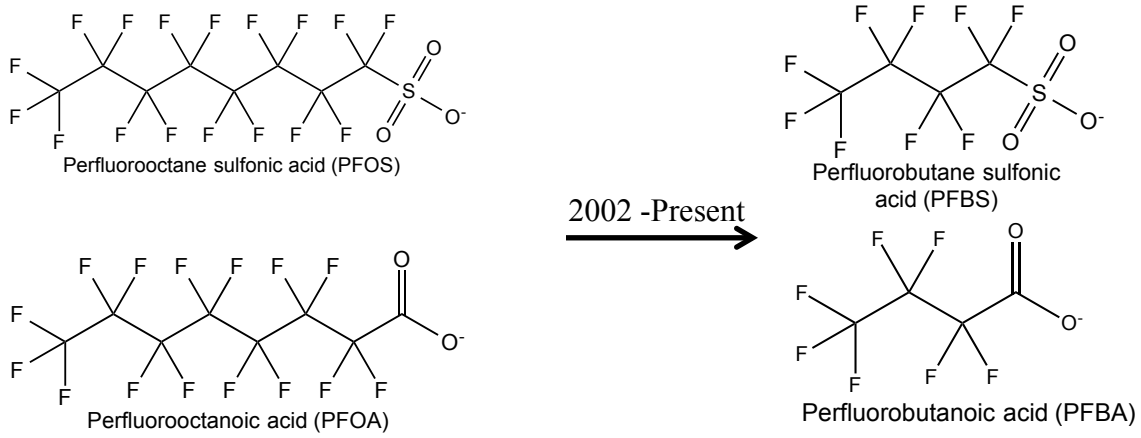


**Figure 1.** The usage of PFAAs in industry has transitioned over time with companies switching to chemicals based on the four-carbon PFAAs, PFBS and PFBA, from the eight-carbon PFAAs, PFOS and PFOA.

Even though the use of longer chain PFAAs has been phased out of by most U.S. industries, the negative impacts of their bioaccumulation and toxicity are still being seen today and will be for decades to come. Notably, case after case of drinking water contamination by PFAAs have recently been revealed. One example that has gained

national attention is the water crisis in Hoosick Falls, NY. The EPA found that the town's drinking water supply was contaminated with PFOA and not potable. The source of the contamination is thought to be a manufacturing plant most recently operated by Saint-Gobain Performance Plastics, which had stopped using PFAAs at its main manufacturing site about 17 years ago. Unfortunately, due to PFAAs being highly persistent within the environment, the levels of PFOA in the drinking water were found to be above 400 parts per trillion.[10] Additionally, the PFOA levels in the blood of Hoosick Falls residents were found to be 15 times the national median.[11] The toxicological effects of this exposure have been noticed. In Hoosick Falls, there is an abnormally large incident rate for kidney cancer, which is one of the cancers associated with PFAA exposure.[11] This recent crisis, among many others, demonstrates just how important it is to study the environmental and biological fates of PFAAs. Even though the longer chain PFAAs have been phased out of most U.S. manufacturing, these chemicals are globally pervasive and will continue to cause problems for decades to come. Additionally, the usage of the less studied short chain molecules is concerning because they, too, could pose adverse environmental and human health effects.

*Properties of perfluoroalkyl acids*

PFAAs are synthetic derivatives of hydrocarbon carboxylates and sulfonates, in which the hydrogen atoms are replaced with fluorine atoms. There are two synthetic pathways for converting hydrogenated carboxylates and sulfonates to fluorinated derivatives. The two pathways developed for commercial production of PFAAs are electrochemical fluorination and telomerization fluorination.[8] The unique hydrophobic

and lipophobic properties of PFAAs are due to the substitution of the hydrogen atoms for the fluorine atoms. The general structures for these compounds are shown in Figure 2.

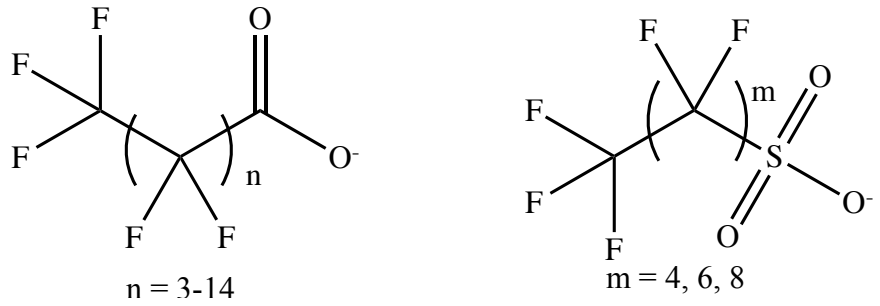


**Figure 2.** General structure of perfluoroalkyl carboxylates (PFCAs, left) and sulfonates (PFSAs, right).

PFAAs are usually shown as deprotonated due to the low pKa values of the chemicals. Studies have found that PFAAs have a pKa around 0.5. Due to this low pKa, it is assumed that PFAAs exist in the environment as the deprotonated carboxylates and sulfonates.[12]

As stated earlier, these chemicals have been shown to resist degradation. This phenomenon can be attributed to the strength of the carbon fluorine bond due to the immense electronegativity of fluorine. In addition to the hydrophobicity and lipophobicity of PFFAs, the electronegativity of the fluorine atom allows PFAAs to resist multiple types degradation such as exposure to strong acids and bases, oxidizing and reducing agents and many more.[13] This resistance to degradation, in part, allows for the bioaccumulation of these chemicals in organisms. The bioaccumulation and toxicity that PFAAs have displayed are the reasons why they are highly studied.

*Bioaccumulation and toxicity of perfluoroalkyl acids*

The bioaccumulation and toxicity patterns of PFAAs are unlike other hydrophobic contaminants due to their unique properties. Hydrophobic contaminants tend to bioaccumulate in fatty tissues while PFAAs mainly bioaccumulate in tissues with high protein content, such as the blood, liver and kidneys of organisms. PFAAs have been found to bioaccumulate in organisms ranging from fish to birds to rats to humans.[1,9,14-25] The anthropogenic sources of PFAAs have been widely studied in order to better understand the accumulation of these chemicals in the environment and in humans. Besides the biomagnification due to exposure from everyday products and consuming contaminated organisms (mainly fish), the bioaccumulation of theses chemicals can be attributed to contaminated drinking water supplies, such as in the Hoosick Falls, NY, case. Recently there have been a few studies examining the sources of PFAAs in drinking water, and they have determined that the proximity of drinking water resources to industrial sites, military fire training areas and wastewater treatment plants are all strong predictors for PFAA water contamination.[25-27] Humans and organisms are exposed to these chemicals in a multitude of ways, and the areas in which they accumulate in the body are vital for human and organismal health and function. Unfortunately, PFAAs have been linked to toxicological effects, which is why the areas in which they bioaccumulate are even more of a concern.

Some of the negative effects that are attributed to PFAAs are adverse changes in membrane function, mitochondrial bioenergetics and peroxisome proliferation. Additionally, PFAAs have been linked to numerous toxic effects, such as thyroid cancer, testicular cancer, liver cancer, neonatal and liver toxicity and tumors in multiple

organisms.[5-7,9] These adverse effects linked to PFAAs are interestingly linked to many of the areas in which they bioaccumulate. Many of the toxicological effects have been seen in areas of high protein content, which is why there have been many studies of PFAA-protein interaction. In many of these studies, the model protein used is serum albumin since it is the most abundant protein found in the blood. One of the main functions of serum albumin is to transport molecules around the body. Many of these molecules are fatty acids, which have a similar structure to PFAAs. Serum albumin is also able to reversibly bind and transport other endogenous and exogenous ligands, which makes it an ideal model protein to study the fate and transport of PFAAs within the body.[28-31]

*Human serum albumin: the model protein*

Human serum albumin (HSA) is the most abundant protein in the human body, at 50 grams per liter. It is produced in the liver and has a half-life of 19 days.[32] Some of the functions of HSA include maintaining blood pH and pressure, transporting fatty acids, removing oxygen free radicals and its previously mentioned reversible binding abilitites.[32]

Due to the ability of HSA to reversibly bind exogenous and endogenous ligands, it is the model protein used in this study. Many of these endogenous ligands are fatty acids, which have similar structures to PFAAs. These similarities may suggest that competitive binding between PFAAs and fatty acids may occur, which means that PFAAs could interfere with the binding of fatty acids to HSA, and possibly other proteins as well. HSA consists of a single polypeptide composed of 585 amino acid residues and

has a molecular weight of approximately 66.5 kDa.[28-31] The structure of HSA, acquired from the protein data bank, can be seen in Figure 3.

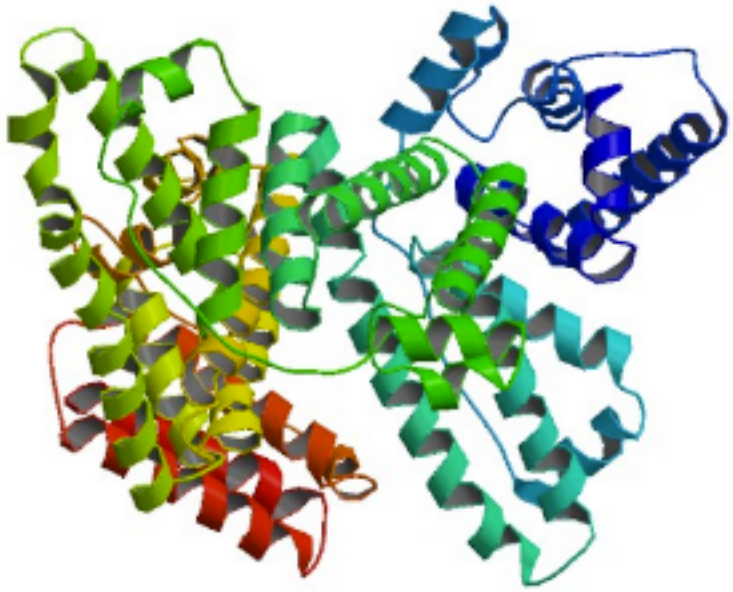

**Figure 3.** Crystal structure of human serum albumin from the protein data bank (ID 1AO6).[30]

HSA is a good model protein for this study due to it having many sites for binding and transporting ligands, as well as the fact that it can be found throughout the entire body. It should be noted that HSA exists as a monomer (a single protein molecule), a dimer (two proteins), a trimer (three proteins), etc. For this study, a commercially available mix of HSA was used, meaning HSA existed as a monomer, dimer, trimer, etc. in the mix.

*Previous work*

Several studies have been conducted to investigate the binding of PFAAs to serum albumin due to the toxicity and bioaccumulative properties of these chemicals. However, there are some limitations to the previous studies. One is that most of the studies primarily focused on the binding of perfluoroctanoic acid (PFOA) and/or

perfluorooctane sulfonic acid (PFOS) to serum albumin. This is a problem because there are more than two PFAAs and these two PFAAs are not the ones in production anymore since they were phased out after 2002.[2] Another limitation of previous studies is that some groups used HSA while others used BSA (bovine serum albumin). If the goal is to understand the fate and transport of PFAAs in the human body, then HSA should be the gold standard for PFAA-protein interaction studies.

The typical way to quantify ligand-protein binding is through the dermination of $K_a$, the association constant. The $K_a$ values for PFOA-HSA and PFOA-BSA binding from previous experiments are displayed in Table 1. The $K_a$ values in this table range from $10^3$ to $10^6$ $M^{-1}$.

**Table 1.** Magnitudes of literature $K_a$ values for perfluorooctanoic acid

| Protein-PFAA System | $K_a$ ($M^{-1}$) | Source |
|---|---|---|
| HSA-PFOA | $10^3$ | Han *et al* (2003)[33] |
| HSA-PFOA | $10^4$ | Wu *et al* (2009)[34] |
| HSA-PFOA | $10^4$ | Hebert *et al* (2010)[35] |
| HSA-PFOA | $10^5$ | Chen *et al* (20)[36] |
| HSA-PFOA | $10^5$ | Messina *et al* (2005)[37] |
| BSA-PFOA | $10^5$ | MacManus-Spencer *et al* (2010)[38] |
| BSA-PFOA | $10^6$ | Bischel *et al* (2010)[39] |

Clearly the vast range of these numbers shows that there is no clear magnitude for how strongly PFOA binds to HSA. Additionally, these values are only for PFOA, so there is no systematic understanding of how PFAAs in general bind to HSA. As stated earlier, PFAAs exist as a variety of chain lengths and with different ionic head groups, and there has been no definitive relationship determined for how chain length or ionic head group affects the binding affinity of PFAAs. Lastly, the methods used to study PFAA-HSA binding have not been ideal. Many of these methods use spectroscopic techniques to study PFAA-HSA binding, which are indirect methods for determining $K_a$. However, the

"gold standard" for protein binding studies is equilibrium dialysis, which is a direct method. The studies that did use equilibrium dialysis were inconsistent because they included few PFAA:HSA mole ratios and obtained different data points on different days due to the tedious nature of equilibrium dialysis. This results in inconsistent and unreliable $K_a$ values. The focus of this study is to expand upon previous research in the MacManus-Spencer lab that has focused on quantifying the binding affinities of PFAAs to HSA using a systematic approach involving a sophisticated, more high-throughput equilibrium dialysis technique.[40-42] The main goal of this study is to determine definitive relationships between HSA-PFAA binding affinity and PFAA chain length and ionic head group.

*Protein-ligand binding kinetics and binding isotherm plots*

Chemical kinetics can be used to characterize the binding interactions between a protein and a ligand. This can be quantified by determine the binding affinity between a protein and a ligand via determining the association constant, $K_a$.

A reversible protein-ligand binding system can be described using the following equilibrium:

$$Protein + Ligand \underset{k_{off}}{\overset{k_{on}}{\longleftrightarrow}} Protein---Ligand$$

The protein and ligand can exist in a bound state as a complex or in an unbound state. The two rate constants $k_{on}$ (association rate constant) and $k_{off}$ (dissociation rate constant) govern the state of the protein and ligand. The rate of the forward reaction is proportional to $k_{on}$:

$$Rate = [Protein][Ligand]k_{on}$$

And the rate of the reverse reaction is proportional to $k_{off}$:

$$Rate = [Protein---Ligand]k_{off}$$

When the system is at equilibrium, the rates of the forward and reverse reactions are equal; this means that that two rate equations are equal at equilibrium:

$$[Protein][Ligand]k_{on} = [Protein---Ligand]k_{off}$$

The association constant, $K_a$, is defined as the ratio between the association and dissociation rate constants at equilibrium:

$$K_a = \frac{k_{on}}{k_{off}}$$

This allows for the rearrangement of the equilibrium expressions to solve for $K_a$:

$$\frac{[Protein][Ligand]}{[Protein---Ligand]} = \frac{k_{on}}{k_{off}} = K_a$$

The $K_a$ value, having units of $M^{-1}$, is a measure of HSA-PFAA binding affinity. This means that a larger $K_a$ value indicates stronger binding interactions because a higher concentration of the HSA-PFAA complex would exist compared to the concentration of unbound PFAA.

In order to determine the $K_a$ value for a specific PFAA, a binding isotherm plot is created. This is done by running an equilibrium dialysis experiment on a 96-well equilibrium dialyzer plate with a range of PFAA:HSA mole ratios. The amount of free PFAA is quantified via liquid chromatography – tandem mass spectrometry (LC-MS/MS) and a binding isotherm plot is created. Plotting the binding coefficient, which is the concentration of bound PFAA divided by the concentration of HSA, versus the concentration of free PFAA creates the binding isotherm plot. An example of a theoretical binding isotherm plot is shown in Figure 4.

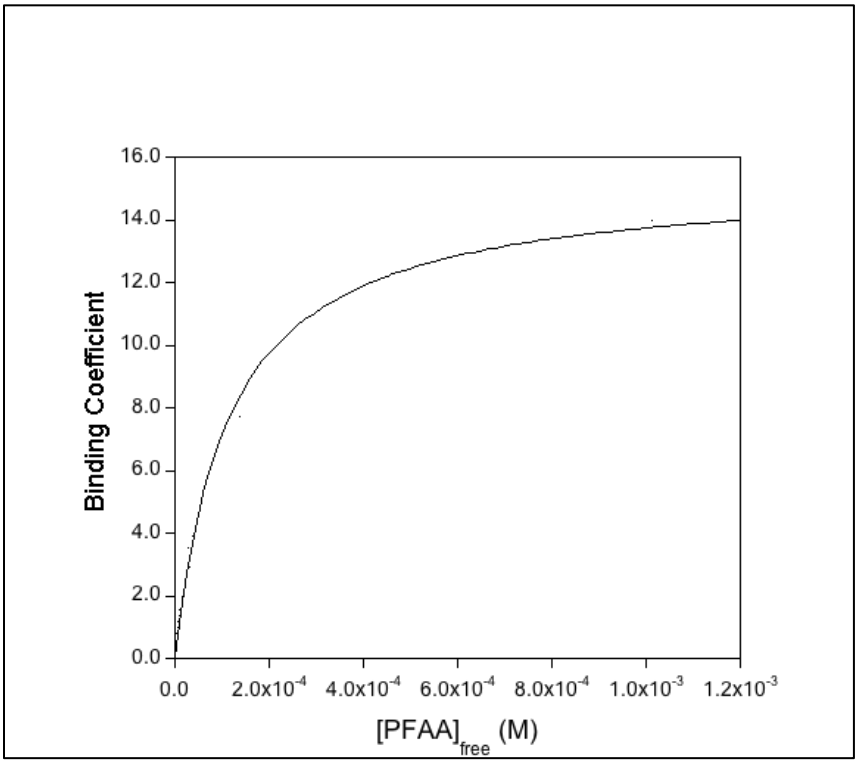


**Figure 4.** An example of a theoretical binding isotherm plot.

To determine the $K_a$ value for a specific PFAA, nonlinear curve fitting software, KaleidaGraph, is then used. This software is able to determine the $K_a$ and the binding stoichiometry (number of PFAAs bound per protein molecule) by fitting the plot with a binding model. There are several possible binding models that can describe the binding of PFAAs to HSA; the models used in this study include: one-class binding (equation 1), two-class binding (equation 2), and one-class that includes a nonspecific binding term (equation 3). A one class binding model means that PFAAs bind at one type of site on the protein with similar affinity; a two class binding model means that PFAAs bind at two types of sites on the protein with different affinities; and a one-class binding model that includes nonspecific binding means that PFAAs bind at one type of site strongly and then weakly associate on the surface of the protein.[42]

$$v = \frac{n_1 K_{a1}[PFAA]_{free}}{1 + (K_{a1}[PFAA]_{free})} \quad (1)$$

$$v = \frac{n_1 K_{a1}[PFAA]_{free}}{1 + (K_{a1}[PFAA]_{free})} + \frac{n_2 K_{a2}[PFAA]_{free}}{1 + (K_{a2}[PFAA]_{free})} \quad (2)$$

$$v = \frac{n_1 K_{a1}[PFAA]_{free}}{1 + (K_{a1}[PFAA]_{free})} + K_{nsb}[PFAA]_{free} \quad (3)$$

Depending on the model fit for each PFAA, certain properties about the bioaccumulation and transport of that PFAA can be determined, for example, the number of sites to which it strongly and weakly associates and how strong these interactions are.

*Experimental Goals*

The main goal of this study is to study PFAA-HSA binding interactions. As stated earlier, there has been no definitive relationship determined for how PFAA:HSA binding association strength is affected by PFAA chain length and ionic head group. The first goal is to determine this relationship using the systematic equilibrium dialysis method developed in the MacManus-Spencer lab.[41,42] This study looks at six different PFAAs varying in chain length and ionic head group in order to determine a relationship by quantifying the $K_a$ values for each of these PFAAs to HSA. The PFAAs used in this study can be seen in Figure 5.

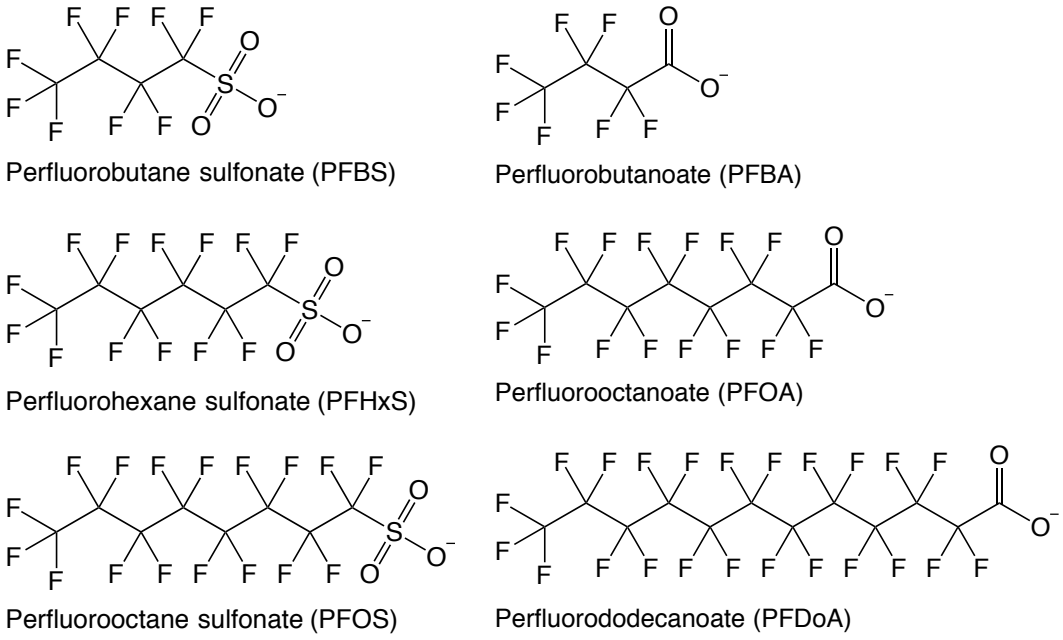


**Figure 5.** The six different PFAAs studied to determine the relationships between chain length and ionic head group and HSA binding constants.

If a relationship between chain length and ionic head group can be established, the fate and transport of PFAAs in the human body will be more generally understood. Currently, the fate and transport of PFOA and PFOS is partially understood, but this study will hopefully allow for the understanding of the fate and transport of all PFAAs. These results will then hopefully allow for stricter regulation of these chemicals if it is seen that chain length doesn't affect the $K_a$ value significantly, meaning the shorter chain PFAAs bind just as strongly as the longer chain PFAAs.

**Materials**

96-well Equilibrium Dialyzer plates and a plate rotator were obtained from Harvard Apparatus (Holliston, MA). Human serum albumin (HSA; ≥ 99%, essentially fatty acid and γ-globulin free) was obtained from Sigma-Aldrich (St. Louis, MO), and 66,437 Da was used as the molecular weight.[43] Perfluorobutanesulfonic acid potassium salt (PFBS-$K^+$; 98%), tridecafluorohexane-1-sulfonic acid potassium salt (PFHxS; ≥ 98%), perfluorooctanoic acid (PFOA; 96%), perfluorooctanesulfonic acid potassium salt (PFOS-$K^+$; ≥ 98%) perfluoroundecanoic acid (PFUnA; 95%), and perfluorododecanoic acid (PFDoA; 95%) were obtained from Sigma-Aldrich. Perfluorobutanoic acid (PFBA; 99%) and perfluorooctanoic acid (PFOA; 96%) were obtained from Acros Organics (Morris Plains, NJ) through Fisher Scientific USA (Pittsburgh, PA). Sodium phosphate monobasic (99.3%, monohydrate), sodium phosphate dibasic (99.3%, anhydrous), ammonium hydroxide (certified ACS Plus), methanol (Optima LC-MS grade, 0.1-micron filtered), water (Optima LC-MS grade, 0.03-micron filtered), ammonium formate, and polypropylene microcentrifuge tubes were from Fisher Scientific. Polypropylene centrifuge tubes (15- and 50-mL) were obtained from Corning Incorporated (Corning, NY). HPLC autosampler vials and caps were obtained from Kinesis, Inc. (Malta, NY). A $C_{18}$ Targa Sprite column (40 x 2.1 mm x 5 μm) was obtained from Higgins Analytical (Mountain View, CA). Mass labeled [$^{13}C_4$]-PFOA and [$^{13}C_4$]-PFBA (each 50 mg/mL in methanol) were obtained from Wellington Laboratories, Inc. (Ontario, Canada).

**Methods**[44]

*Sample preparation*

A pH 7 sodium phosphate buffer was prepared from sodium phosphate monobasic and sodium phosphate dibasic in Optima LC-MS grade water with a total phosphate concentration of 50 mM. Stock solutions of all the PFAAs were prepared in either pure Optima LC-MS grade methanol or a 70:30 mixture of Optima LC-MS grade methanol and 0.01% ammonium hydroxide in 10 mL volumetric flasks. For the PFAA stock solutions in 50-mM sodium phosphate buffer (pH 7), PFOA, PFHxS PFBS and PFBA were prepared in 10-mL volumetric flasks, but due to solubility issues PFDoA and PFOS were prepared in 100-mL volumetric flasks. If any of the PFAA stock solutions were not soluble, the solutions were sonicated in a water bath between 35-40°C until dissolved using a Bransonic Tabletop Ultrasonic Cleaner 3510 (Branson Ultrasonic Corporation; Danbury, CT). Purity-corrected stock solutions of 500-µM and 50-µM HSA in 50-mM sodium phosphate buffer (pH 7) were prepared in 50-mL polypropylene centrifuge tubes. The 500-µM HSA solution was used for the PFBA, PFBS, PFHxS and PFOA experiments; the 50-µM HSA solutions were used for the PFOS and PFDoA experiments. This was done to keep the mole ratios of PFAA to HSA (0.01-16) constant from experiment to experiment. The HSA solution was prepared fresh for every experiment.

*Equilibrium dialysis (ED)*

Equilibrium dialysis plates from Harvard Apparatus were used to study PFAA-HSA binding. The equilibrium dialysis plates contained 96 wells spread out over 12 columns and 8 rows, and the plate was dual-sided. On the protein side (HSA side), 200

μL of either 500-μM or 50-μM HSA in 50-mM sodium phosphate buffer (pH7) was pipetted into the amount of wells used for each experiment (usually 57 wells). On the opposite side, the "ligand" side, 200 μL of a known concentration of PFAA in 50-mM sodium phosphate buffer (pH 7) was pipetted into the amount of wells used for each experiment. Furthermore, for each ED experiment run, positive and negative controls were tested in triplicate. A positive control had 200 μL of a known concentration of PFAA on the "ligand" side, while on the protein side there was 200 μL of 50-mM sodium phosphate buffer with no HSA. A negative control had 200 μL of either 500-μM or 50-μM HSA in the protein side of the plate, while the PFAA side contained 200 μL of 50-mM sodium phosphate buffer. Once the wells were prepared, the plate was secured onto a plate rotator and rotated at room temperature for 48 hours. Once equilibrated, samples were collected from the PFAA side of the plate and stored in a cold room (4°C) until analyzed by LC-MS/MS for free PFAA concentration quantification.

*LC-MS/MS quantitative analysis*

Samples from the ED experiments were analyzed by LC-MS/MS in order to determine the concentration of free PFAA, which led to the determination of the $K_a$ for each PFAA. For each LC-MS/MS vial, 100 μL of sample was pipette in along with 100 μL of 50-mM sodium phosphate buffer and 50 μL of internal standard in Optima LC-MS grade water, resulting in a total volume of 250 μL. The internal standard used for PFOA was [$^{13}C_4$]-PFOA, which was used at a final concentration of 50 nM. The internal standard for PFOS was PFHxS, which was used at a final concentration of 1000 nM. The internal standard for PFHxS was PFOS, which was used at a final concentration of 1000

nM. The internal standard used for PFBA was [$^{13}C_4$]-PFBA, which was used at a final concentration of 10 nM. The internal standard used for PFBS was PFHxS, which was used at a final concentration of 1000 nM. Lastly, the internal standard used for PFDoA was PFUnA, which was used at a final concentration of 100 nM. The reason PFDoA, PFBS and PFOS did not have mass labeled internal standards is that these were not unknown environmental samples, so it wasn't necessary to use expensive mass labeled internal standards since the contents of each sample were known throughout the experiment.

Sample analysis was done with an Agilent 1200 series HPLC and Agilent 6410B triple quadrupole mass spectrometer, and the samples were run on a Targa Sprite $C_{18}$ column from Higgins Analytical. All HPLC separations were isocratic elutions. The solvents used for LC-MS/MS analysis, with the exception of PFOA, were 2 mM ammonium formate in either Optima LC-MS grade methanol or Optima LC-MS grade water. For PFOA, 2 mM ammonium acetate in either Optima LC-MS grade methanol or Optima LC-MS grade water was used. The PFAAs that were studied using equilibrium dialysis were PFOA, PFOS, PFHxS, PFBA, PFBS and PFDoA. For each of these six PFAAs, different isocratic elution conditions were determined for optimal LC-MS/MS quantification (Table 2).

**Table 2.** LC-MS/MS solvent conditions used for the various PFAAs

| PFAA Analyte | Percent 2 mM Ammonium Formate in Methanol | Percent 2 mM Ammonium Formate in Water |
|---|---|---|
| PFOA* | 60% | 40% |
| PFOS | 60% | 40% |
| PFHxS | 50% | 50% |
| PFBA | 10% | 90% |
| PFBS | 50% | 50% |
| PFDoA | 70% | 30% |

*For PFOA, 2 mM ammonium acetate was used instead of 2 mM ammonium formate.

For all methods, a 40-µL injection volume, 0.5 mL/min flow rate and 400 bar maximum pressure were used as parameters. Additionally, the mass spectrometer was operated in negative mode for electrospray ionization (ESI) with multiple reaction monitoring (MRM) analysis. For all analyses a 50 psi nebulizer pressure, 10 L/min gas flow and 350 °C gas temperature were used.

MassHunter optimizer software was used to determine the relevant precursor and fragment ions for each analyte and internal standard in order to quantify the amount of free PFAA. Also, the MassHunter optimizer software determined the fragmentor voltages and collision energies for the MRM methods used for each analyte. The relevant transition ions monitored, fragmentor voltages and collision energies for each analyte and internal standard used are shown in Table 3

**Table 3.** Parameters used for MRM methods in quantification of free PFAA

| **Analyte** | **Transition Type** | **MRM Transition (m/z)** | **Fragmentor Voltage (V)** | **Collision Energy (V)** |
|---|---|---|---|---|
| PFOA | Quantifier | 413 → 369 | 50 | 4 |
| | Qualifier | 413 → 169 | 50 | 16 |
| [$^{14}C_4$]-PFOA | Qualifier | 417 → 372 | 50 | 4 |
| PFOS | Quantifier | 499 → 80 | 180 | 60 |
| | Qualifier | 499 → 99 | 180 | 56 |
| | Qualifier | 499 → 298 | 180 | 16 |
| PFHxS | Qualifier | 399 → 80 | 150 | 44 |
| PFHxS | Quantifier | 399 → 80 | 130 | 52 |
| | Qualifier | 399 → 90 | 130 | 44 |
| PFOS | Qualifier | 499 → 80 | 180 | 60 |
| PFBA | Quantifier | 213 → 169 | 50 | 0 |
| [$^{13}C_4$]-PFBA | Qualifier | 217 → 79 | 140 | 48 |
| PFBS | Quantifier | 299 → 80 | 160 | 36 |
| PFHxS | Qualifier | 399 → 80 | 150 | 44 |
| PFDoA | Quantifier | 613 → 569 | 70 | 8 |
| | Qualifier | 613 →269 | 70 | 16 |
| | Qualifier | 613 →219 | 70 | 20 |
| | Qualifier | 613 →169 | 70 | 24 |
| PFUnA | Qualifier | 563 →519 | 60 | 8 |

Additionally, calibration curves were generated for each PFAA in order to quantify the amount of free PFAA. A calibration curve ranging from 15 nM – 6000 nM was used for PFOA, while for PFOS, PFBA, PFBS and PFDoA calibration curves ranging from 150 nM – 6000 nM were used. Standards for each of these PFAAs were made within this concentration range. Stock solutions of known PFAA concentration were prepared in Optima LC-MS grade methanol with the calibration standards being made according to a nested dilution scheme in a 70:30 mixture of Optima LC-MS grade methanol and 0.01% ammonium hydroxide in Optima LC-MS grade water. Each of these standards were pipetted into an autosampler vial with the respective internal standard and 50-mM sodium phosphate buffer (pH 7). These standards where then analyzed via LC-MS/MS, and MassHunter Quantitative Analysis software was used to quantify the concentration of the standards, produce a calibration curve and utilize the curve to quantify the concentration of PFAAs from the ED samples.

*Human serum albumin binding for perfluorooctanoic acid*

In this experiment, seventeen PFOA to HSA mole ratios were tested through equilibrium dialysis with 500-μM HSA. For each experiment the seventeen concentrations of PFOA tested were: 5, 25, 100, 250, 400, 600, 800, 1000, 1500, 1800, 2000, 2500, 3000, 3500, 4000, 6000 and 8000 μM. After the 48-hour equilibrium dialysis, samples were collected from the PFOA side of the plate and then diluted in an autosampler vial for LC-MS/MS analysis. During LC-MS/MS analysis, the concentration of free PFOA was determined via the PFOA standards and calibration curve. Once the free PFOA concentration was determined, the bound PFOA concentration was calculated

by subtracting the free PFOA concentration from the initial PFOA concentration. The binding coefficient was determined for each initial PFOA concentration by dividing the bound PFOA concentration by the concentration of HSA. A binding isotherm plot was created by plotting the binding coefficients versus the average free PFOA concentration. Curve fitting software, KaleidaGraph, was then used to determine the binding class model and binding constant ($K_a$).

*Human serum albumin binding for perfluoroctanesulfonic acid*

In this experiment, seventeen PFOS to HSA mole ratios were tested through equilibrium dialysis with 50-μM HSA. For each experiment the seventeen concentrations of PFOS tested were: 0.5, 2.5, 10, 25, 40, 60, 80, 100, 150, 180, 200, 250, 300, 350, 400, 600 and 800 μM. After the 48 hour equilibrium dialysis, the same procedure as above was followed.

*Human serum albumin binding for perfluorobutanoic acid*

In this experiment, seventeen PFBA to HSA mole ratios were tested through equilibrium dialysis with 500-μM HSA. For each experiment the nineteen concentrations of PFBA tested were: 5, 25, 100, 250, 400, 600, 800, 1000, 1500, 1800, 2000, 2500, 3000, 3500, 4000, 6000 and and 8000 μM. After the 48 hour equilibrium dialysis, the same procedure as PFOA was followed.

*Human serum albumin binding of perfluorbutanesulfonic acid*

In this experiment, seventeen PFBS to HSA mole ratios were tested through equilibrium dialysis with 500-μM HSA. For each experiment the seventeen concentrations of PFBS tested were: 5, 25, 100, 250, 400, 600, 800, 1000, 1500, 1800,

2000, 2500, 3000, 3500, 4000, 6000 and 8000 μM. After the 48 hour equilibrium dialysis, the same procedure as PFOA was followed.

*Human serum albumin binding for perfluorohexanesulfonic acid*

In this experiment, nineteen PFHxS to HSA mole ratios were tested through equilibrium dialysis with 500-μM HSA. For each experiment the nineteen concentrations of PFHxS tested were: 5, 25, 100, 250, 400, 600, 800, 1000, 1500, 1800, 2000, 2500, 3000, 3500, 4000, 6000 and 8000 μM. After the 48 hour equilibrium dialysis, the same procedure as PFOA was followed.

*Human serum albumin binding of perfluorododecanoic acid*

In this experiment, seventeen PFDoA to HSA mole ratios were tested through equilibrium dialysis with 50-μM HSA. For each experiment the seventeen concentrations of PFOS tested were: 0.5, 2.5, 10, 25, 40, 60, 80, 100, 150, 180, 200, 250, 300, 350, 400, 600 and 800 μM. After the 48-hour equilibrium dialysis, the same procedure as PFOA was followed.

*Negative and positive control tests*

Negative control tests were conducted (in triplicate) in order to test whether the experimental conditions had any effect on measured PFAA concentration. In this experiment, either 500 or 50 μM HSA was pipetted into an equilibrium dialysis well with 50-mM sodium phosphate buffer (pH 7) in the opposite well. After a 48-hour equilibrium dialysis, samples were collected from the buffer side of the plate and prepared for LC-MS/MS analysis. During the LC-MS/MS analysis, it was determined whether any measurable amount of PFAA could be detected.

Positive control tests were conducted (in triplicate) in order to test if the PFAAs were passing through the membrane and not sticking to the walls of the equilibrium dialysis wells. This was tested by calculating percent recovery values for each PFAA using the equilibrium dialysis method. In this experiment, 200 μM of each PFAA was pipette into an equilibrium dialysis well with 50-mM sodium phosphate buffer (pH 7) in the opposite well. After a 48-hour equilibrium dialysis, samples were collected from the PFAA side of the plate and then diluted in an autosampler vial for LC-MS/MS analysis. During LC-MS/MS analysis, the concentration of PFAA was determined via standards and calibration curves. Once the PFAA concentrations were determined, the sample dilutions were accounted for to determine the concentration in the PFAA side of the well. These values were then used to determine the percent recovery values for each PFAA using the equilibrium dialysis method.

## Results

*Perfluorooctanoic acid*

The PFOA binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFOA. This was done in previous work by Mike Morris ’14, Andrew Glaser ’16 and replicated in this study. It was determined that PFOA binds to HSA according to a two-class binding model. It was determined that the $K_{a1}$ was 1.1 (± 0.9) × $10^5$ $M^{-1}$ with n, the approximate number of PFOA molecules binding to that class of sites (the strong association sites), equaling 1.5 (± 0.9). It was also determined that the $K_a$ value for the second class of sites ($K_{a2}$) was 5.5 (± 0.9) × $10^3$ $M^{-1}$ with about 14.7 (± 0.6) PFOA molecules binding at that class of sites (the weak association sites). The binding isotherm for PFOA constructed by Andrew Glaser ’16 is shown in Figure 6.[42]

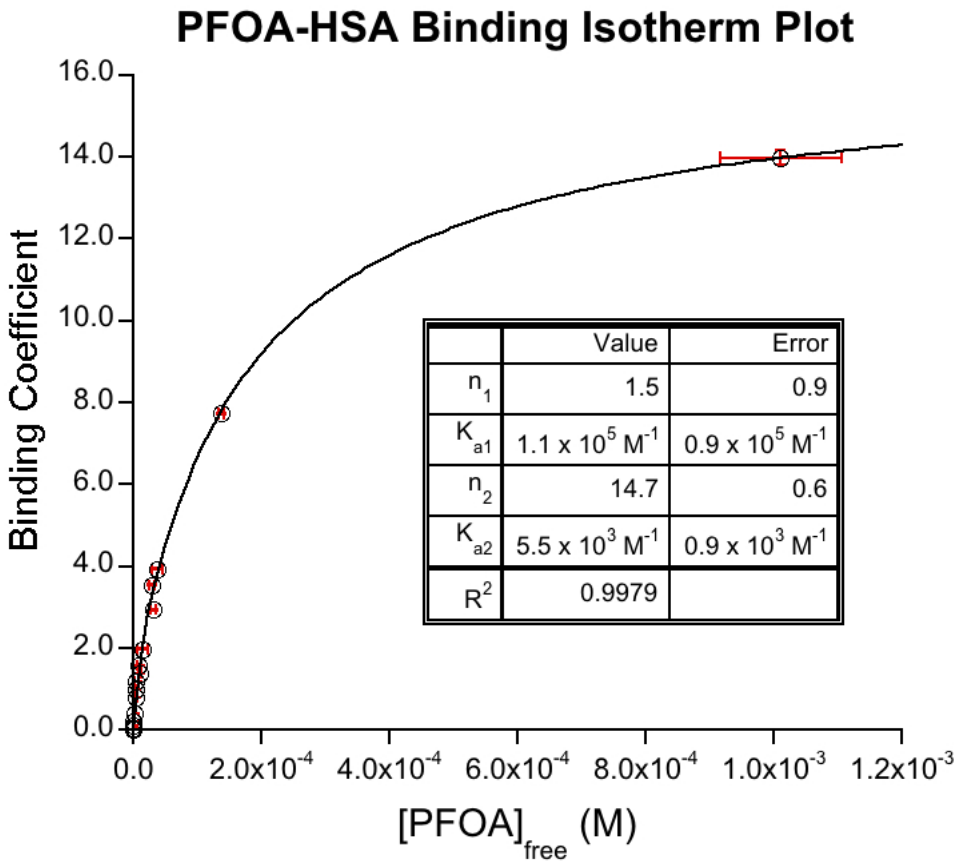


**Figure 6.** PFOA-HSA two-class binding isotherm determined via equilibrium dialysis by Andrew Glaser '16. This plot was created by plotting the binding coefficients versus the average free concentrations of PFOA. From this plot the $K_{a1}$ was determined to be 1.1 (± 0.9) × $10^5$ $M^{-1}$ and $n_1$ being 1.5 (± 0.9). Additionally the $K_{a2}$ was determined to be 5.5 (± 0.9) × $10^3$ $M^{-1}$ with $n_2$ being 14.7 (± 0.6).

*Perfluoroocatanesulfonic acid*

The PFOS binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFOS. It was determined that PFOS binds to HSA according to a two-class binding model. The $K_{a1}$ value was determined to be 5 (± 4) × $10^6$ $M^{-1}$ with an $n_1$ value of 1.4 (± 0.4) PFOS molecules binding at the first class of site. Additionally, the $K_{a2}$ was determined to be $9._6$ (± 2) × $10^4$ $M^{-1}$ with an $n_2$ value of 9.6 (± 0.5) PFOS molecules binding at the second class of sites. The binding isotherm determined for PFOS is shown in Figure 7.

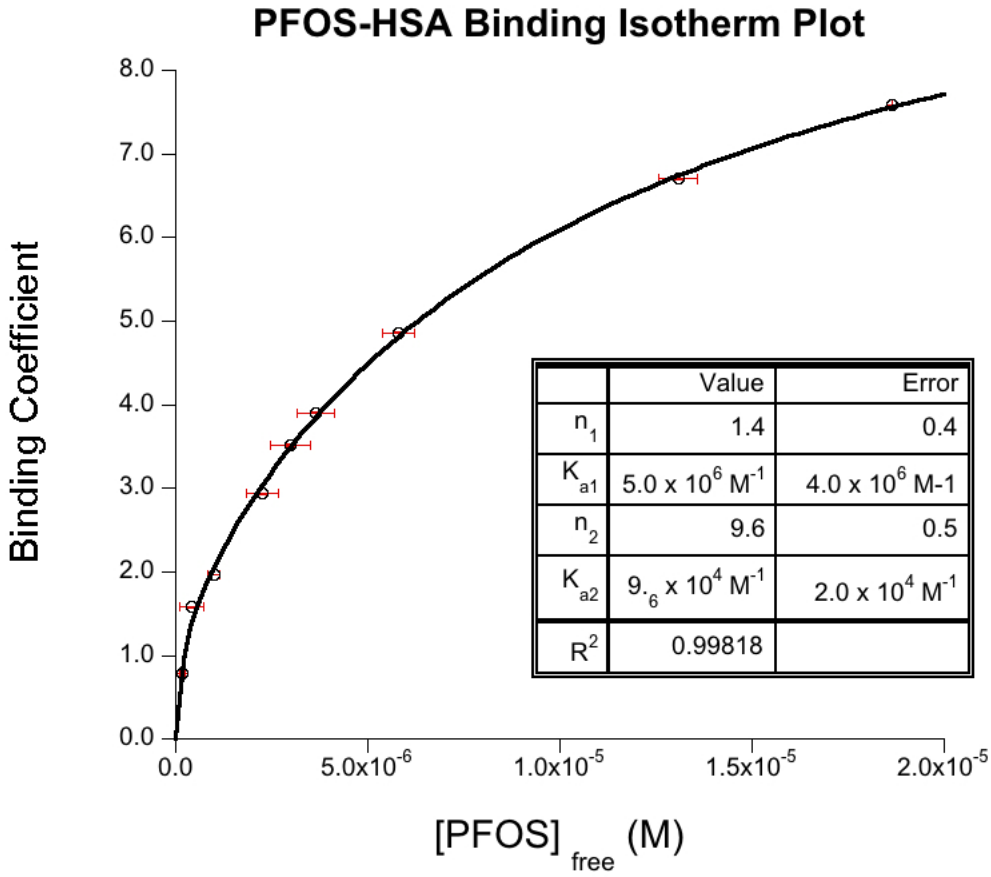


**Figure 7.** PFOS-HSA two-class binding isotherm plot determined via equilibrium dialysis. This plot was created by plotting the binding coefficient versus the average concentration of PFOS. From this plot the $K_{a1}$ was determined to be 5 (± 4) × $10^6$ $M^{-1}$ and $n_1$ value of 1.4 (± 0.4). Additionally the $K_{a2}$ was determined to be $9._6$ (± 2) × $10^4$ $M^{-1}$ with an $n_2$ value of 9.6 (± 0.5).

*Perfluorobutanoic acid*

The PFBA binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFBA. It was determined that PFBA binds to HSA according to a two-class binding model. The $K_{a1}$ value was determined to be 1.5 (± 0.9) × $10^5$ $M^{-1}$ with an $n_1$ value of 1.4 (± 0.3) PFBA molecules binding at the first class of sites. Additionally, the $K_{a2}$ was determined to be 9 (± 1) × $10^2$ $M^{-1}$ with an $n_2$ value of 11.2 (± 0.5) PFBA molecules binding at the second class of sites. The binding isotherm determined for PFBA is shown in Figure 8.

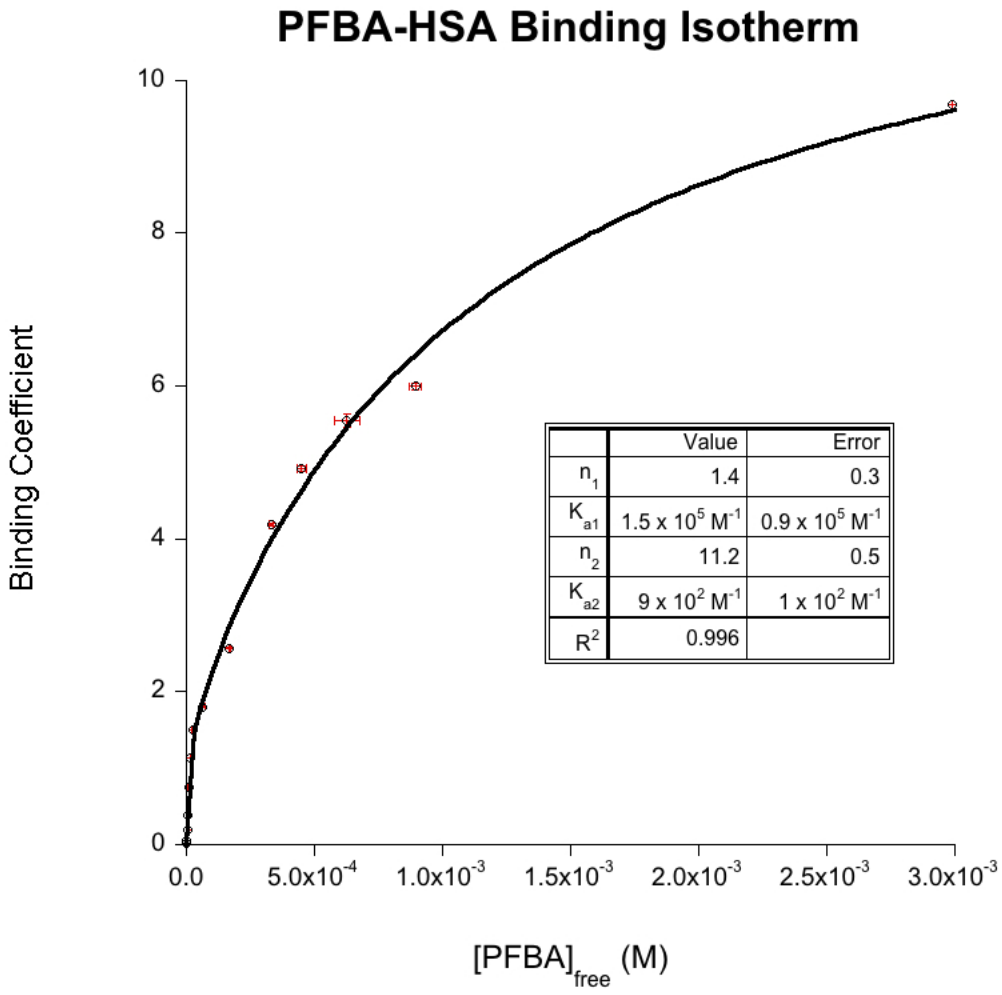


**Figure 8.** PFBA-HSA two-class binding isotherm plot determined via equilibrium dialysis. This plot was created by plotting the binding coefficient versus the average concentration of PFBA. From this plot the $K_{a1}$ was determined to be 1.5 (± 0.9) × $10^5$ $M^{-1}$ and $n_1$ value of 1.4 (± 0.3). Additionally the $K_{a2}$ was determined to be 9 (± 1) × $10^2$ $M^{-1}$ with an $n_2$ value of 11.2 (± 0.5).

*Perfluorobutanesulfonic acid*

The PFBS binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFBS. It was determined that PFBS binds to HSA according to a two-class binding model. The $K_{a1}$ value was determined to be 4 (± 1) × $10^4$ $M^{-1}$ with an $n_1$ value of 2.7 (± 0.3) PFBS molecules binding at the first class of sites. Additionally, the $K_{a2}$ was determined to be 1.8 (± 0.8) × $10^2$ $M^{-1}$ with an $n_2$ value of 24 (± 7) PFBS molecules binding at the second class of sites. The binding isotherm determined for PFBS is shown in Figure 9.

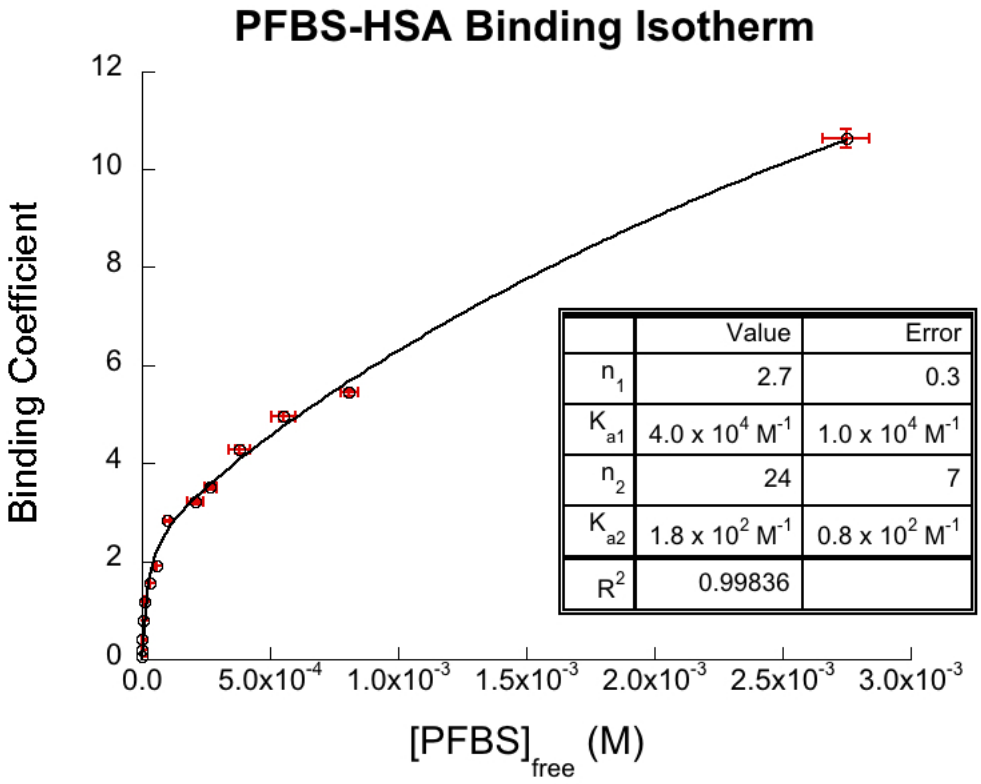


**Figure 9.** PFBS-HSA two-class binding isotherm plot determined via equilibrium dialysis. This plot was created by plotting the binding coefficient versus the average concentration of PFBA. From this plot the $K_{a1}$ was determined to be 4 (± 1) × $10^4$ $M^{-1}$ and $n_1$ value of 2.7 (± 0.3). Additionally the $K_{a2}$ was determined to be 1.8 (± 0.8) × $10^2$ $M^{-1}$ with an $n_2$ value of 24 (± 7).

*Perfluorohexanesulfonic acid*

The PFHxS binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFHxS. It was determined that PFHxS binds to HSA according to a two-class binding model. The $K_{a1}$ value was determined to be 1.6 (± .7) × $10^5$ $M^{-1}$ with an $n_1$ value of 2.5 (± 0.5) PFHxS molecules binding at the first class of sites. Additionally, the $K_{a2}$ was determined to be 2.0 (± 0.6) × $10^3$ $M^{-1}$ with an $n_2$ value of 24 (± 3) PFHxS molecules binding at the second class of sites. The binding isotherm determined for PFHxS is shown in Figure 9.

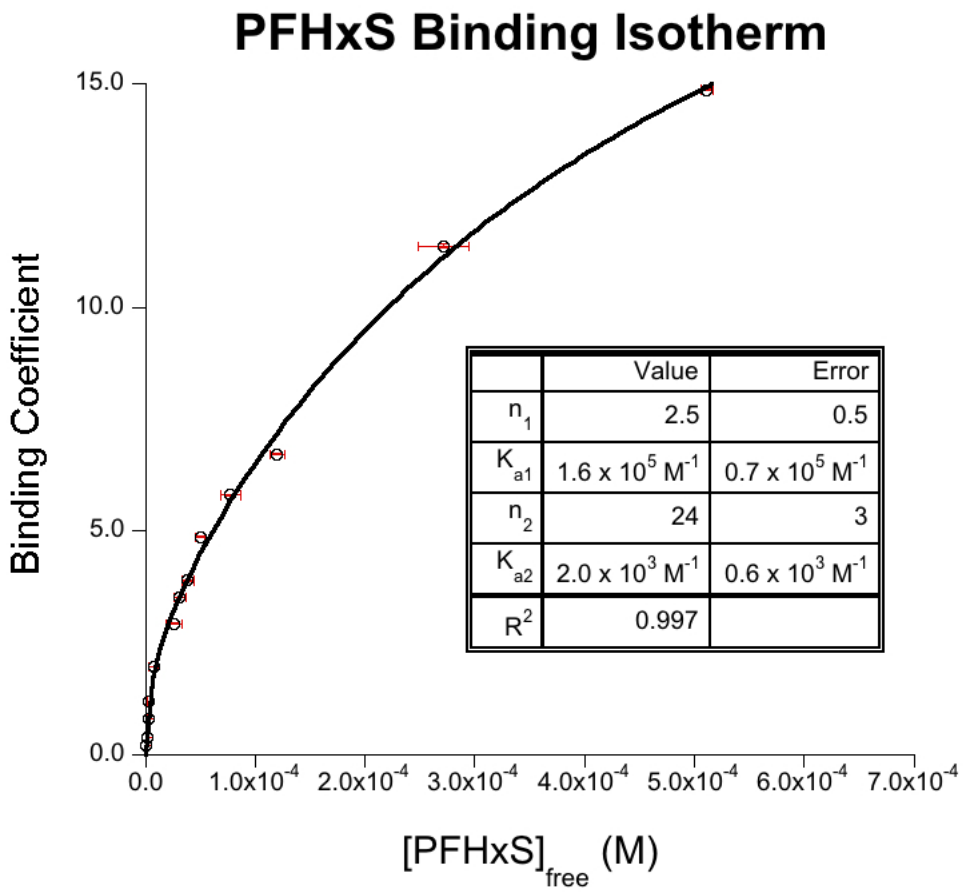


**Figure 10.** PFHxS-HSA two-class binding isotherm plot determined via equilibrium dialysis. This plot was created by plotting the binding coefficient versus the average concentration of PFBA. From this plot the $K_{a1}$ was determined to be 1.6 (± 0.7) × $10^5$ $M^{-1}$ and $n_1$ value of 2.5 (± 0.5). Additionally the $K_{a2}$ was determined to be 2.0 (± 0.6) × $10^3$ $M^{-1}$ with an $n_2$ value of 24 (± 3).

*Perfluorododecanoic acid*

The PFDoA binding isotherm was generated using the developed equilibrium dialysis method. The plot was created by plotting the binding coefficients versus the average free concentrations of PFDoA. It was determined that PFDoA binds to HSA according to a two-class binding model. The $K_{a1}$ value was determined to be 1.3 (± 0.8) × $10^6$ $M^{-1}$ with an $n_1$ value of 3.0 (± $0.9_6$) PFDoA molecules binding at the first class of sites. Additionally, the $K_{a2}$ was determined to be 2.9 (± $0.9_7$) × $10^4$ $M^{-1}$ with an $n_2$ value of 13.8 (± 0.9) PFDoA molecules binding at the second class of sites. The binding isotherm plot determined for PFDoA is shown in Figure 10.

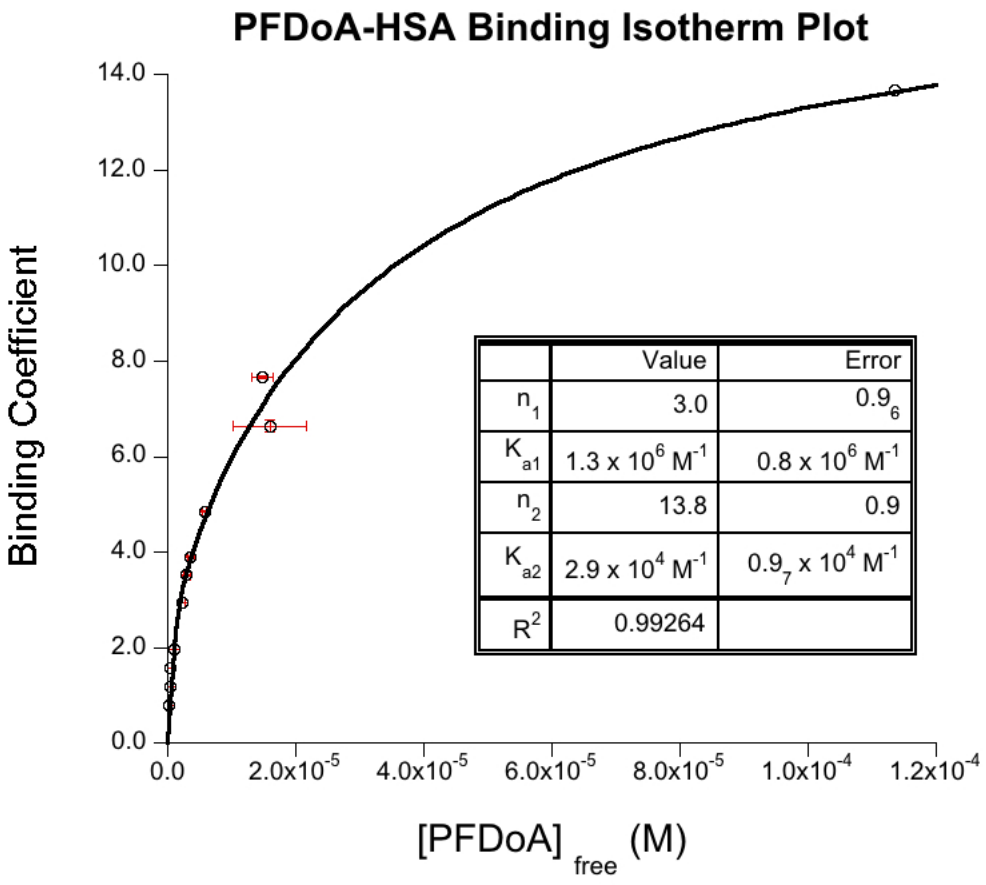


**Figure 11.** PFDoA-HSA two-class binding isotherm plot determined via equilibrium dialysis. This plot was created by plotting the binding coefficient versus the average concentration of PFDoA. From this plot the $K_{a1}$ was determined to be 1.3 (± 0.8) × $10^6$ $M^{-1}$ and $n_1$ value of 3.0 (± $0.9_6$). Additionally the $K_{a2}$ was determined to be 2.9 (± $0.9_7$) × $10^4$ $M^{-1}$ with an $n_2$ value of 13.8 (± 0.9).

*Summary of binding isotherm results*

Since each PFAA exhibited a two-class binding model, it allows for the comparison of all of the values derived from the binding isotherm plots. The $K_{a1}$, $n_1$, $K_{a2}$ and $n_2$ values for each PFAA are shown in Table 4.

**Table 4.** Summary of the $K_a$ and n values derived for each PFAA using the two-class binding model

| **PFAA** | **$K_{a1}$ ($M^{-1}$)** | **$n_1$ (sites)** | **$K_{a2}$ ($M^{-1}$)** | **$n_2$ (sites)** |
|---|---|---|---|---|
| PFBA | 1.5 (± 0.9) x $10^5$ | 1.4 (± 0.3) | 9.0 (± 1.0) x $10^2$ | 11.2 (± 0.5) |
| PFOA | 1.1 (± 0.9) x $10^5$ | 1.5 (± 0.9) | 5.5 (± 0.9) x $10^3$ | 14.7 (± 0.6) |
| PFDoA | 1.3 (± 0.8) x $10^6$ | 3 (± $0.9_6$) | 2.9 (± $0.9_7$) x $10^4$ | 13.8 (± 0.9) |
| PFBS | 4.0 (± 1.0) x $10^4$ | 2.7 (± 0.3) | 1.8 (± 0.8) x $10^2$ | 24.0 (± 7.0) |
| PFHxS | 1.6 (± 0.7) x $10^5$ | 2.5 (± 0.5) | 2.0 (± 0.6) x $10^3$ | 24.0 (± 3.0) |
| PFOS | 5.0 (± 4.0) x $10^6$ | 1.4 (± 0.4) | $9._6$ (± 2.0) x $10^4$ | 9.6 (± 0.5) |

Since each PFAA displayed a two-class binding model, each type of $K_a$ value could be compared to see if a relationship between association strength and chain length and ionic head group could be determined. The $K_{a1}$ and $Ka_2$ values for the carboxylates and sulfonates are compared in Figures 12 and 13, respectively.

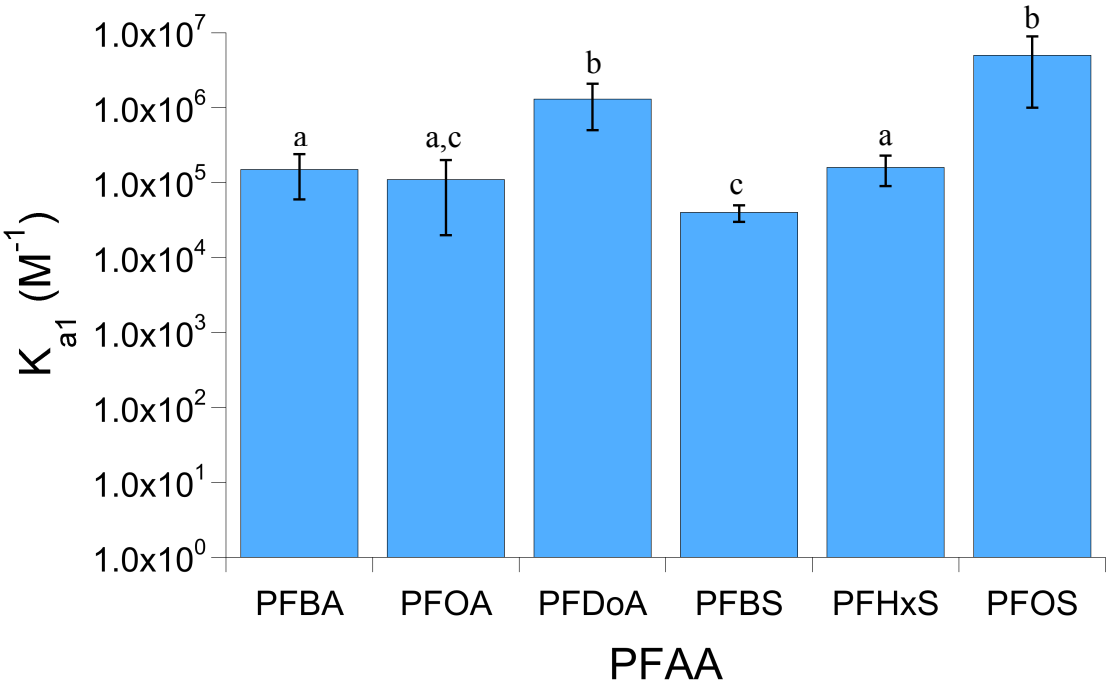


**Figure 12.** Bar graph displaying the $K_{a1}$ values for the six PFAAs tested in this study. A distinct relationship can be seen for the $K_{a1}$ values between increasing chain length and increasing association strength amongst the sulfonates, while the carboxylates do not display this relationship. The replacement carboxylate, PFBA, seems to associate just as strongly as the previously used carboxylate, PFOA. Different letters denote statistically significant differences among $K_{a1}$ values ($p < 0.05$).

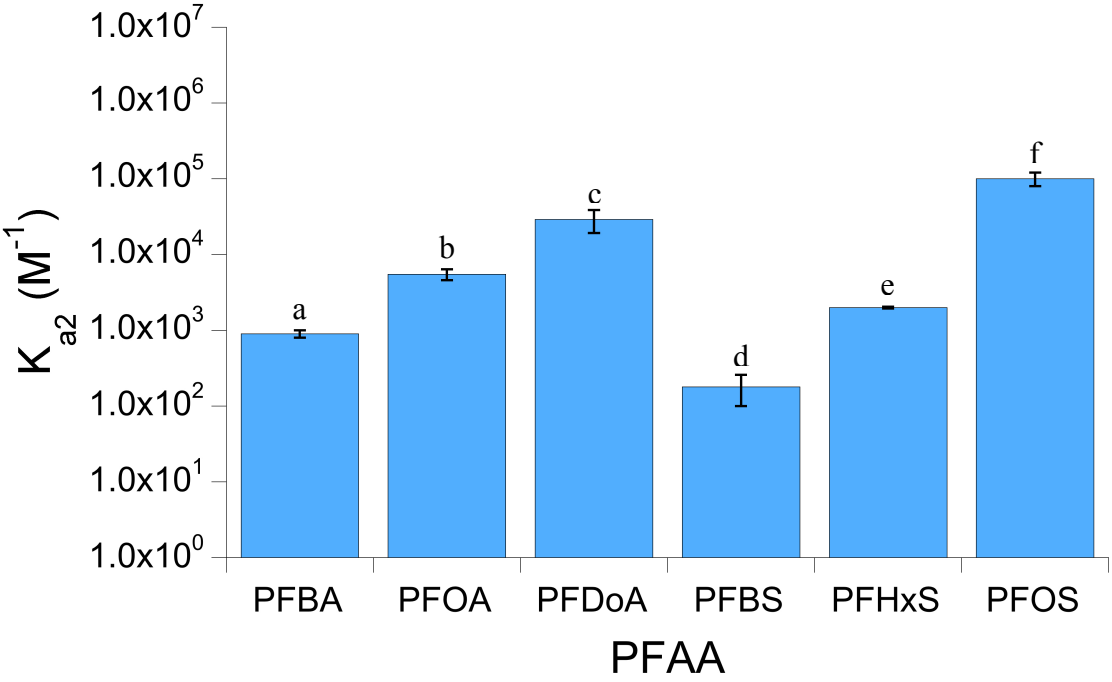


**Figure 13.** Bar graph displaying the $K_{a2}$ values for the six PFAAs tested in this study. A distinct relationship between increasing chain length and increasing association strength can be seen for the $K_{a2}$ values amongst both the carboxylates and sulfonates used in this study. Different letters denote statistically significant differences among $K_{a2}$ values ($p < 0.05$).

*Negative and positive control results*

Equilibrium dialysis samples containing no added PFAAs were run as negative controls through the full equilibrium dialysis experiment. No PFAAs were detected in any of the negative control tests. Positive control equilibrium dialysis samples containing a concentration of 200 μM for PFBA, PFBS, PFHxS and PFOA, and 20 μM for PFOS and PFDoA were run through the method. The measured concentrations and percent recoveries for each PFAA can be seen in Table 5.

**Table 5.** Concentrations and percent recoveries for each PFAA from the positive control tests

| | **PFBA** | **PFOA** | **PFDoA** | **PFBS** | **PFHxS** | **PFOS** |
|---|---|---|---|---|---|---|
| **Concentration (μM)** | 207.6 | 196.6 | 2.03 | 211.18 | 195.3 | 12.58 |
| **Percent Recovery** | 104% | 98% | 10%* | 106% | 98% | 63%* |

*These PFAAs will be run through a positive control test again.

## Discussion

*Understanding the strong and weak association strengths of the various PFAAs*

All the PFAAs tested in this study associate to HSA according to a two-class binding model, meaning there are two types of sites at which PFAAs bind to HSA with different affinities ($K_{a1}$ and $K_{a2}$) and numbers of PFAA molecules ($n_1$ and $n_2$).[42] The two types of sites may be classified as high-affinity binding sites ($K_{a1}$ and $n_1$) and lower-affinity binding sites ($K_{a2}$ and $n_2$). The $n_1$ values for the 6 PFAAs range from ~1-3, which means that ~1-3 PFAA molecules bind at the high-affinity site(s) on HSA. For the lower-affinity site(s), many more PFAAs bind demonstrated by the $n_2$ values ranging from ~10-24 PFAA molecules at the lower-affinity site. The $n_1$ values demonstrated here agree with the crystal structure of PFOS bound to HSA. Previous work has shown that approximately 2 PFOS molecules bind to HSA with high-affinity,[45] which agrees with what was seen for all PFAAs.

As each PFAA binds to HSA according to a two-class binding model, comparisons of both $K_a$ values can be done to see if a relationship between association strength and PFAA chain length and ionic head group exists. It should also be noted that only $K_{a1}$ values are available in the literature, and the values derived in this experiment agree with the higher reported $K_{a1}$ values. These discrepancies can be attributed to the other studies either using spectroscopic binding studies (which are indirect methods and not the gold standard for protein binding studies) or due to the fact that many of these studies had limited data points to the inconsistencies in the equilibrium dialysis methods used. Both of these factors make it hard to compare the calculated $K_{a1}$ values to any previously determined values.

There is a clear relationship between increasing chain length and increasing association strength at the high-affinity sites ($K_{a1}$) for the PFSAs (Figure 12). For the PFCAs, this relationship is not as distinct (Figure 12). PFBA and PFOA display similar $K_{a1}$ values while PFDoA has a larger $K_{a1}$ value, demonstrating that the same relationship between increasing chain length and increasing association strength as seen for the PFSAs does not exist at the high-affinity sites for the PFCAs. For the lower-affinity sites, there is a clear relationship between increasing chain length and increasing association strength for both PFCAs and PFSAs (Figure 13). As the PFAA chain length increases for both ionic head groups, the association strength increases as well, though all of the $K_{a2}$ values are significantly lower than the $K_{a1}$ values.

The observed relationship of increasing chain length and increasing association strength amongst PFSAs can be related to the hydrophobicity of the chemicals. Previous work has shown that the soil adsorption strength of PFAAs increases with increasing hydrophobicity.[46] Additionally, previous work with PFOA and PFDA (perfluorodecanoic acid) has shown that increasing hydrophobicity was a major contributor to increasing association strength to BSA.[47] This previous work supports some of the data presented in here and help to explain some of the observed relationships, specifically for the PFSAs. For the PFCAs, a systematic relationship between chain length and association strength was only observed at the lower-affinity sites. This relationship may also be explained by the increasing hydrophobicity of the longer chain PFAA molecules.

However, the systematic relationship between chain length and association strength is not observed for the PFCAs at the high-affinity sites. PFDoA does have a stronger association constant when compared to PFOA, which is comparable to previous

studies,[47] but PFBA associates just a strongly as PFOA. This result is comparable to previous $K_{a1}$ data derived for PFBA. Bischel et al. found that PFBA and PFPnA (perfluoropentanoic acid, another short chain PFAA) had high association constants when binding to BSA,[48] which were comparable to the association constants derived for PFOA in an earlier paper.[39] This can possibly be explained by the size and rigidity of PFBA compared to PFOA. PFBA is a smaller molecule, possibly allowing it to more easily fit into binding pockets of HSA, resulting in the apparent comparable $K_{a1}$ values. Previous work has shown that the size and shape of PFAAs affect association strengths, showing that branched isomers of PFAAs have weaker association strengths than the linear PFAAs.[49] Additionally, previous work with fluorinated compounds has found that fluorinated molecules are extremely rigid in nature, but longer fluorinated molecules are more rigid then shorter fluorinated molecules.[50] PFBA is a much shorter chain PFAA than PFOA, meaning that it may be less rigid, and this lack of rigidity may cause the apparent comparable $K_{a1}$ value. To compare to another short chain PFAA, PFBS has a slightly lower $K_{a1}$ than PFBA and PFOA. It is both less hydrophobic than PFOA, but more rigid and larger than PFBA. This can help to explain how rigidity, size and hydrophobicity of PFAAs play a role in PFAA-HSA association at the high-affinity sites.

The results presented here can potentially provide insight into what factors affect the strength of association of PFAAs at each of the types of association sites on HSA. Due to the distinct relationship between chain length and association strength displayed for both the PFCAs and PFSAs at the lower-affinity sites, hydrophobicity seems to be the main factor affecting association at this site. Due to the differences seen in the association strengths for the various PFAAs at the high-affinity sites, other factors besides

hydrophobicity seem to be affecting association strengths. Based on what is known about fluorinated chemicals, it seems that size, rigidity and hydrophobicity are affecting the association strengths at the high-affinity sites on HSA.

*Evaluating the association strengths of the replacement PFAAs*

There is a general lack of sufficient data on the association of PFBA and PFBS to HSA, which is concerning since these are two of the replacement PFAA chemicals used in industry. Here we present new data on the high-affinity association of the short-chain PFAAs to HSA, as well as the first data on the lower-affinity association of these chemicals. Understanding the association constants for both of these PFAAs allows for their evaluation as replacement chemicals in the manufacture of everyday products, such as non-stick pans and oil- and water-repellant clothing. The $K_{a1}$ values presented here for PFBA and PFBS are slightly lower than those previously reported for short-chain PFAAs in one previous equilibrium dialysis study.[48] However, the lack of data points and smaller range of PFAA:HSA mole ratios tested (and, therefore, much larger errors) in the previous study make it difficult to compare to the $K_{a1}$ values derived here. Additionally, the previously reported $K_{a1}$ values were determined using BSA as the model protein instead of HSA, also limiting our ability to make comparisons to prior data.

The magnitudes of the $K_{a1}$ values for these chemicals allows for the determination of whether or not PFBA and PFBS are smart choices as replacement chemicals. The $K_{a1}$ values are more important for understanding whether or not these chemicals are good replacements because these values represent high-affinity association to HSA and most likely represent how PFAAs are transported in the human body. PFBA binds to HSA

with a $K_{a1}$ of 1.5 (± 0.9) x $10^5$ $M^{-1}$, which is similar to the high-affinity association strength of PFOA (1.1 (± 0.9) x $10^5$ $M^{-1}$), which is the chemical PFBA replaced. Therefore, PFBA may not be a suitable replacement for PFOA. PFBS binds to HSA with a $K_{a1}$ of 4.0 (± 1.0) x $10^4$ $M^{-1}$, which is weaker than the high-affinity association strength of PFOS (5.0 (± 4.0) x $10^6$ $M^{-1}$), the chemical that PFBS replaced. However, PFBS binds only moderately weaker to HSA than PFOA, which calls into question whether PFBS is a suitable PFAA replacement chemical to use. Additionally, since both of these replacement chemicals do bind to HSA, they still have the potential to bioaccumulate in the body and could potentially display adverse effects.

*Evaluation of the negative and positive control tests*

The negative control tests were conducted to see if the developed method was subject to any background PFAA signals. The test resulted in each PFAA having a concentration of 0 μM, which means is accurately reporting the free PFAA concentration. This means that the measured concentrations for the various PFAAs evaluated using this method are due to the amount of PFAA left over in the equilibrium dialysis well and are not influenced by other factors in the method.

The positive control tests were conducted to ensure that the measured free concentrations of PFAAs at equilibrium were representative and not affected by the possible binding of the PFAAs to the walls of the equilibrium dialysis plates. In order to validate the method, a goal percent recovery of 80-120% was established for each PFAA. PFBA, PFOA, PFHxS and PFBS resulted in percent recoveries of 104%, 98%, 98% and 106%, respectively. These results mean that these PFAAs are traveling through the

membrane and coming to equilibrium with the other well and are not adhering to the walls of the plates. PFDoA and PFOS resulted in percent recoveries of 10% and 63%, which is not ideal. These 2 chemicals will be run again to ensure that all 6 of the PFAAs are traveling through the membrane.

**Conclusions**

In this study, the factors affecting the association of PFAAs to HSA at two different classes of sites were observed. Due to the chain length dependent relationship displayed at the lower-affinity sites, it seems that hydrophobicity plays a major role in association at those sites, with the longer and more hydrophobic PFAAs having higher association constants. At the high-affinity sites, it seems that additional factors affect the association of each PFAA. The association strengths of the PFSAs display the same chain length dependence, while those for the PFCAs do not. Due to PFBA having a higher than expected association constant when looking at hydrophobicity alone, it seems that factors such as rigidity, shape and size play a role in the strength of association at these sites. Additionally, based on the strength of the observed $K_{a1}$ values of PFBA and PFBS, these chemicals may not be suitable replacements, especially PFBA since it associates just as strongly as PFOA at the high-affinity sites. Lastly, this is the first study to use a high-throughput method to systematically investigate the effects of chain length and ionic head group on PFAA-HSA binding, and the data gathered here represent the type of data that are needed for additional replacement chemicals and other poly- and perfluoroalkyl substances that are still in use.

**Future Work**

Immediate future work involves positive control tests will be run with PFDoA and PFOS, to determine if these chemicals travel across the equilibrium dialysis membrane. These are vital in testing the validity of the developed method. Additionally, future work involves studying the binding of PFAAs under varying conditions such as pH, temperature and ionic strength in order to test the affects of these variables on association strength. Long-term goals involve exploring PFAA-HSA binding through competitive binding studies in order to test whether the presence of fatty acids inhibits the binding of PFAAs to HSA.

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## Appendix

*Sample chromatograms of PFAAs*

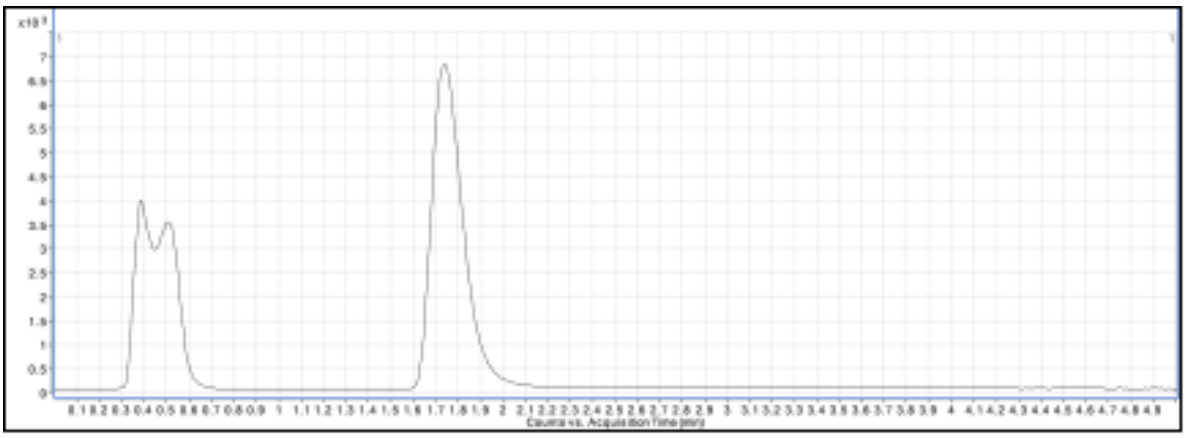

**Figure 14.** Sample chromatogram of PFBA with internal standard mPFBA. The peaks on the left are mPFBA with the peak on the right being PFBA.

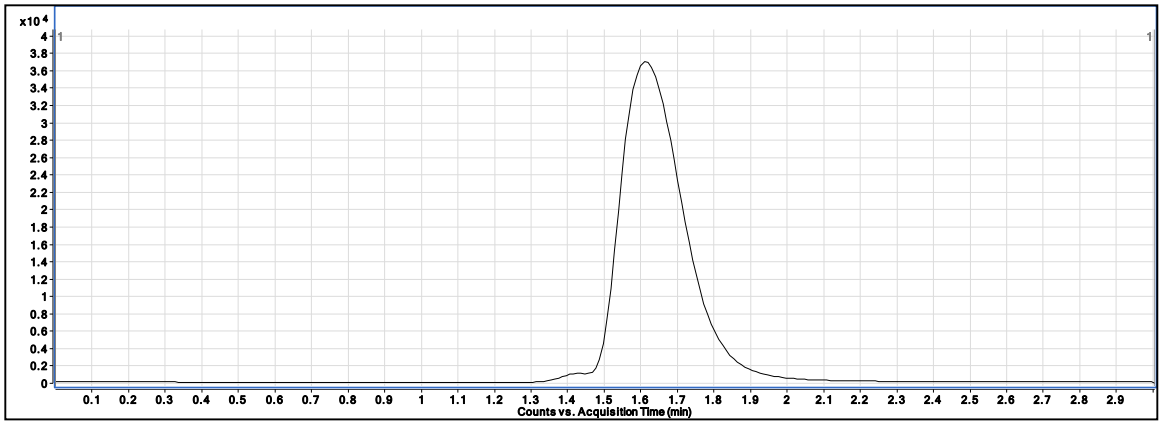


**Figure 15.** Sample chromatogram of PFOA with internal standard mPFOA. Both elute at similar times resulting in a singular peak.

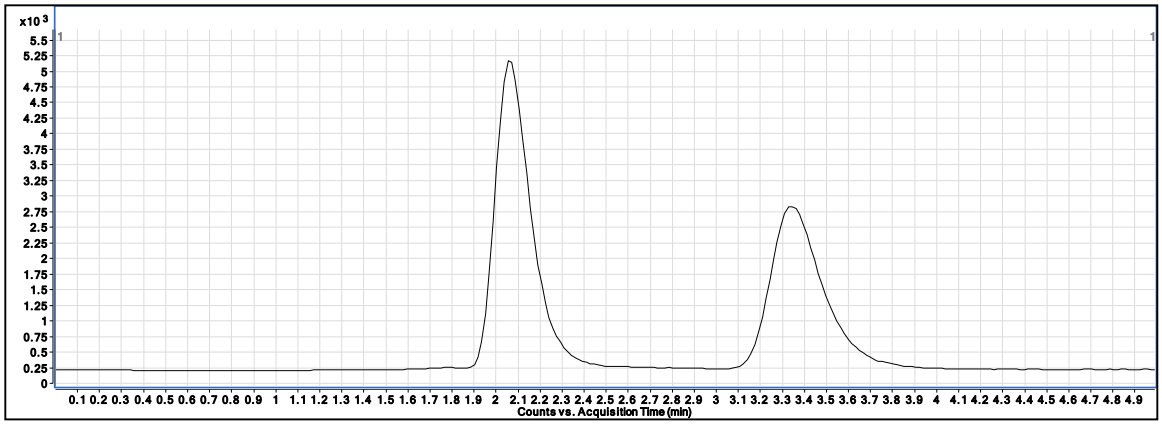


**Figure 16.** Sample chromatogram of PFDoA with PFUnA as the internal standard. PFUnA is the peak on the left and PFDoA is the peak on the right.

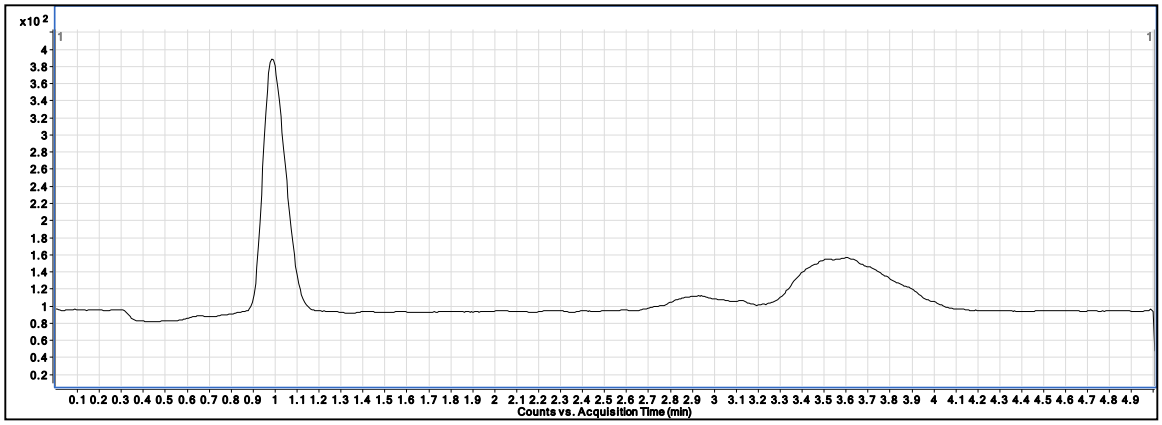


**Figure 17.** Sample chromatogram of PFBS with PFHxS as the internal standard. PFBS is the peak on the left and PFHxS is the peak on the right.

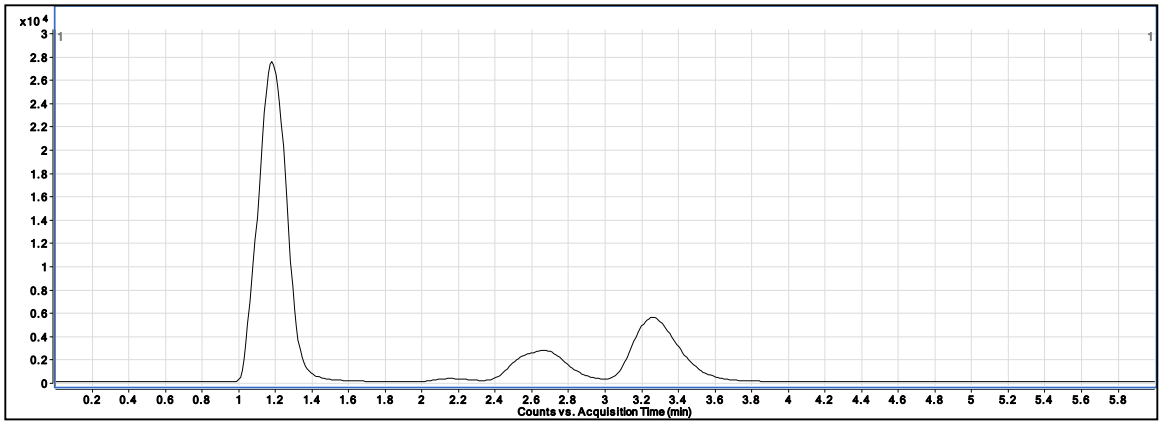


**Figure 18.** Sample chromatogram of PFHxS with PFOS as the internal standard. PFHxS is the peak on the left and PFOS is the group of peaks on the right.

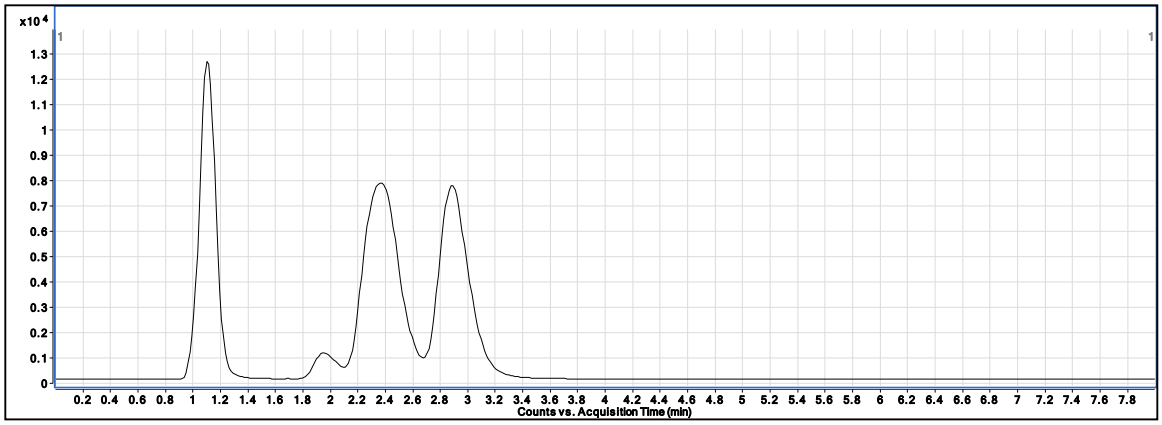


**Figure 19.** Sample chromatogram of PFOS with PFHxS as the internal standard. PFHxS is the peak on the left and PFOS is the group of peaks on the right.

*Sample calibration curves for PFAAs*

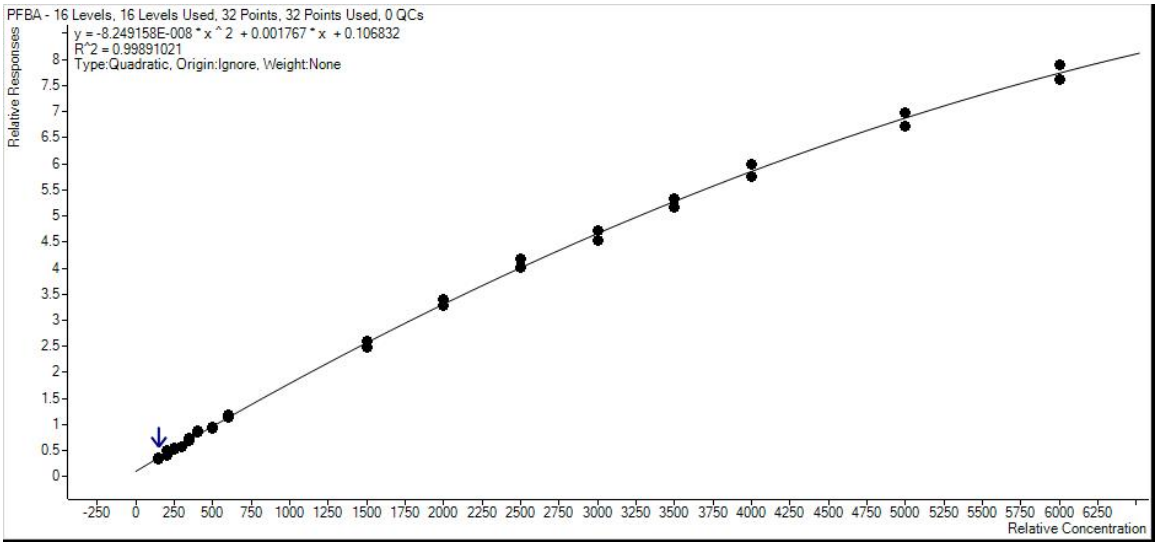


**Figure 20.** Sample calibration curve used to quantify the concentration of free PFBA post equilibrium dialysis. The fit is quadratic and has an $R^2$ of 0.9989.

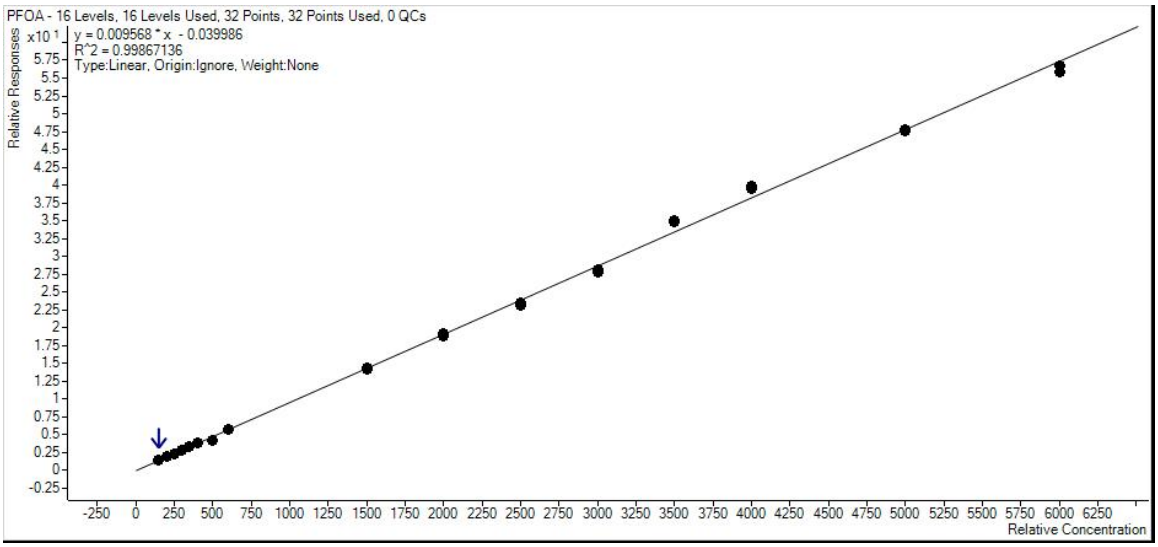


**Figure 21.** Sample calibration curve used to quantify the concentration of free PFOA post equilibrium dialysis. The fit is linear and has an $R^2$ of 0.9986.

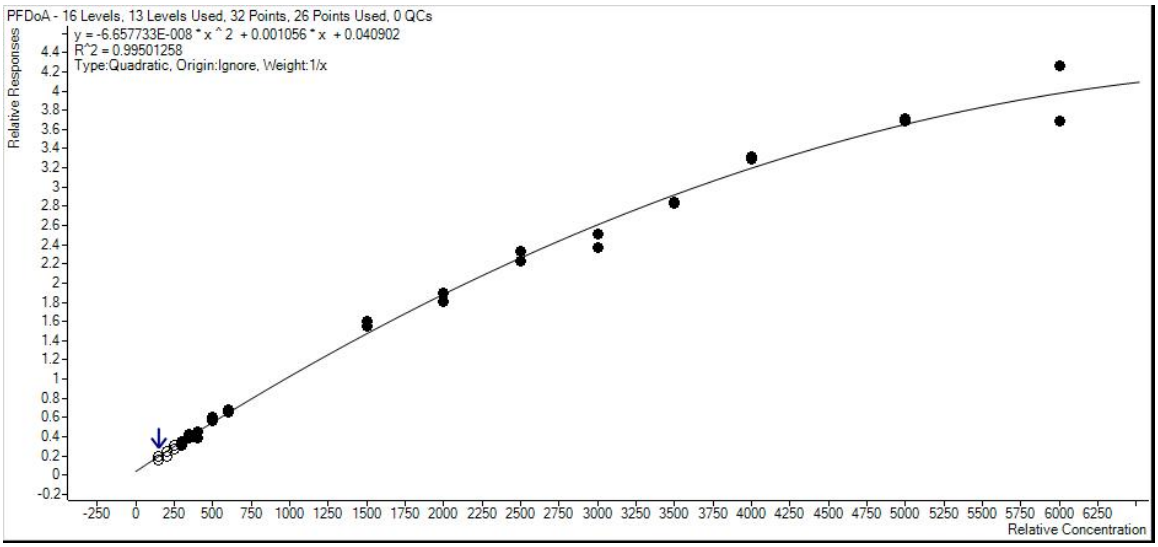


**Figure 22.** Sample calibration curve used to quantify the concentration of free PFDoA post equilibrium dialysis. The fit is quadratic and has an $R^2$ of 0.995.

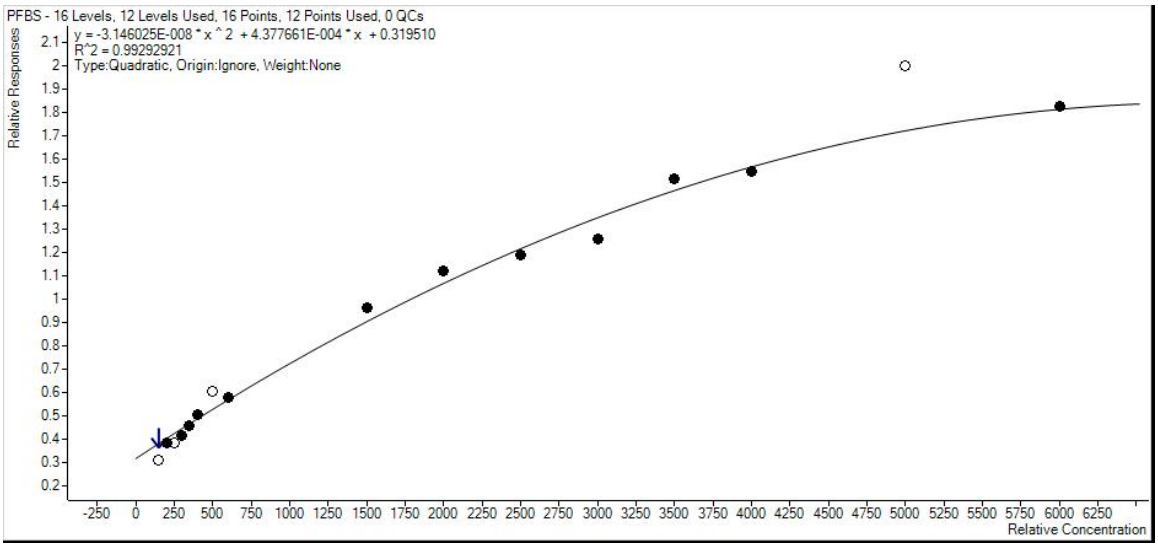


**Figure 23.** Sample calibration curve used to quantify the concentration of free PFBS post equilibrium dialysis. The fit is quadratic and has an $R^2$ of 0.9929.

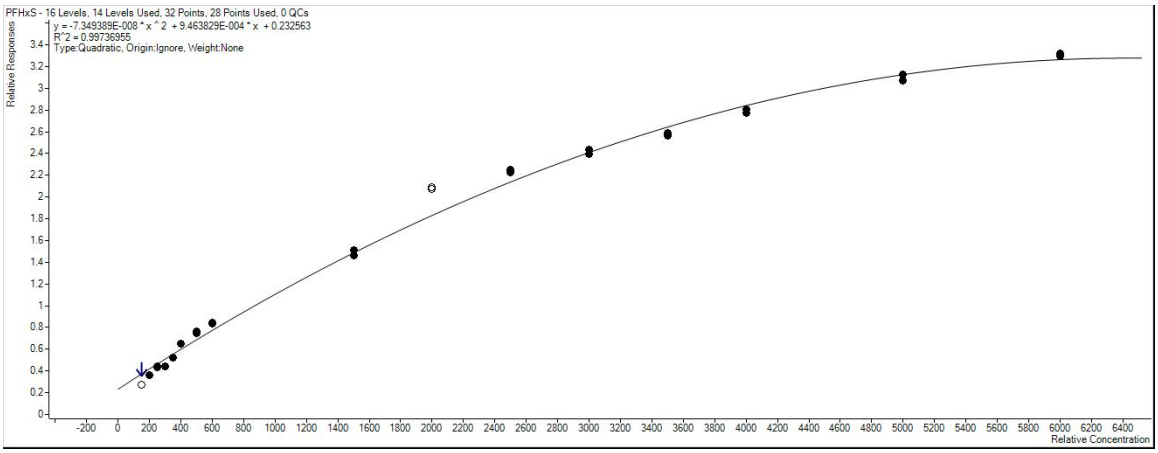


**Figure 24.** Sample calibration curve used to quantify the concentration of free PFHxS post equilibrium dialysis. The fit is quadratic and has an $R^2$ of 0.9973.

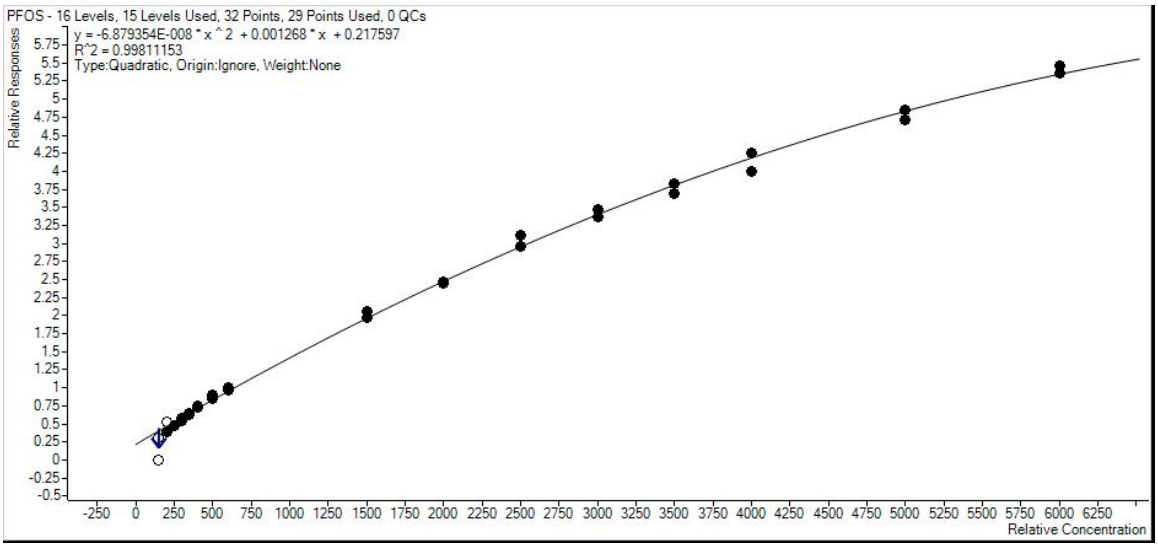


**Figure 25.** Sample calibration curve used to quantify the concentration of free PFOS post equilibrium dialysis. The fit is quadratic and has an $R^2$ of 0.9981.