# DETERMINATION OF SINGLE AND COMPETITIVE BINARY ADSORPTION ISOTHERMS FOR R AND S OPTICAL ISOMERS OF FLUOXETINE

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# ABSTRACT

The pharmaceutical industry has come to rely on isotherm data when working with preparative chromatography. The data derived during isotherm measurement is used to optimize experimental conditions which will be used in the making of drugs.

For the R- and S- enantiomers of Fluoxetine, four different methods were used to determine the single component isotherms and this data was fitted to the Langmuir Isotherm Model. Both enantiomers, when tested singly, fit the model using the Frontal Analysis Method and Retention Time Method, but did not fit the model when using the FACP and ECP methods.

The major objective of this research was to determine whether the single component isotherm measurement can be used to predict the multi-component isotherm in order to avoid the cumbersome, tedious and very time consuming procedures for measuring the multi-component isotherm. Prediction of a multi-component isotherm using single component isotherms is in most cases impossible due to the competition between the two solutes and the difference in column saturation capacity of the two compounds. The two components of a chiral compound have very similar physical properties and therefore they should have similar column saturation capacities. The competitive binary isotherm was determined on the racemic mixture of Fluoxetine using the Frontal Analysis Method for Multi-Component Isotherms. It was then compared to values predicted from the Single Component studies and it was determined that the Multi-Component isotherm could be predicted from data obtained from the isotherms of the single components.

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# DEDICATION

I would like to dedicate this work to Gayle, without whom the process would not have even gotten started.

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# INTRODUCTION

A large amount of research has been devoted to the study of thermodynamic function in the form of adsorption isotherms as they relate to chromatographic separation. The usefulness of the findings has become accepted in a variety of different areas, including preparative chromatography, production scale chromatography and chromatographic purification. The practical importance of liquid chromatography deals almost exclusively with the separation of multi-component mixtures, especially with enantiomeric separation and also the separation of impurities from the active pharmaceutical ingredient (API) of a drug product. Adsorption isotherms quantitatively describe the equilibrium distribution of a solute between the two phases involved in the chromatographic process (mobile and stationary) over a wide concentration range. They give information about the solvent, solute, and adsorbent, and the interactions between these three. Adsorption isotherms are becoming a major tool for the investigation of the processes involved in chromatographic retention. (Jacobson et all, 1987) They also play a key role in the analysis of preparative and production scale chromatography. Determination of the equilibrium isotherms can be the starting point for computerized optimization of the preparative chromatographic process (Mihlbachler et all, 2002). An extensive examination of the isotherm will also provide the needed data for a chromatographic purification and/or separation process. Although this technique for optimization of chromatographic purification is useful and important in the pharmaceutical and biotechnology industries, it is not an easy task and as of yet has not become an efficient and routine part of the preparative or production process (Seidel-Morgenstern, 2004).

Chiral compounds are difficult to separate. The two components are mirror images of each other and therefore they have many of the same physical and chemical properties.

However, the need to have optically pure enantiomers for many modern pharmaceuticals is evident (Zhou et all, 2003). Because of the differences in toxicity and effectiveness between the two enantiomers, separation on a large scale is very important for the pharmaceutical industry. Frequently, one enantiomer will be effective, while the other has little or no activity, and might even be toxic. Therefore, it is necessary to obtain large amounts of the pure chiral isomer through preparative chromatographic separation. Preparative chromatography makes use of either large volumes (column overloading) or high concentrations (concentration overloading) of the racemic mixture of the API being used. The concentration overloading is more efficient and economical. There are two types of preparative chromatography, large scale and semi-preparative. Isolating and using a large amount of the purified compound is performed during large scale preparative chromatography. Large scale preparative chromatography is performed when purification and production of enantiomers, peptides and proteins are needed (Guiochon et all, 2006). Semi-preparative scale chromatography is used when a small amount of pure minor compound is needed for characterization, structural identification toxicological or pharmacological data. Because the preparation of the optically pure enantiomer is a difficult and expensive process, methods based on computer-assisted optimization are used to aid in the chiral separation for increased speed, productivity and reduced cost. In order to optimize the separation using chromatographic modeling with the aid of a computer, the competitive equilibrium isotherms of the two enantiomers are needed.

Adsorption Isotherms

An adsorption isotherm of a compound represents the adsorption equilibrium of that compound from a solution. This adsorption equilibrium is what regulates the separation of two enantiomers in a chiral compound, especially at high concentration. It is needed for the design

and optimization of preparative chromatography. When the solution being considered has more than one sorbable component at high concentration, the components will compete with each other and influence each other's adsorption. Isotherms obtained under these conditions are called "competitive isotherms" (Jacobson, 1987). While trying to separate multi-component mixtures, the isotherms of the individual components can not always be used, due to competition for the adsorption sites between the components. In ideal cases the multi-component isotherms can be predicted from single component isotherms; however in a majority of cases this is not possible. A multi-component isotherm must be measured in a way that describes the behavior of the individual solutes and the influence they have over one another. There are many different methods for determining single component isotherms. The most widely used chromatographic methods for determining single component isotherms are the following: Frontal Analysis (FA), Frontal Analysis by Characteristic Point (FACP), Elution by Characteristic Points (ECP) and the Retention Time Method (RTM) (Guiochon, 2006). For multi-component isotherms, the determining method is Frontal Analysis for multi-component isotherms also called Competitive Frontal Analysis. The FACP and ECP methods can not be extended to determine multi-component isotherms.

Adsorption Isotherm Measurement

Isotherms are measured so that the mechanisms of retention of a system can be understood and used in a practical way to achieve separation, and it is necessary to use it in preparative chromatographic modeling to optimize the separation and achieve maximum production with minimum cost. Equilibrium in liquid-solid chromatography occurs between the solid stationary phase and the liquid mobile phase and their interactions between the components of the compound being studied. The equilibrium isotherm is a plot of the concentration of the

component in the stationary phase versus the concentration of the component in the mobile phase.

The interface between a gas and a solid is much simpler than the liquid-solid interface; therefore more scientific literature can be found describing the gas-solid equilibria. There are two theories for gas-solid equilibria that provide insight and give more understanding of liquid-solid equilibria. The first theory is the Gibbs equation. It relates the amount of the component adsorbed at the interface to interface tensions or surface tension. A theory put forth by Brunauer, Emmett, and Teller (BET) describes the phase equilibria of multilayer adsorption at the gas-solid interface (Gritti, 2003). Due to competition by the mobile phase and sometimes other components of the system for binding sites on the solid phase, it is more difficult to determine the mechanism of component retention in liquid-solid chromatography.

Langmuir Isotherm Model

The Langmuir isotherm model is the most common equlilbrium isotherm model (equation 1). It was developed by Irving Langmuir in 1916 to describe the dependence of the surface coverage of an adsorbed gas on the pressure of the gas above the surface. It has since been shown to be useful in modeling equilibrium isotherms (Nix, 2003).

$$\frac{Q}{Q_s} = \frac{bC}{1 + bC} \qquad (1)$$

or

$$Q = \frac{aC}{1 + bC} \qquad (2)$$

Q = adsorbed amount of solute in the stationary phase in equilibrium with the concentration of solute in the mobile phase.

b = an experimental constant related to the energy of adsorption

C = concentration of solute

Qs = column saturation capacity

a = slope of isotherm at very low concentration

This equation is derived with the assumptions that the solution is ideal, the solute gives monolayer coverage, there are no solute-solute interactions in the monolayer, there are no solvent-solute interactions, and the adsorbed layer is ideal. Even though most of these assumptions may not be completely valid for liquid-solid adsorptions, there is experimental data that shows that the Langmuir isotherm is an excellent approximation for single component adsorption equilibriums in liquid-solid chromatography. Many examples can be found in scientific literature showing that the model is valid at low concentrations. Originally used for single layer adsorption, the Langmuir adsorption isotherm gives a curve that depicts the fraction of adsorbent covered with the solute as a function of concentration. The Langmuir isotherm is a curve which is sharper while the surface of the adsorbent is not yet covered with solute, and flattens out when that coverage is complete. The Langmuir isotherm for double layer adsorption has also come into use and is similar to single layer adsorption with the initial portion of the curve being sharper.

Methods for Determination of Single Component Isotherms

Frontal Analysis (FA) was first used to determine adsorption isotherms in the 1950's. Frontal Analysis can be defined as the relationship of the solute concentration in the liquid phase to the concentration at the surface of the solid phase over a concentration range. It is performed by making abrupt step changes of increasing concentration at the column inlet and determining the breakthrough curves (Guiochon et all, 2006). The retention time of the breakthrough curve is

used to calculate the amount of the compound adsorbed (Q) at concentration (C) using equation 3.

$$\frac{\Delta Q}{\Delta C} = \frac{V_r - V_0}{V_a} = \frac{V_0}{V_a} \times \frac{t_r - t_0}{t_0} = \frac{1}{F} \times \frac{t_r - t_0}{t_0} \quad (3)$$

$t_r$ = retention time of the breakthrough curve (corrected for system delay time)

$t_0$ = void time of the column

F = phase ratio of the chromatographic system.

$V_o$=column void volume

$V_a$=volume of adsorbent in the column

The phase ratio is defined by equation 4.

$$F = \frac{V_a}{V_0} = \frac{1-\varepsilon}{\varepsilon} \quad (4)$$

ε = porosity of the column.

More than one experiment must be performed in order to determine the single component isotherm using the FA method. Each experiment gives one point on the isotherm. This method is more accurate than the others because the inflection point of the breakthrough curve is independent of kinetics, it depends only on the thermodynamic properties of the system; however the preparation of the series of solutions at known concentrations can be time consuming and require a large amount of the pure compound (Guiochon et all, 2006). Using a liquid chromatograph with a gradient delivery system that can accurately deliver precise concentrations of the solute in a stepwise fashion can reduce the experimental error and make the experimental

design simpler; however it will not eliminate the need for a large amount of material. FA works best with narrow bore columns, which help to decrease the amount of chemicals and solvents needed to perform the testing. Frontal Analysis is the most accurate chromatographic method for the determination of adsorption isotherms.

Another procedure for determining a single component isotherm is Frontal Analysis by Characteristic Point (FACP). The rear boundary of the elution profile is used to determine Q and C. This method can use the rear boundary recorded during FA analysis or by making a negative step change from a certain concentration back to pure mobile phase. A large concentration step is pumped onto the column and allowed to plateau. Then the solution is replaced with pure mobile phase. The isotherm is derived from the profile that is recorded during this wash. Another way to measure the isotherm using FACP is by using a wide rectangular injection. Only one experiment is needed to obtain the entire isotherm measurement, because the concentration is changed only once. The main disadvantage of FACP comes from the fact that the calculation ignores the kinetic effect and assumes the elution profile is dependant only on thermodynamics (Guiochon et all, 2006). This is not a correct assumption because the elution profile depends on both thermodynamics and kinetics. This will allow for error at especially low concentrations where the kinetic effect is more significant. The problem can be alleviated (error can be reduced) if a column with high theoretical plates is used because it has less kinetic effect. The adsorption isotherm is calculated from the points on the rear profile of the curve using equation 5. This equation is derived assuming that there is no kinetic effect, that the number of theoretical plates of a column is infinite.

$$Q = \frac{1}{V_a}\int_0^c (V - V_0)dc = \frac{V_0}{V_a}\int_0^c \left(\frac{t_r - t_0}{t_0}\right)dc = \frac{1}{F}\int_0^c \frac{t_r - t_0}{t_0}dc \qquad (5)$$

$V_a$ = volume of adsorbent in the column

$V_0$ = void volume of the column

F = phase ratio ($F = V_a/V_0$)

V = retention volume of the diffuse boundary at concentration C

$t_0$=column void time

$t_r$=retention time of diffuse boundary at concentration C (corrected for system delay time)

Since the integration is calculated from zero concentration of solute, data points at low concentrations cannot be disregarded and thus error is inherent in the isotherm.

Elution by Characteristic Point (ECP) is yet another method for determining a single point isotherm. ECP is similar to FACP however it only requires that a limited amount of solute be injected on the system to give an elution peak. This method is based on the idea that velocity can be associated with concentration and it, in fact, depends on the concentration. In this method, the isotherm is derived from the back portion of an overloaded elution profile. This profile is obtained by injecting a large sample. Just as with the FACP method, the biggest disadvantage of the ECP method is the assumption that the elution profile is dependant only on thermodynamics. It is based on the assumption that the column is infinitely efficient, which is not the case in laboratory situations. Another disadvantage of both the FACP and ECP methods is the need for concentration of the rear boundary. Unfortunately, the detector response is not linear at the higher concentrations needed for isotherm measurement. A separate experiment is needed to determine the UV responses of a series of steps of known concentration. Next, the concentration versus absorption is fit to a polynomial equation to derive another equation. This final equation is used to calculate the concentration from the UV response measurement of the

rear boundaries. Equation 4 is also used to calculate the isotherm using data from the ECP method with one exception. Since the sample is directly injected on the column $t_r$ is not corrected for system delay time.

The last commonly used method for single component analysis that will be discussed here is the Retention Time Method (RTM) (Golshan-Shirazi, 1987). The RTM will only work if an isotherm follows the Langmuir model. If the isotherm to be determined by the RT method is Langmuirian, using a very small and a very large size sample injection can determine the isotherm parameters. The retention time of the small injection under linear conditions can be used to determine the capacity factor ($K'_0$) and the retention time of the front of the large injection can be used to determine the column saturation capacity. Adsorption isotherms that are Langmuirian are linear at low concentrations. When a small amount is injected, the retention time of the peak is obtained under linear conditions. This retention time is proportional to the initial slope of the isotherm. This method will fail if the isotherm does not follow the Langmuir model. It is the easiest and quickest way to determine a single component isotherm and does not need a large amount of sample. However, it cannot be applied to the determination of any other type of isotherm.

There are many isotherm models available if it is found that the Langmuir model is not appropriate. The data from any of these methods, except the Retention Time Method, can be fitted to the appropriate isotherm models and then the resulting isotherm model can be used in order to study a wide rage of factors influencing the separation of enantiomers of chiral compounds or any other preparative separation.

Methods for Determination of Multi-Component Isotherms

Frontal Analysis for Multi-Component (Competitive Frontal Analysis) is performed by preparing a solution containing a constant relative composition of the two components that are being studied. The solutions are pumped through the HPLC column starting with the low concentration and moving up step by step to the high concentration, using several steps. Then the curves are recorded. The breakthrough curves are complex and the isotherm is related to the breakthrough retention times as well as the concentration of each of the solutes in the plateaus; therefore it is necessary to obtain the concentrations at each of the intermediate plateaus. This can be done by collecting the fraction at each intermediate plateau and injecting in a second chromatograph with a short high-efficiency column to determine the concentration of each solute at each intermediate plateau ($C_{i,m}$) using an external standard method. From the breakthrough times and the plateau concentrations, the competitive isotherm can be determined by writing a mass balance equation for each solute around the breakthrough curve therefore this method is called the Method of Mass Balance (MMB) (Jacobson 1987). The isotherms of the two components can be determined by writing the mass balance equation of each component around each breakthrough curve using Equation 6 or 7.

$$Q_i = Q_{i-1} + \frac{(V_2 - V_0)(C_{i,b} - C_{i,a}) - (V_2 - V_1)(C_{i,m} - C_{i,a})}{V_{ads}} \tag{6}$$

$$Q_i = Q_{i-1} + \frac{1}{F}\left[\left(\frac{t_{r2} - t_0}{t_0}\right)(C_{i,b} - C_{i,a}) - \left(\frac{t_{r2} - t_{r1}}{t_0}\right)(C_{i,m} - C_{i,a})\right] \tag{7}$$

$V_0$ = column dead volume

$V_1$ = elution volume of the first breakthrough front

$V_2$ = elution volume of the second breakthrough front

$V_{ads}$ = the volume of adsorbent in the column

$C_{i,a}$ = the concentration of component *i* in the mobile phase in the column ahead of the first front

$C_{i,b}$ = the concentration of component *i* in the mobile phase in the column behind the second front

$C_{i,m}$ = the concentration of component *i* in the mobile phase in the intermediate plateau

F = phase ratio = $\frac{Va}{V_o} = \frac{1-\varepsilon}{\varepsilon}$

$t_{R1}$ = the retention time of the intermediate breakthrough curve (corrected for system delay time)=$t_{Rm1}$-$\tau$

$t_{R2}$ = the retention time of the final breakthrough curve (corrected for system delay time)=$t_{Rm2}$-$\tau$

$t_0$ = void time of the column

$Q_i$ = concentration of component i in the stationary phase

Competitive frontal analysis is the only method available for determination of competitive multi-component isotherms. Method of Mass Balance (MMB) is the only general procedure which can be used for calculation of competitive isotherms from the resulting breakthrough curves. When the isotherm of the two solutes is constructed they can be fitted to a variety of available isotherm models. The measured isotherm is for a certain constant relative concentration. If the isotherm is needed for another composition ratio of two solutes, the study must be repeated entirely and it is very burdensome and time consuming.

There are several other procedures which can be used for calculation of competitive isotherm of multi-component from the resulting breakthrough curves of competitive frontal

analysis if the multi-component isotherm is assumed to follow the competitive Langmuir isotherm model.

$$Q = \frac{a_i C_i}{1 + \sum b_i C_i} \tag{8}$$

These procedures are the Method of Composition Velocity (MCV), the Method of Mezzanine Concentration (MMC) and the Hybrid Method of Mass Balance (HMMB) (Jacobson, 1990). The experimental procedure is the same for all of these methods by performing frontal analysis of a mixture of solutes at constant relative composition.

The Method of Composition Velocity (MCV) is a procedure used to determine the best coefficients of a competitive Langmuir isotherm using a set of data obtained through competitive frontal analysis. The competitive velocity of the breakthrough fronts (shock waves) will be measured therefore the method is called The Measurement of Composition Velocity (MCV) (Jacobson 1987). These velocities are related to the competitive Langmuir isotherm parameters ($a_i$ and $b_i$). Therefore by assuming Langmuir isotherms $a_i$ and $b_i$ will be determined from the velocity of each shock wave. The advantage of this procedure is that there is no need to collect and measure an intermediate plateau, but it is only valid if the isotherm follows the competitive Langmuir isotherm model.

The Method of Mezzanine Concentration (MMC) takes a non-liner regression of the intermediate plateau concentrations (mezzanines) obtained during the frontal analysis experiment. This regression is fitted to the intermediate plateau equation and the concentration is derived assuming a Langmuir isotherm. This regression gives estimates of the isotherm, assuming it is Langmuirian. Like MMB, the intermediate plateau concentration is needed to determine the isotherm. MMC is only valid for Langmiurian isotherm models. There is no point

in using the MMC procedure over MMB, since in both procedures the concentration of solutes must be measured on an intermediate plateau. After these measurements, it is much easier to use Equation 7 (MMB method) which is independent of the isotherm method rather than using MMC which is only valid for Langmuir isotherms.

The Hybrid Method of Mass Balance (HMMB) uses equation 7 which is also used for the Method of Mass Balance (MMB), however instead of collecting fractions and determining $C_{i,m}$ in each intermediate plateaus, these concentrations will be estimated from the equations derived assuming it is a Langmuir isotherm. This method is much simpler than MMB because there is no need to collect and measure the concentrations of solute in the intermediate plateaus. The isotherm can be determined from the retention times of the breakthrough fronts, but it is only valid if the isotherm follows the Langmuir isotherm model.

Purpose

In this research, the following methods were examined in more detail: Frontal Analysis, Frontal Analysis by Characteristic Point, Elution by Characteristic Point, Retention Time Method for single component isotherms, Frontal Analysis Method for Multi-Component Isotherms, implementing the Method of Mass Balance (MMB). Data was tested to determine if it fit the Langmuir Isotherm Model.

In preparative chromatography, determining the absorption isotherm is very important. If the material being used has more than one component, the isotherm will be a competitive binary isotherm. Determination of the competitive binary isotherm is very cumbersome and time consuming. By determining the single and multi-component isotherms of a chiral compound, this research attempted to show that the multi-component isotherm of a chiral compound could be predicted from the single component isotherms of the individual enantiomers.

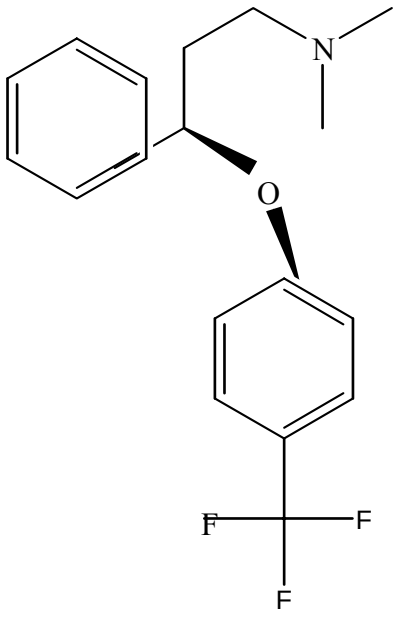


Figure 1: Chemical structure of Fluoxetine

The single component isotherms were determined on the R- and S- enantiomers of Fluoxetine, and the muti-component isotherm was determined using the racemic mixture.

Fluoxetine is a selective serotonin-reuptake inhibitor. It is used for the treatment of obsessive-compulsive disorders and depression. The most common form of fluoxetine used is the racemate form; however the individual isomers do not have the same activity. The racemic drug has established safety and clinical data, therefore measures need to be taken to ensure that each batch of Active Pharmaceutical Ingredient (API) is racemic and does not have an excess of either enantiomer.

## EXPERIMENTAL

### Equipment

The High-Performance Liquid Chromatography (HPLC) equipment used for this research was a Hewlett-Packard 1100 equipped with a gradient pump, temperature controlled column compartment, temperature controlled autosampler, and ultraviolet (UV) variable wavelength detector.

The column used for chiral separation was a Chiralcel OD-R (Cellulose tris(3,5 – dimethylphenyl carbamate) on a 10 μm silica-gel substrate) , 250 x 4.6 mm, 10 μm particle size column supplied by Daicel (Chiral Technologies, Inc.). This column was chosen because of its availability and versatility for chiral separation.

To determine if the materials tested had an acceptable UV absorbance, a Ultraviolet/Visible Spectrophotometer equipped with both Tungsten and Deuterium lamps was used.

### Chemical Information

The following chemicals were purchased from Sigma-Aldrich and were used throughout this research:

Penicillamine

Naproxen Sodium

Cycloserine

R-Fluoxetine

S-Fluoxetine

Fluoxetine Hydrochloride

Hexafluorophosphate buffer (Potassium Hexafluorophosphate) was used as the buffer portion of the mobile phase for the reverse phase HPLC.

Organic phases used were

Acetonitrile (Mallinckrodt, HPLC grade)

Hexane (Fisher, ACS grade)

Isopropyl Alcohol (Fisher, HPLC grade)

Diethylamine (DEA) (Fisher, ACS grade)

Computer Software

Chromatographic data was collected by Waters Millennium acquisition software, version 4.0. The software was used to measure retention times of the peaks, peak areas, and the retention times of the breakthrough curves. Microsoft Excel 2003 was used to calculate Q using the experimental data. It was used to determine the linear regression of the data using a Scatchard plot. It was also used to plot Q (concentration of compound on adsorbed phase) vs. C (concentration in the mobile phase).

Column Void Time Determination

The column void time, $t_0$, was required for measurements of the isotherm. The void time of the column is the length of time required for an inert component to pass through the void volume of the column and elute after it is injected onto the column. Column void time can be estimated from the solvent front peak of the chromatogram or it can be measured by injecting a non-retained material onto the column. Void time was measured by injecting a non-retained material, in this case sodium nitrate (1 mg/mL), on to the column. A peak will appear in the chromatogram at the time that it takes the sodium nitrate to flow through all the lines of the system and freely through the column. The retention time of the peak was equal to the void time of the column ($t_0$). See Figure 2 for an example chromatogram of the solution used to determine column void time.

System Delay Time Determination

In order to accurately determine the retention time of the breakthrough curve during frontal analysis, the system delay time, $\tau$, was needed. This time represented the amount of time required for the compound to travel from the solvent reservoir through the HPLC system to the beginning of the column. The system delay time was measured by replacing the column with a zero-volume union and determining the breakthrough

time of acetone. Pure methanol was pumped through the system. At the start time of the determination, the pump was switched to a mixture of acetone and methanol so that a breakthrough curve of acetone was produced (see Figure 3). The retention time of the breakthrough curve of acetone was used to determine the system delay time. The measured retention time of the fronts in frontal analysis was then corrected by subtraction from the system delay time.

Porosity and Phase Ratio Determination

In order to calculate the amount of a compound absorbed by the stationary phase given the concentration in the mobile phase, the porosity and phase ratio of the column must be determined. The porosity (ε) is defined as the ratio of the volume of the column not occupied by the stationary phase (Vo) to the total volume of the column (V column)

$$\varepsilon = \frac{Vo}{Vcolumn} \tag{9}$$

Vo = column void volume

The volume of the column not occupied by the stationary phase (column void volume) can be calculated using Equation 10.

$$Vo = t_0 \times F_v \tag{10}$$

$t_0$ = void time of column

$F_v$ = mobile phase flow rate

The total volume of the column can be calculated using the column dimensions.

$$Vcolumn = \frac{\pi \times D^2 \times L}{4} \tag{11}$$

D = column diameter

L = column length

If the column dimension, flow rate and $t_0$ are known, the column porosity (ε) can be calculated. Then, the phase ratio (F) is calculated using equation 4.

Frontal Analysis Method for Single Component Isotherm

The FA method determines the isotherm through a series of stepwise increases in the concentration of Fluoxetine. Using the HPLC system, one line was inserted into a

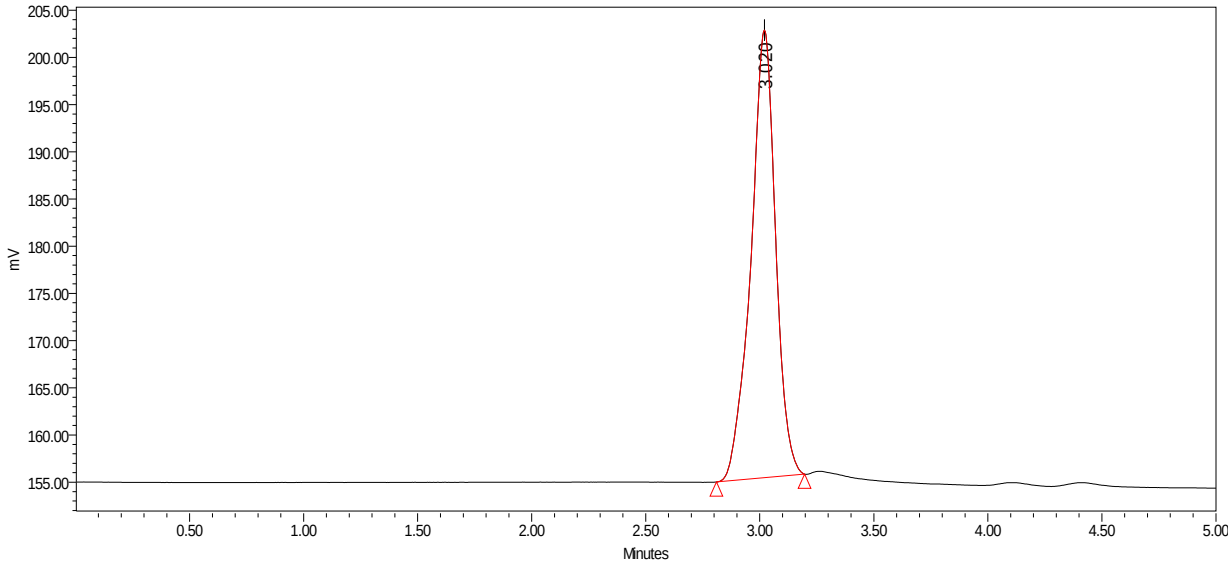


Figure 2: Example Chromatogram of Sodium Nitrate used to determine column void time

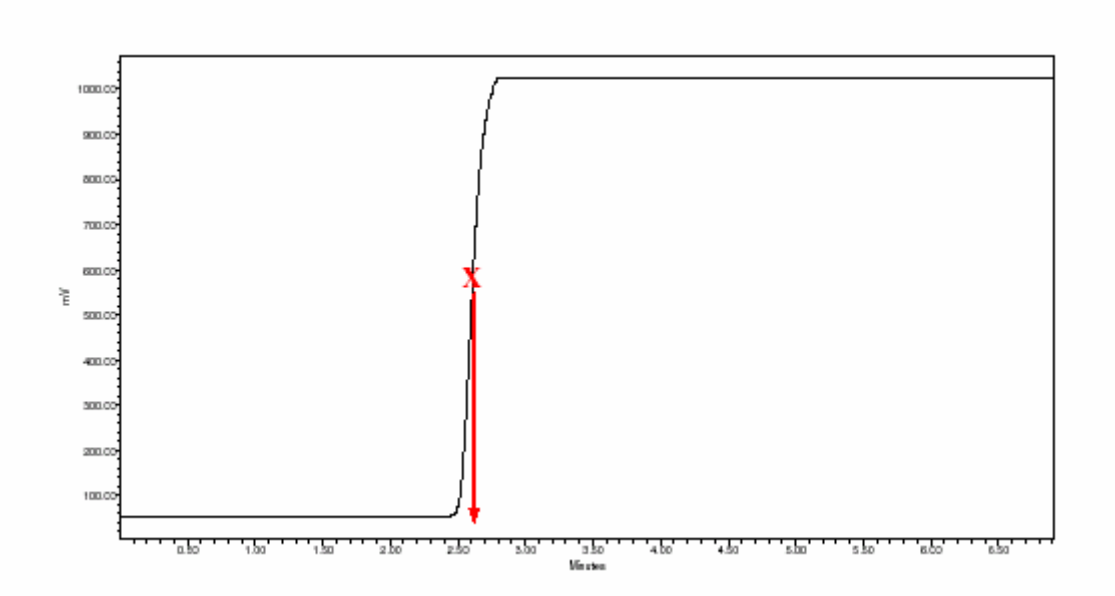


Figure 3: Example Chromatogram of breakthrough curve of acetone used to determine system delay time

reservoir of 100% Mobile Phase (line A), and the other was inserted into a reservoir containing a predetermined concentration of one or the other enantiomers of Fluoxetine in Mobile Phase (line B). The amount pumped through the system from line B was increased step-wise at 10% intervals from 0% until it reached 100% and the retention time of the breakthrough curve was recorded. These retention times relate to $\Delta Q/\Delta C$.

Because the concentration of the solute in mobile phase and retention times are known, Q was determined using equation 3, and from the values of Q, the isotherm was also constructed.

FACP Method

Isotherm measurements were also performed using the FACP method. The chromatographic system set up for the FA method was also used for the FACP method. After obtaining the plateau for the final concentration, 100%, the pumps were adjusted so that pure Mobile Phase was being pumped through the system. The retention times of the resulting curve correlated to dQ/dC and the isotherm was derived using equation 5. Calculating an isotherm using the FACP method requires the concentration of the diffuse boundary at each time point. Unfortunately, at high concentrations the detector is not linear, therefore it is necessary to perform additional experiments measuring the UV absorbance at successive step increases of the concentrate and fitting the concentration versus the UV absorbance to a polynomial to determine the equation which will be used to determine concentration for the UV absorbance of the diffuse boundary. Closed integration of equation 5 is impossible, therefore it can be written as a sum of integrals.

$$Q = \left( \left( \frac{\left(X_n + X_{n-1}\right)}{2} \right) \times \left(C_n - C_{n-1}\right) \right) + Q_{n-1} \qquad (12)$$

with

$$Xn = \frac{1}{F} \times \left( \frac{\left( t_{Rn} - t_0 - \tau \right)}{t_0} \right) \qquad (13)$$

C = concentration of solute

$t_{Rn}$ = retention time of diffuse boundary at concentration $C_n$

$t_o$ = column void time

$\tau$ = system delay time

F = phase ratio

ECP Method

The single component isotherm was also measured using the ECP method. A large, known concentration of Fluoxetine was injected onto the HPLC system and the back end of the resulting peak was analyzed. The curve produced on the back end of the peak corresponds to dQ/dC and was used, just like the FACP method, to derive the isotherm. As with the FACP method, the ECP method relies on measuring absorbance data and converting it to concentration using an additional experiment of measuring UV absorbance at successive step increases of the concentration and fitting the concentration to a polynomial for the calculation. Also, as in the FACP method, the baseline absorbance was subtracted from all absorbencies used in the calculation. Equation 12 is used for the calculating the isotherm using the ECP method, except since in ECP the isotherm is determined by injection a high concentration of solute rather than by switching from the reservoir there is no delay time. $\tau$ is needed to correct the retention time of the rear boundary; therefore equation 13 in the ECP method will be replaced with:

$$X_n = \frac{1}{F} \times \frac{\left( t_{Rn} - t_0 \right)}{t_0} \qquad (14)$$

Retention Time Method

Injections of both enantiomers of Fluoxetine at a low (0.00016 mg/mL) and high (1.0 mg/mL) concentrations were made onto the HPLC system. The retention times of peaks at low and high concentrations for each enantiomer were determined and used to calculate the isotherms.

Frontal Analysis Method for Multi-Component Isotherms

A multi-component isotherm was determined using the FA method for Multi-Component isotherms. The principle is the same as for the single component isotherm; an HPLC system with a binary pump was used. One pump introduced 100% Mobile Phase onto the system, and the other introduced a solution made from the racemic mixture with a known concentration of both enantiomers of Fluoxetine. The amount of this solution that was placed onto the system was increased stepwise at 10% intervals, starting at 0% until reaching 100%. The difference between the FA for single component isotherms and for multi-component isotherms is that with the two components, two plateaus will be obtained for each step up in concentration (an intermediate and final plateau). These curves can be used to determine the isotherm by measuring the retention time of each front and concentration of both solutes in the intermediate plateaus and by implementing the mass balance equation for bother isomers. Due to competition the concentrations of both enantiomers at the intermediate plateaus and the retention times of the fronts depends on the isotherm parameters of both components. The next step with the multi-component system was to determine the individual concentrations of each enantiomer in the intermediate plateau in order to determine the isotherms. After the solution passed through the HPLC system, the fractions were collected at each plateau. In order to collect solution from each plateau separately as it came off the system, care had to be taken to only collect the solution

corresponding to each plateau. The UV absorbance plot was monitored and test tubes were placed to collect the solution needed leaving the UV Spectrophotometer at the end of the HPLC system. These solutions were then injected back on the HPLC system, bracketed by a standard with a known concentration of each enantiomer. The concentration of each enantiomer at the intermediate plateaus ($C_{i,m}$) could then be calculated. The retention times of the fronts were also measured. Then, the data was entered in to equation 7 to determine $Q_1$ and $Q_2$ at each breakthrough curve ($C_1$ and $C_2$) and from the values of $Q_1$ and $Q_2$ the isotherm for each compound could be constructed

## RESULTS AND DISCUSSION

### HPLC Method Development and Optimization

The first part of this research was determining whether a chosen chiral compound met the needed requirements; a good UV chromophore, give good separation by HPLC, and the availability of both enantiomers and the racemic mixture. The next goal was to develop a suitable HPLC method that would give good separation and quantitation of enantiomers selected.

There were many different compounds researched (Stinson, 1998 and 2001) in order to find a suitable chiral compound. The initial objective was to develop a normal phase HPLC method that would give good, reliable separation of the R- and S- enantiomers of the chiral compound chosen using a Chiracel OD column. The initial Mobile Phase chosen, which would also be used as the diluent, was (90:10) hexane:isopropyl alcohol (IPA). A racemic mixture of penicillamine was dissolved in the diluent at a concentration of 0.1 mg/mL. The UV spectrum of this solution was tested using an Ultraviolet/Visible spectrophotometer. No absorbance was detected between 200 nm and 1100 nm, therefore penicillamine was not a viable compound for this study due to the lack of chromophore and its inability to be detected by a UV detector. The

next compound tested was naproxen sodium, which was not soluble in the chosen diluent (90:10 hexane:IPA); therefore it could not be separated using the nonpolar mobile phase. Cycloserine was the next compound tested. It was soluble in the diluent, and also had noticeable absorbance; therefore an attempt was made to separate the enantiomers of this compound using the Chiracel OD column. A reliable normal phase HPLC method could not be obtained to separate the R- and S- enantiomers of cycloserine. Other chiral compounds were researched and discussed, however due to the limitations in obtaining the individual enantiomers of these compounds, they were dismissed. Fluoxetine was finally chosen as a chiral compound that was soluble in the diluent, had a good absorbance, and R- and S- isomers of it could be easily obtained.

After a compound for study was determined, an HPLC method was developed. Using the (90:10) hexane:IPA mobile phase that was originally chosen, and a Chiracel OD column, separation of the enantiomers could not be achieved. The parameters of the HPLC system were modified. The mobile phase composition was varied, the column temperature was varied, and DEA was added in an attempt to reduce the tailing, but still no separation was achieved. Much reading and research was done on the HPLC separation of chiral compounds (Feng, 2000);(Perrin, 2002);(Migliorini, 2000);(Jacobson, 1990);(Cavazzini, 2001);(Miller, 1989);(Wainer, 1988);(Matthijs, 2004)and(Olsen et all, 1997). Finally, a reverse phase method was chosen and tested (Gatti et all, 2003). The mobile phase was a buffer system using hexafluorophosphate mixed with an organic, in this case Acetonitrile, the chiracel column was used in reverse phase mode (Chiracel OD-R), and separation of the R- and S- forms of Fluoxetine was obtained. The method was then optimized. The optimum chromatographic conditions for reversed phase separation of R- and S-Fluoxetine were as follows:

Chromatographic Conditions:

Column: Chiralcel OD-R, 250 x 4.6 mm, 10 µm particle size

Column Temperature: 25º C

Injection Volume: 50 µL

Flow Rate: 1.0 mL/min

Mobile Phase: 60:40 50 mM hexafluorophosphate buffer (pH 5.0): Acetonitrile

Detection: 217 nm

The R-Fluoxetine peak eluted first, at ~15 minutes, and the S-Fluoxetine peak eluted at ~17 minutes.

Figures 4-6 are example chromatograms of R- and S-Fluoxetine and a racemic mixture after optimizing the chromatographic conditions.

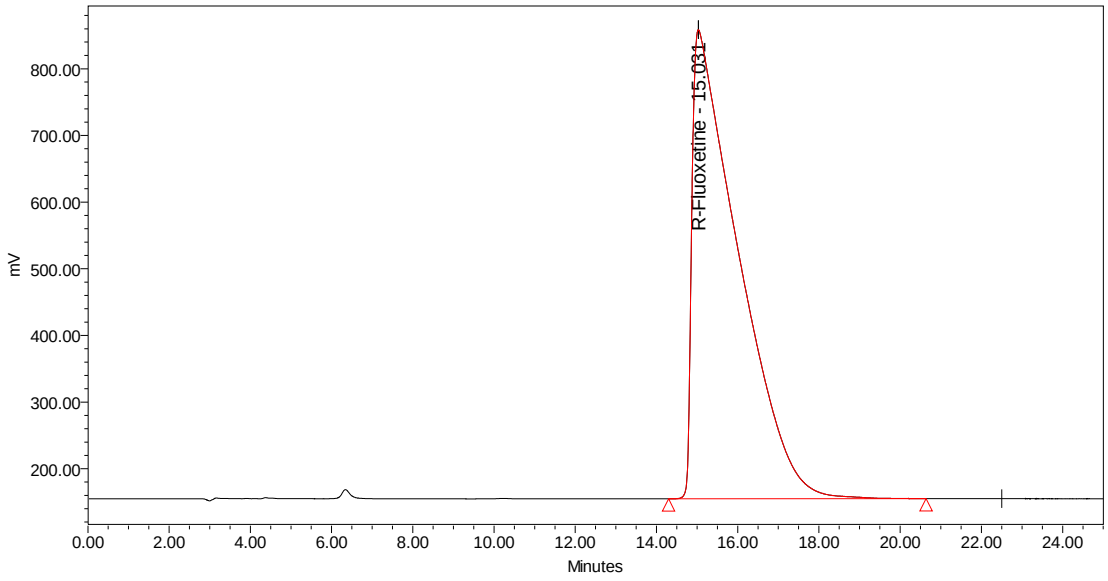


Figure 4: Example Chromatogram of 0.5 mg/mL R-Fluoxetine, chromatographic conditions on pg 26

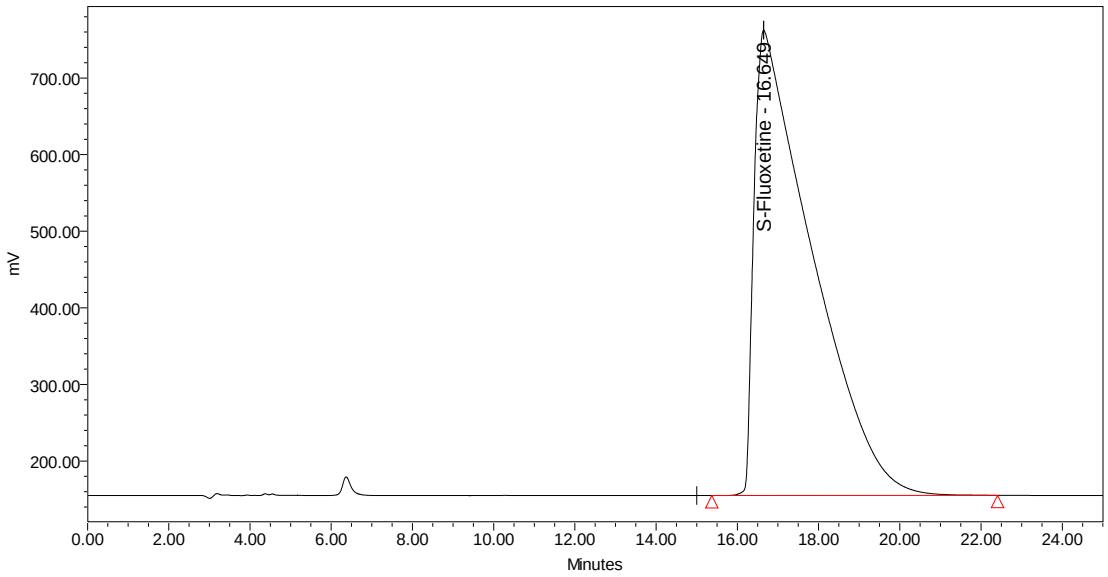


Figure 5: Example Chromatogram of 0.5 mg/mL S-Fluoxetine, chromatographic conditions on

pg 26

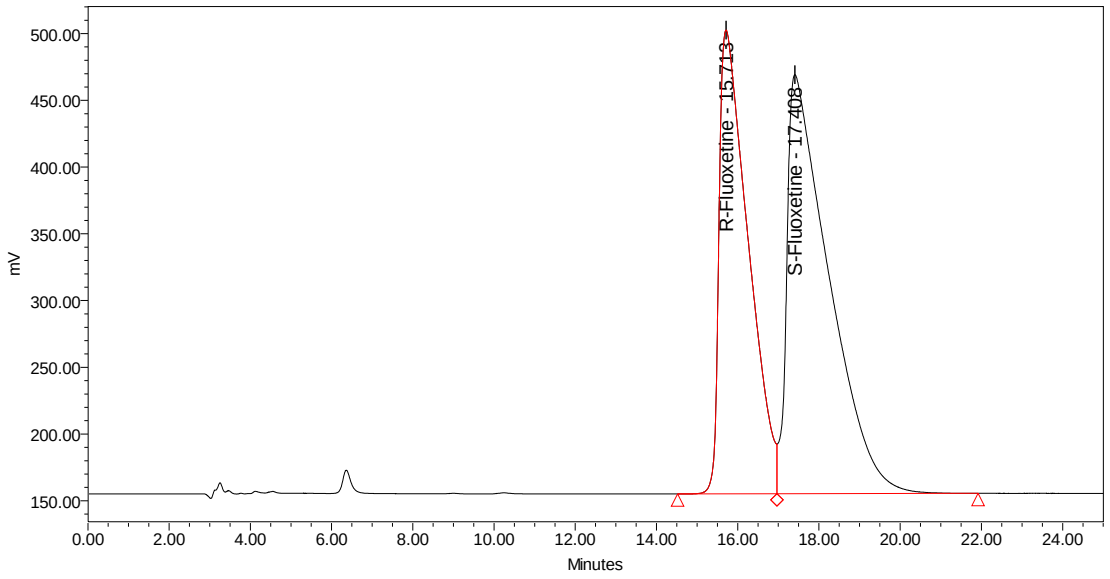


Figure 6: Example Chromatogram of 0.25 mg/mL Racemic Mixture of Fluoxetine, chromatographic conditions on pg 26

## Measurement of Single Component Isotherms

### Frontal Analysis Method

Figures 7-8 show the example experimental breakthrough curves for the Frontal Analysis determination of both R- and S- Fluoxetine for determination of the isotherms. Data from these breakthrough curves (retention times of the fronts) was obtained and Q (concentration of the solute in the stationary phase) for the isotherm was calculated at each concentration step using equation 3 and the isotherm was constructed (Figure 9).

### FACP Method

Example chromatograms for FACP method can be seen in Figures 10-11. Q for the FACP method is determined from the UV absorption and retention times collected at the end of the FA steps in the resulting diffuse boundary until the solute elutes completely and the concentration reaches zero. Q as a function of concentration in mobile phase (C) was then determined using equations 12 and 13 and the isotherm was constructed. (Figure 12)

### ECP Method

Figures 13-14 show the example chromatograms for R- and S- Fluoxetine. Similar to the FACP method, data points from these chromatograms, plugged into equations 12 and 14 give the results for Q for each concentration in the Mobile Phase and the isotherm was constructed (Figure 15).

### Retention Time Method

The Retention Time method, assuming the single component follows the Langmuir isotherm, can be performed by making just two chromatographic injections,

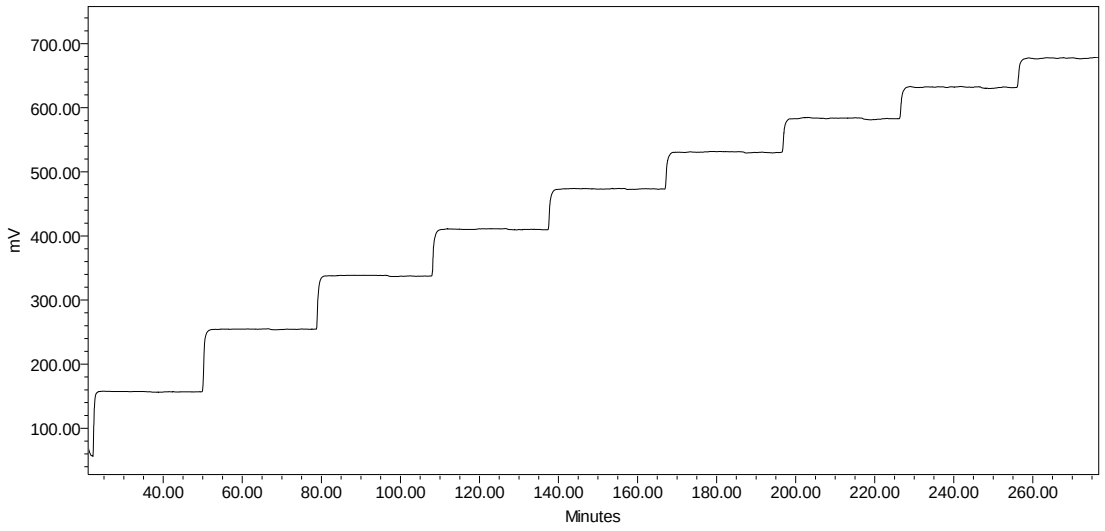


Figure 7: Example Breakthrough Curve of FA Method for R-Fluoxetine, chromatographic conditions on pg 26

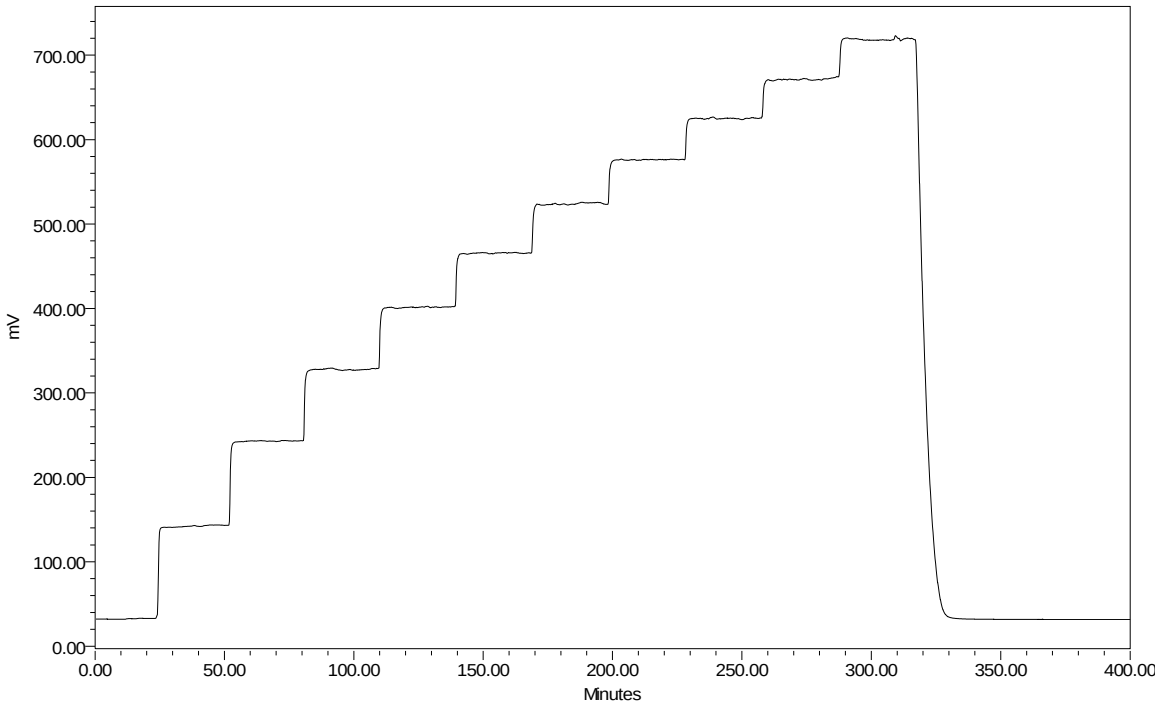


Figure 8: Example Breakthrough Curve of FA Method for S-Fluoxetine,

chromatographic conditions on pg 26

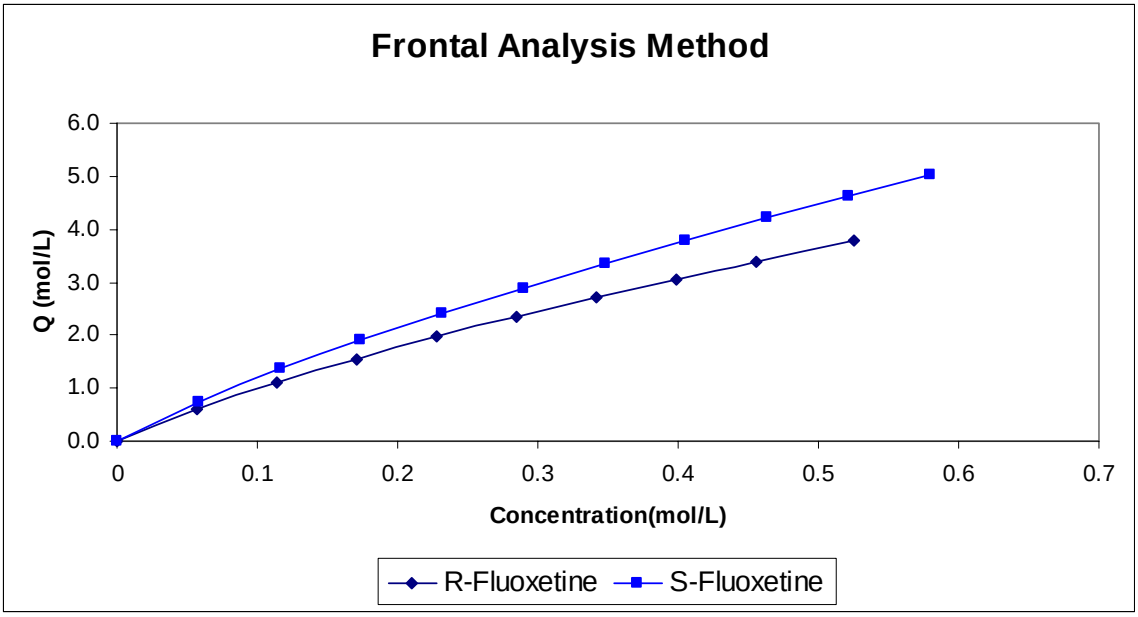


Figure 9: Adsorption Isotherm calculated using Frontal Analysis Method

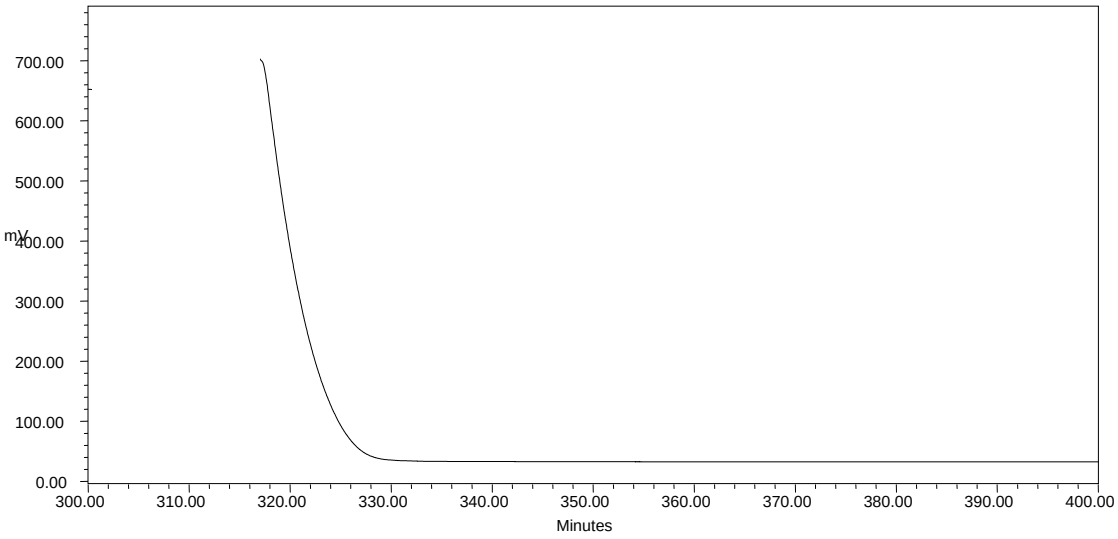


Figure 10: Example Chromatogram of the FACP method for R-Fluoxetine,

chromatographic conditions on pg 26

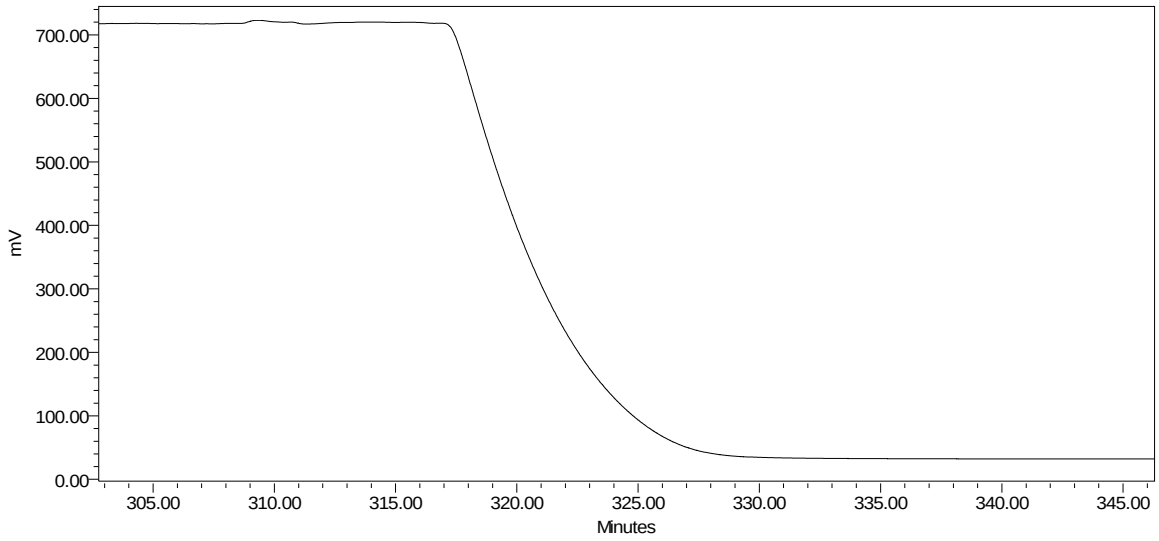


Figure 11: Example Chromatogram of the FACP method for S-Fluoxetine,

chromatographic conditions on pg 26

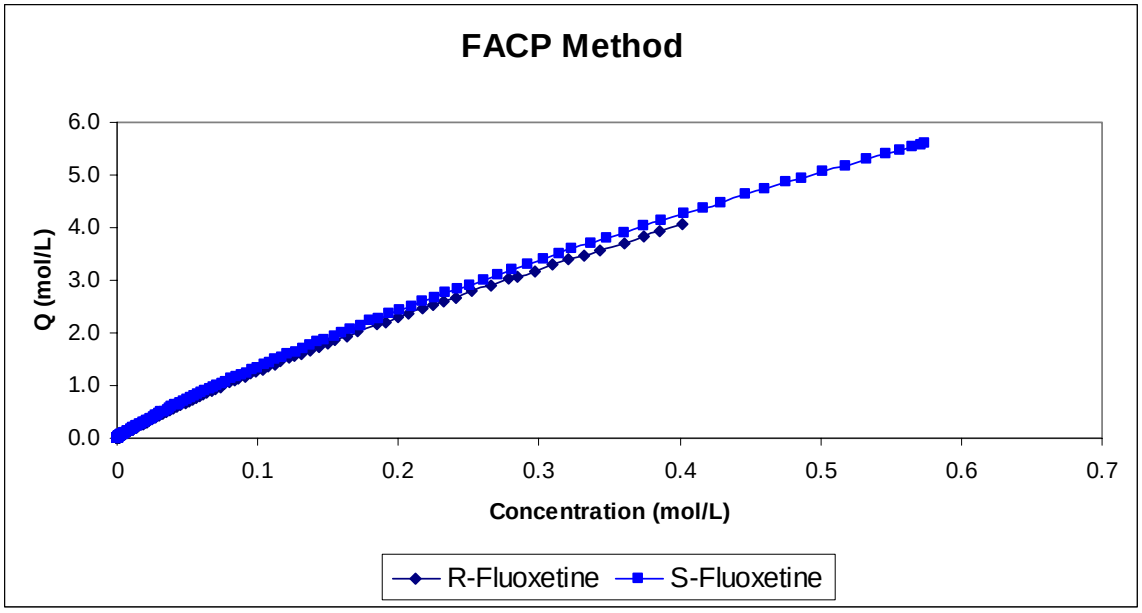


Figure 12: Adsorption Isotherm calculated using FACP Method

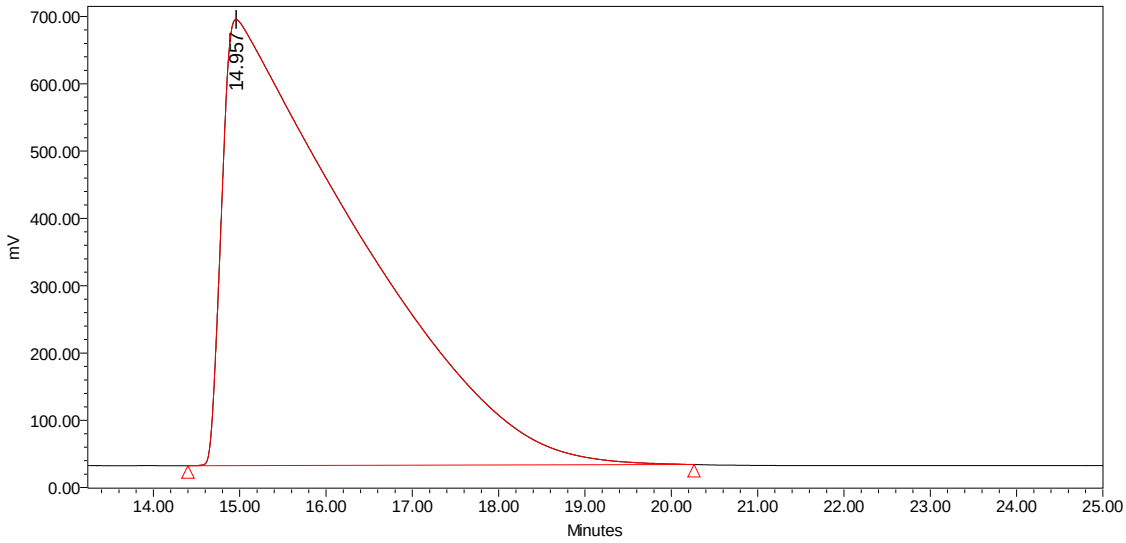


Figure 13: Example Chromatogram of ECP Method for R-Fluoxetine

(1.0 mg/mL solution), chromatographic conditions on pg 26

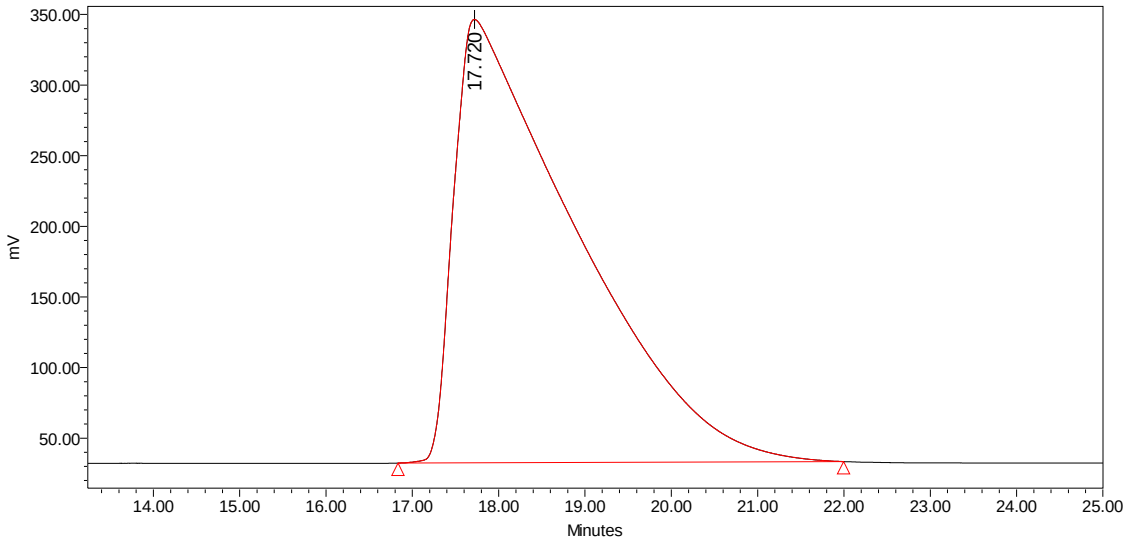


Figure 14: Example Chromatogram of ECP Method for S-Fluoxetine

(1.0 mg/mL solution), chromatographic conditions on pg 26

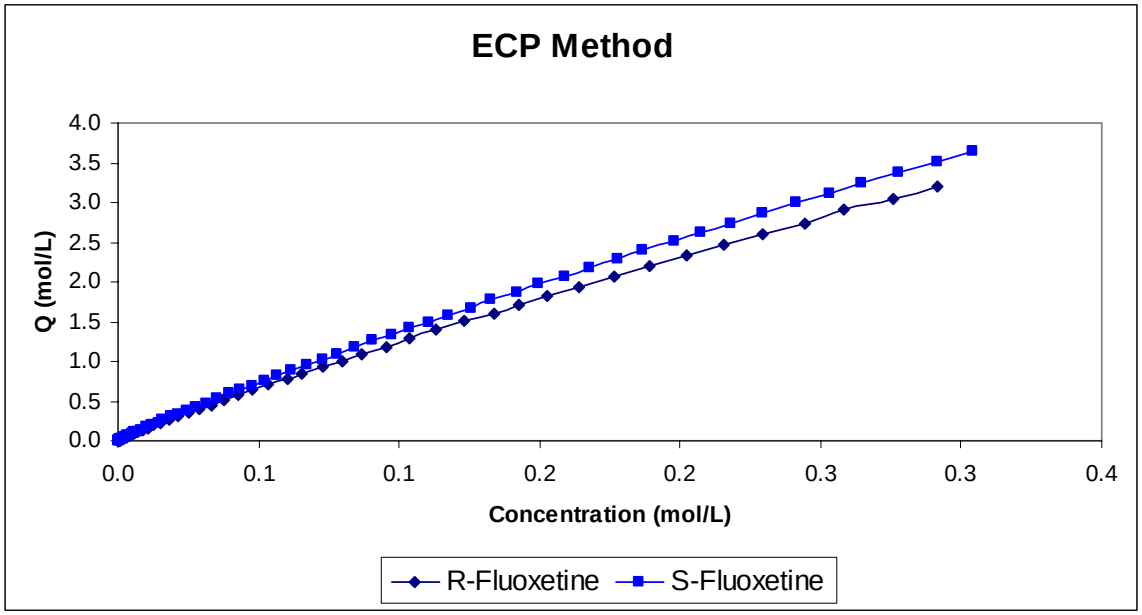


Figure 15: Adsorption Isotherm calculated using ECP Method

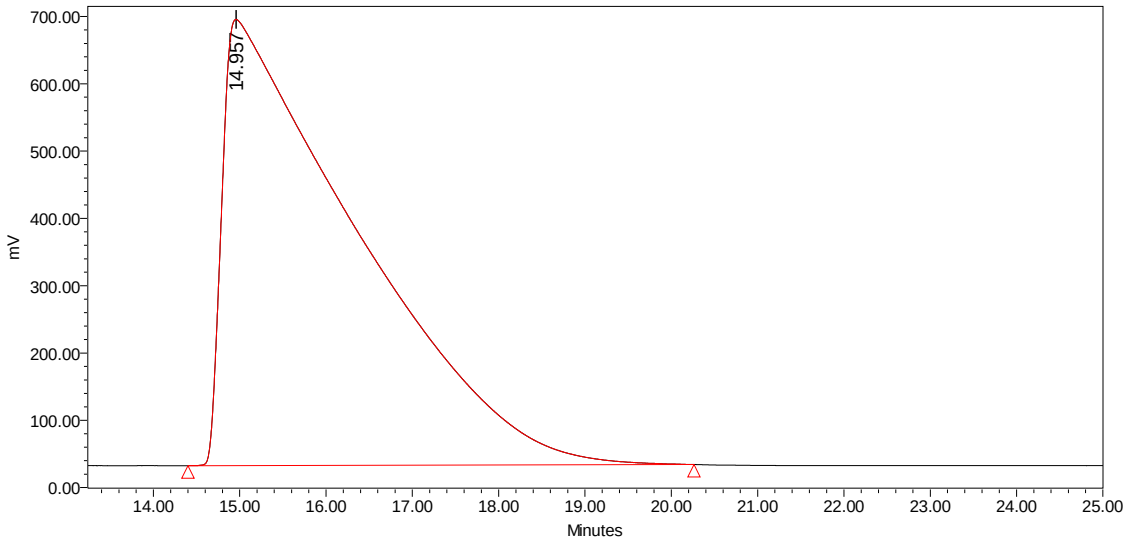


Figure 16: Example Chromatogram of RT Method for R-Fluoxetine (1.0 mg/mL solution), the retention time of overloaded chromatogram used to calculate parameter b (ratio of rate constant of solute being adsorbed vs. rate constant of solute be desorbed) of isotherm, chromatographic conditions on pg 26

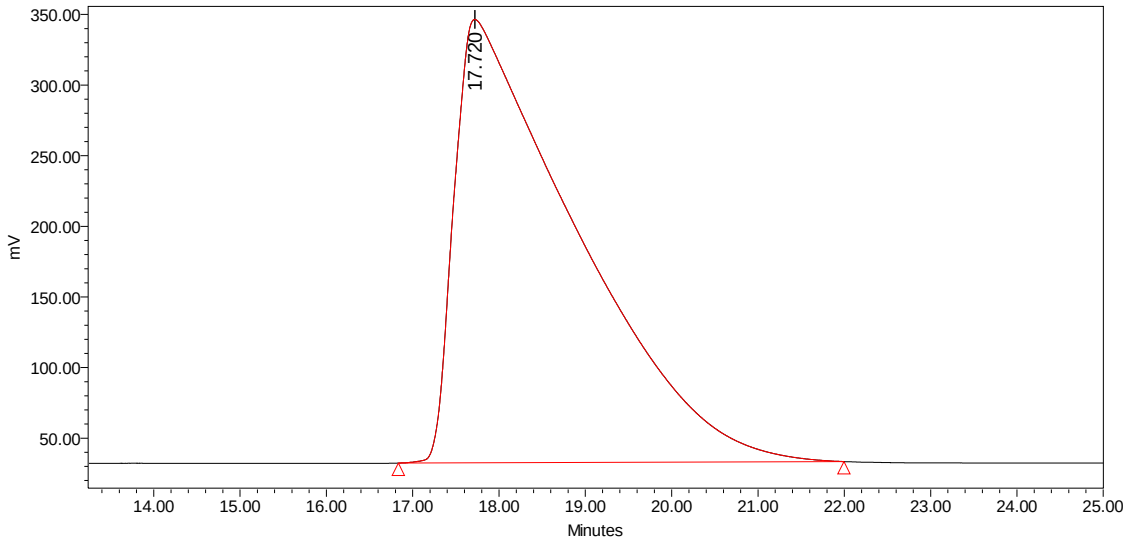


Figure 17: Example Chromatogram of RT Method for S-Fluoxetine (1.0 mg/mL solution), the retention time of overloaded chromatogram used to calculate parameter b (ratio of rate constant of solute being adsorbed vs. rate constant of solute be desorbed) of isotherm, chromatographic conditions on pg 26

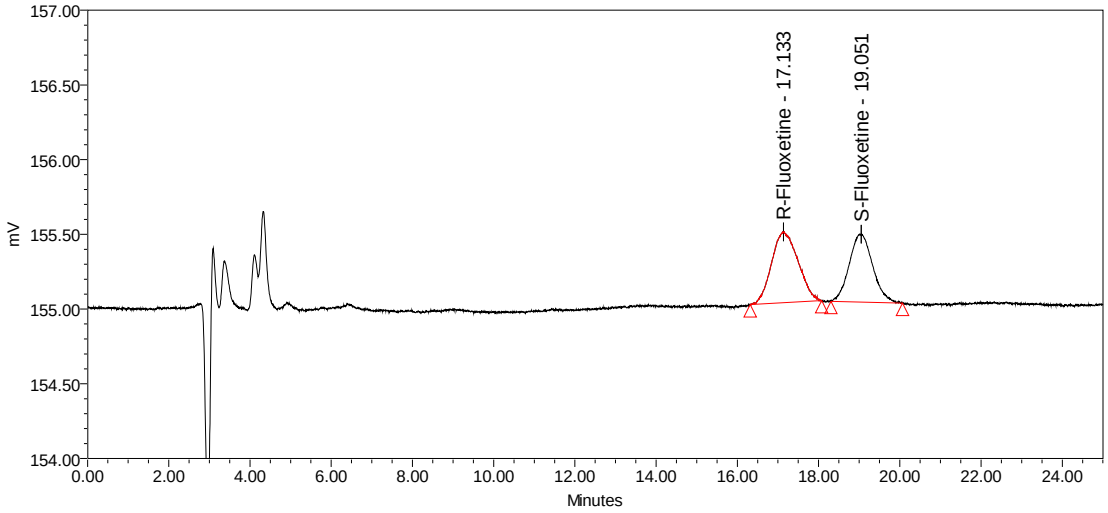


Figure 18: Example chromatogram of RT method for R- and S- Fluoxetine, the retention time of very low concentration (0.00016 mg/mL solution) (linear chromatogram) used to calculate parameter a (slope of isotherm at low concentration) of R- and S-Fluoxetine isotherms, chromatographic conditions on pg 26

one with a very dilute solution and one with a concentrated solution. The retention times of these injections are then used to calculate parameters "a" and "b" which can then be used with equation 2 to determine Q and the isotherm. The parameter "a" is calculated from the retention time of a very dilute solution using equation 15.

$$a = \frac{t_{R0} - t_0}{F} \qquad (15)$$

$t_{R0}$ = retention time of dilute solution

$t_0$ = void time of the column

F = phase ratio (equation 4)

The parameter "b" is related to the loading factor of the column and calculated using equation 16a and b (Golshon-Sharizi, 1987)

$$b = \frac{L_F \times Fv \times (t_{R0} - t_0)}{n_m} \qquad (16a)$$

$$L_F = \text{loading factor} = \left[1 - \sqrt{\frac{t_f - t_0 - t_p}{t_{R0} - t_0}}\right]^2 \qquad (16b)$$

$t_f$ = retention time of the front of concentrated solution (minutes)

$t_p$ = time it takes the solutes to travel through the injection loop ( $t_P = \frac{V_P}{F_V}$ ), where $V_P$ is the injection loop size (mL)

Fv = mobile phase flow rate (mL/min)

$t_{R0}$ = retention time of dilute solution (minutes)

$t_0$ = void time of the column (minutes)

$n_m$ = the amount of sample injected (mol/L)

b = Langmuir parameter

Summary of Single Component Isotherm Methods

Tables 1 and 2 show the concentrations in the mobile phase, in mol/L of R- and S-Fluoxetine, respectively, and calculated Q (concentration in the stationary phase) values for the isotherm determinations using different methods. Figures 19 and 20 give the graphical representations of this data. As can be seen from the graphs, the results from the ECP and FACP methods do not compare favorably to the results from the FA method. They do, however compare favorably to each other.

In order to easily use the isotherm data for optimization of preparative chromatography, they must be fitted to an isotherm model to obtain a proper isotherm model which will be used for optimization. The simplest model is the Langmuir isotherm; therefore fitting the single component isotherm data to this model was attempted. This can be done using Scatchard plots where

$$Q = \frac{aC}{1 + bC} \text{ or } Q + QbC = aC \text{ or } \frac{Q}{C} = a - bQ \qquad (17)$$

Scatchard Plots

A Scatchard plot is a linear representation of the Langmuir model that can then be analyzed by linear regression. Scatchard plots of the data obtained by Frontal Analysis, FACP and ECP for Q vs. Q/C were created (Figures 21-23). If the system studied is Langmuirian, the graph obtained should be linear with a negative slope. The slope of the line produced by graphing the data is equal to "–b" and the y-intercept is equal to "a". The Scatchard plot for the isotherm data obtained by the Frontal Analysis method is linear, indicating that this data follows the Langmuir isotherm model. The Scatchard

| Frontal Analysis | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 0.057 | 0.600 |
| 0.114 | 1.103 |
| 0.171 | 1.550 |
| 0.228 | 1.962 |
| 0.285 | 2.349 |
| 0.342 | 2.715 |
| 0.399 | 3.055 |
| 0.456 | 3.385 |
| 0.526 | 3.773 |

| ECP | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 0.00006 | 0.001 |
| 0.00039 | 0.007 |
| 0.0013 | 0.022 |
| 0.0037 | 0.060 |
| 0.0104 | 0.160 |
| 0.0250 | 0.310 |
| 0.0480 | 0.638 |
| 0.0797 | 1.005 |
| 0.1229 | 1.505 |
| 0.1763 | 2.065 |
| 0.2446 | 2.738 |
| 0.2917 | 3.204 |

| FACP | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 0.0001 | 0.002 |
| 0.0009 | 0.016 |
| 0.0029 | 0.049 |
| 0.0067 | 0.110 |
| 0.0135 | 0.213 |
| 0.0245 | 0.368 |
| 0.0402 | 0.575 |
| 0.0640 | 0.869 |
| 0.0982 | 1.261 |
| 0.1503 | 1.809 |
| 0.2326 | 2.605 |
| 0.3433 | 3.576 |
| 0.4016 | 4.051 |

Table 1: Calculated values of Q and C for R-Fluoxetine using Single Isotherm Methods

| Frontal Analysis | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 0.057 | 0.600 |
| 0.114 | 1.103 |
| 0.171 | 1.550 |
| 0.228 | 1.962 |
| 0.285 | 2.349 |
| 0.342 | 2.715 |
| 0.399 | 3.055 |
| 0.456 | 3.385 |
| 0.526 | 3.773 |

| ECP | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 0.00003 | 0.002 |
| 0.00091 | 0.018 |
| 0.0031 | 0.056 |
| 0.0037 | 0.060 |
| 0.0083 | 0.144 |
| 0.0186 | 0.301 |
| 0.0349 | 0.539 |
| 0.0567 | 0.816 |
| 0.0974 | 1.329 |
| 0.1497 | 1.971 |
| 0.2179 | 2.744 |
| 0.3042 | 3.646 |

| FACP | |
|---|---|
| **C(mol/L)** | **Q(mol/L)** |
| 8.40E-05 | 0.001 |
| 2.94E-04 | 0.005 |
| 6.74E-04 | 0.012 |
| 1.73E-03 | 0.031 |
| 3.96E-03 | 0.069 |
| 8.69E-03 | 0.146 |
| 1.72E-02 | 0.276 |
| 3.04E-02 | 0.465 |
| 5.00E-02 | 0.729 |
| 7.78E-02 | 1.080 |
| 1.17E-01 | 1.540 |
| 1.73E-01 | 2.145 |
| 2.51E-01 | 2.914 |
| 3.60E-01 | 3.903 |
| 5.02E-01 | 5.059 |
| 5.75E-01 | 5.603 |

Table 2: Calculated values of Q and C for S-Fluoxetine using Single Isotherm Methods

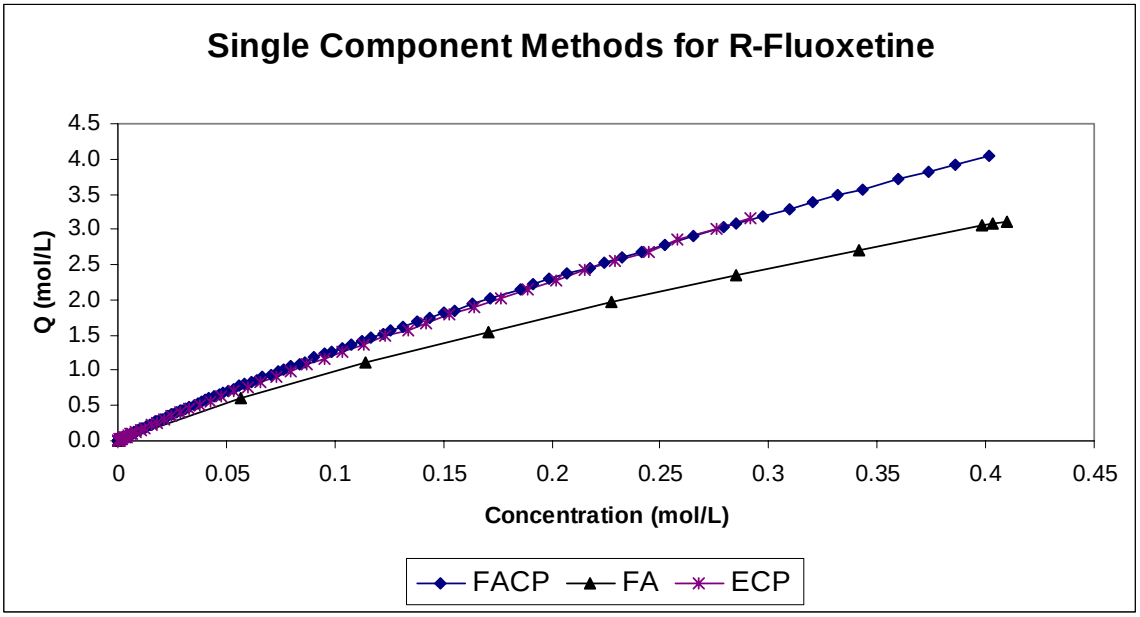


Figure 19: Experimental Data of Single Component Isotherms of R-Fluoxetine

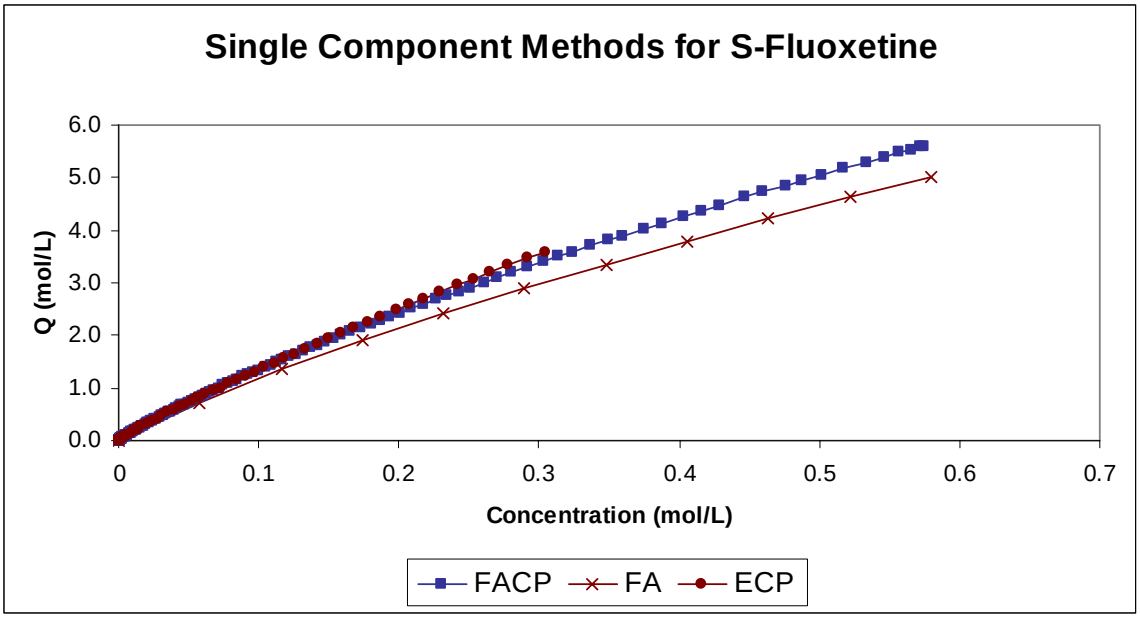


Figure 20: Experimental Data of Single Component Isotherms of S-Fluoxetine

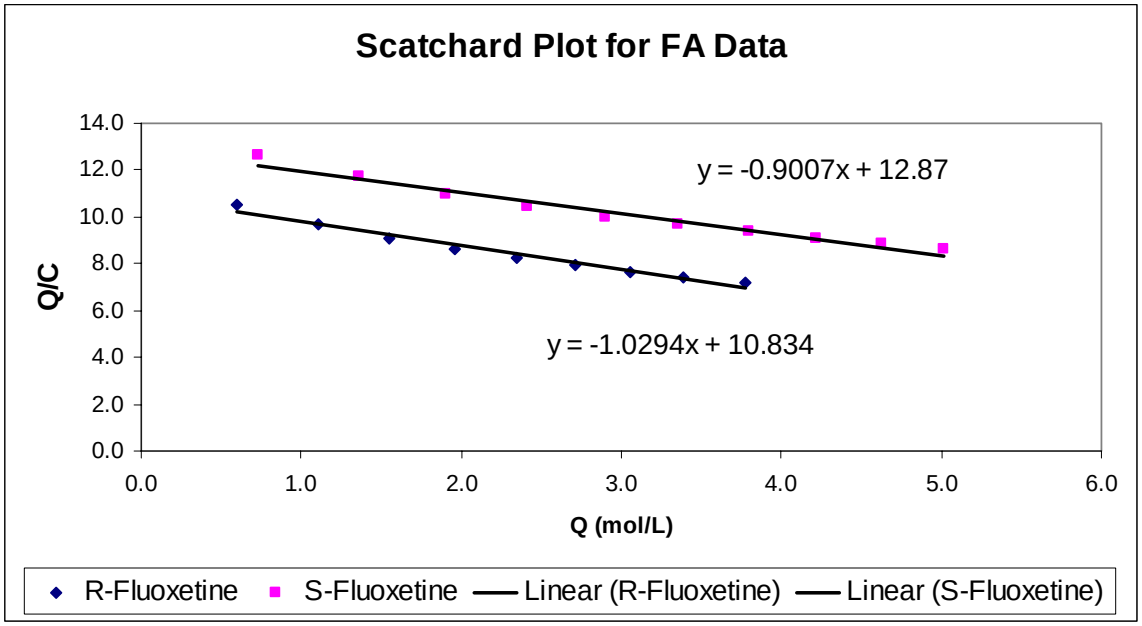


Figure 21: Frontal Analysis Scatchard data fitted to Langmuir Model (Equation 17)

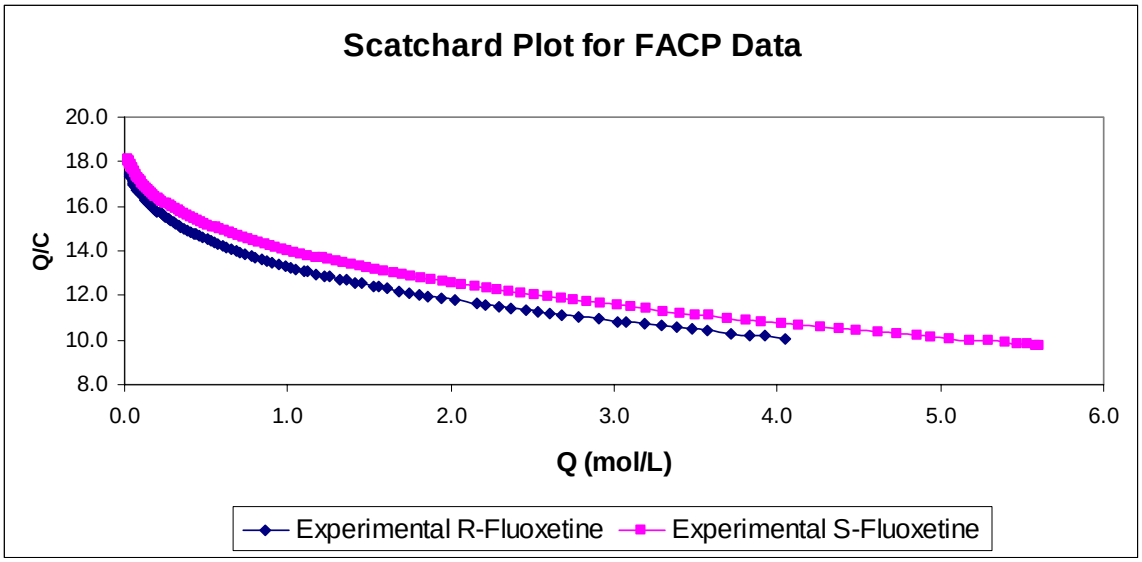


Figure 22: Low Concentration FACP data does not fit Langmuir Model (Equation 17)

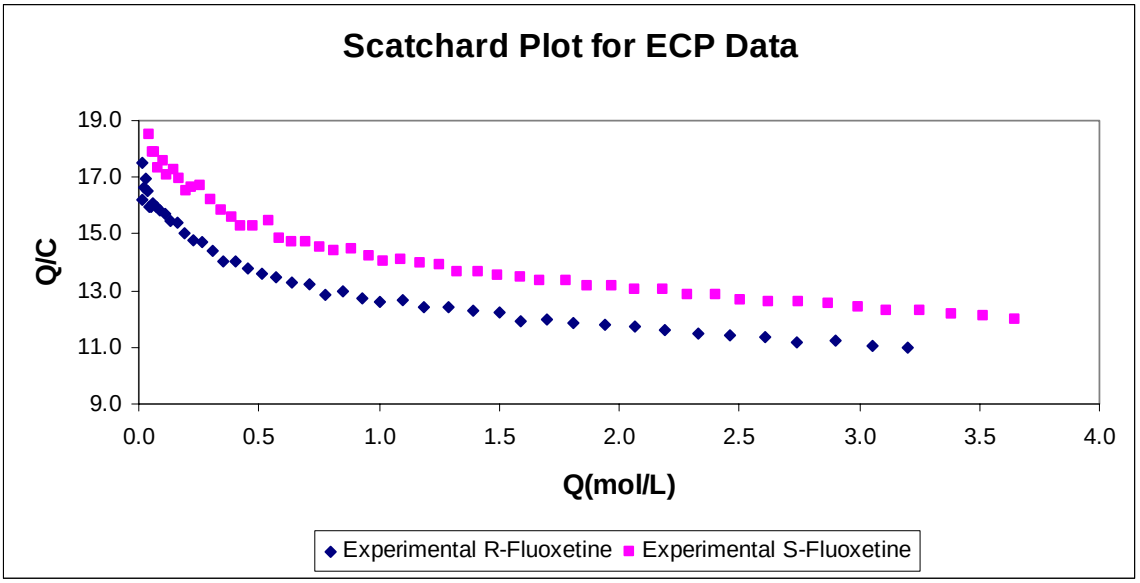


Figure 23: Low concentration ECP Scatchard data does not fit Langmuir Model (Equation 17)

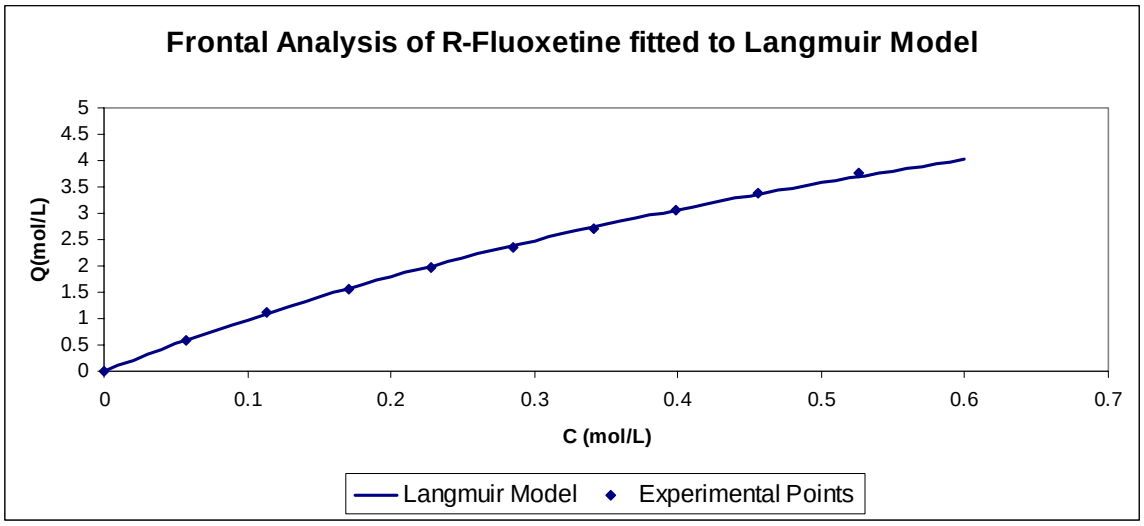


Figure 24: Single Component Isotherm of R-Fluoxetine fitted to Langmuir Model

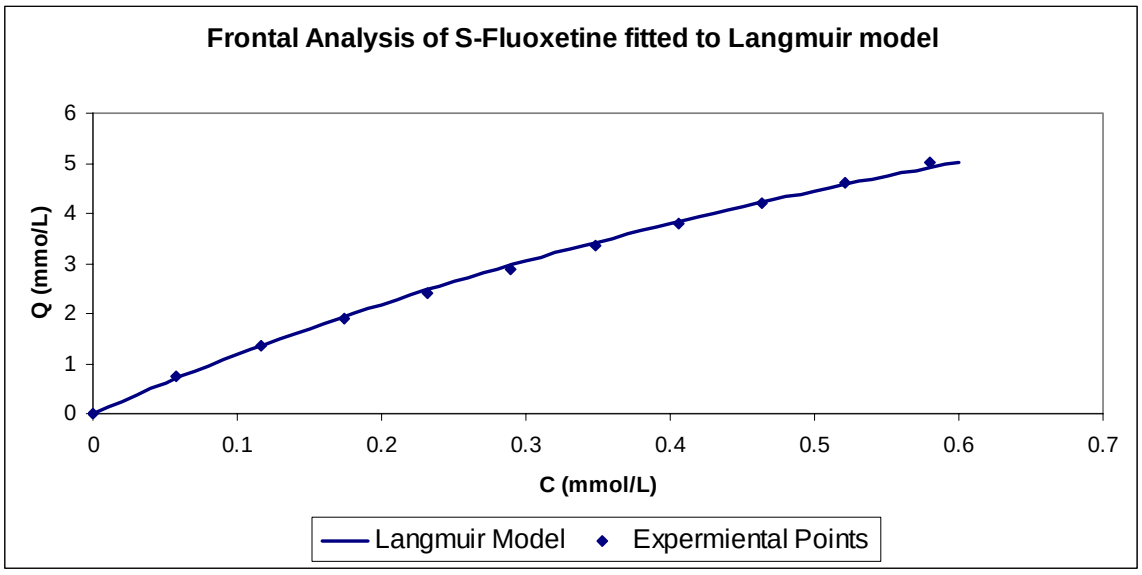


Figure 25: Single Component Isotherm of S-Fluoxetine fitted to Langmuir Model

R-Fluoxetine

| | a | b (L/mol) | Qs (mol/L) |
|---|---|---|---|
| FA | 10.8 | 1.0 | 10.8 |
| RT | 10.3 | 0.9 | 11.4 |

S-Fluoxetine

| | a | b (L/mol) | Qs (mol/L) |
|---|---|---|---|
| FA | 12.9 | 0.9 | 14.3 |
| RT | 12.7 | 1.0 | 12.7 |

Table 3: Scatchard data for Single Component Isotherm Measurements that fit the Langmuir model

plots for the isotherm data obtained by FACP and ECP are not linear, indicating that these data do not match the Langmuir model.

Discussion of Single Component Isotherm Experiments

The data obtained from the Frontal Analysis measurement of R- and S-Fluoxetine was fitted to the Langmuir isotherm model to see whether the isotherm of the chiral components of Fluoxetine followed the model. Figures 24-25 show the experimental data obtained during the Frontal Analysis compared to theoretical values determined by calculating a and b using equation 17 and determining the theoretical value for Q using generalized values for C. The experimental points match well with the line representing the Langmuir model. The data points also fit the model demonstrated in Figure 21 by the linear graph with a negative slope. The results from the FACP and ECP measurements do not compare favorably to the Langmuir isotherm model. Because the RT method assumes from the start that the isotherm will be Langmuiran, fitting to the model is not needed. It will be equal to the model. Table 3 shows a and b results from the Scatchard plots for the Retention Time and Frontal Analysis methods. From the data in this table it can be seen that the results for a and b determined using the FA and RT methods are very similar. This is further evidence that the single component systems for both R- and S- Fluoxetine are Langmuiran as shown by the Frontal Analysis and Retention Time methods. The Scatchard plots for the ECP and FACP methods are not linear, especially at low concentrations.

There are two possible reasons for the results from the FACP and ECP methods not following the Langmuir model. The first is that when the isotherm is calculated for these methods, the kinetic effect is disregarded. This can result in a more significant error at low concentrations when the kinetic effect is more pronounced. This is especially true in chiral

separation where column efficiency is low and the kinetic effect is significant. The second reason is that the stationary phase has two different sites with different energy levels. The ECP and FACP methods are not accurate methods for isotherm determination. This is especially true for chiral compounds.

Measurement of Binary Isotherm by Frontal Analysis

The determination of Multi-component Isotherm data from Frontal Analysis is similar to the determination for the single component; through a series of stepwise increases in the concentration of a mixture of R- and S-Fluoxetine. For this experiment the racemic mixture of Fluoxetine was used. Using the HPLC system, one line was inserted into a reservoir of 100% Mobile Phase (line A), and the other was inserted into a reservoir containing a predetermined concentration of the racemic mixture of Fluoxetine in Mobile Phase (line B). The amount pumped through the system from line B was increased step-wise at 10% intervals from 0% until it reached 100% and the retention time of the breakthrough curve was recorded. Because there are two components competing for adsorption sites, each breakthrough curve will have two plateaus, an intermediate and final plateau. The retention time of these fronts as well as the concentration of each solute in the intermediate plateau are a complex function of both isomers due to the competitive nature of isotherms. The retention times of these fronts can be measured from the breakthrough curves. The concentration of each intermediate plateau must be measured by collecting the fractions on these intermediate plateaus and reinjecting them in another system and determining the concentrations of the two isomers using external standards by elution chromatography. Figures 26 a and b show the example chromatography of a fraction collected at intermediate plateaus and the standard used for the determination of the concentration of R- and S-Fluoxetine in this fraction..

Because the concentration of the initial and final plateaus are known and the retention times of the intermediate and final plateaus was measured and the concentration of the intermediate plateau ($C_{i,m}$) can be determined by collection fractions and determining by reinjection and quantitation, $Q_i$ can be determined using equation 7 and an isotherm curve can be constructed for each compound in the mixture. Figures 27 shows an example of one step of the breakthrough curve for a binary mixture and Figure 28 shows the multi-component isotherm data. Figure 29 shows the binary isotherms of R- Fluoxetine determined by the Method of Mass Balance (MMB). It also includes the binary isotherm of R- Fluoxetine calculated using Equation (8) which uses the single component isotherm parameters (the values in Table 3 calculated using the FA Method). Figure 30 shows the same information for S-Fluoxetine.

Discussion of Multi-Component Isotherm Experiments

Only in very rare cases can multi-component isotherms be fitted to the Langmuir model. This is due to the fact that when in a mixture, the compounds in the mixture compete for binding sites on the stationary phase. Due to the differences in size and shape, the column saturation capacities for compounds in the mixture are different. The Langmuir Isotherm Model is derived assuming that the column saturation capacities for each component will be equal; otherwise it violates the law of thermodynamics. Determining the multi-component isotherm experimentally is very time consuming and cumbersome. The ability to predict the multi-component isotherm for the single component isotherms of the compounds in the mixture makes the task much easier, as the

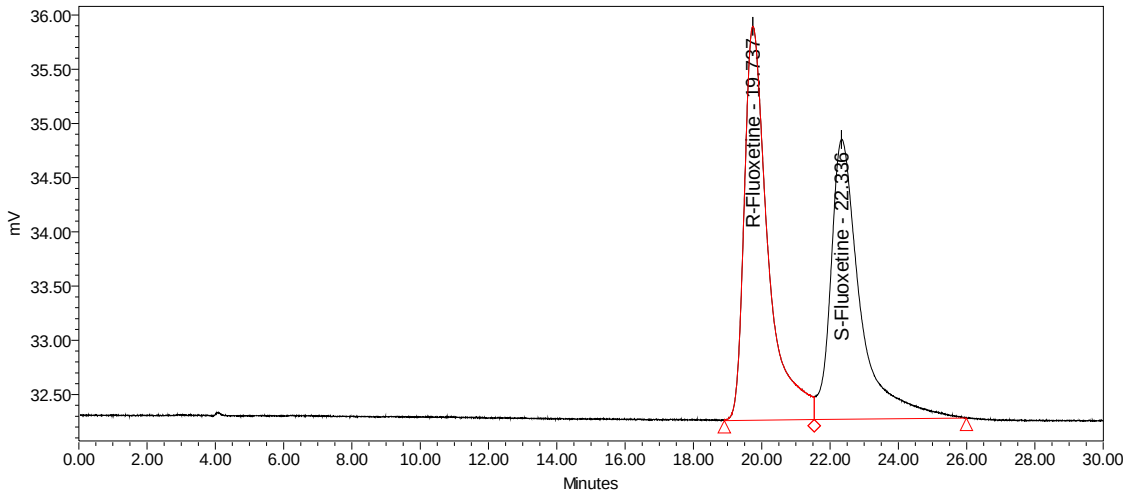


Figure 26a: Example Chromatogram of Fraction collected at Intermediate Plateau, chromatographic conditions on pg 26

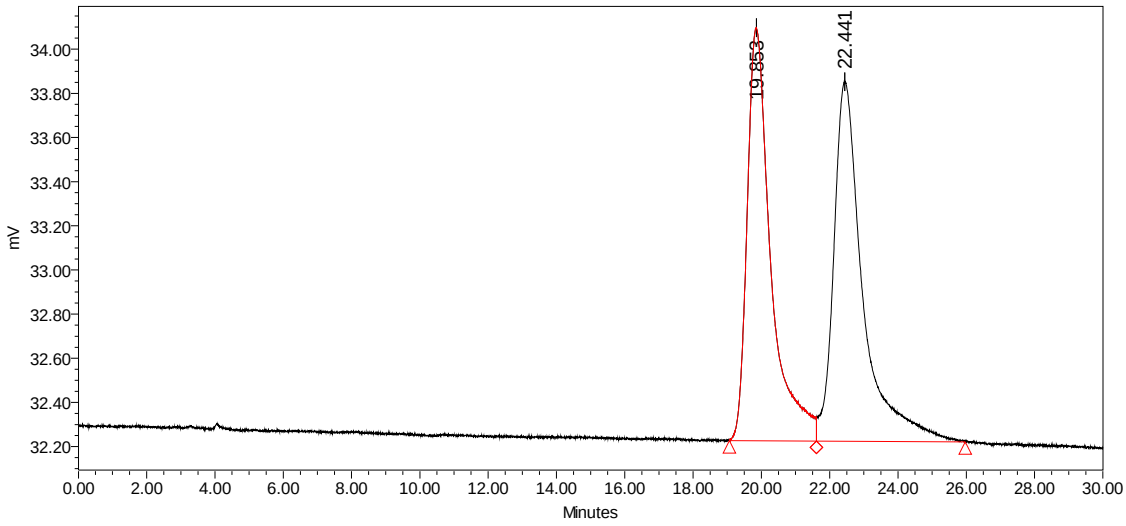


Figure 26b: Example Chromatogram of Standard used to Calculate Concentration of R- and S-Fluoxetine at Intermediate Plateau, chromatographic conditions on pg 26

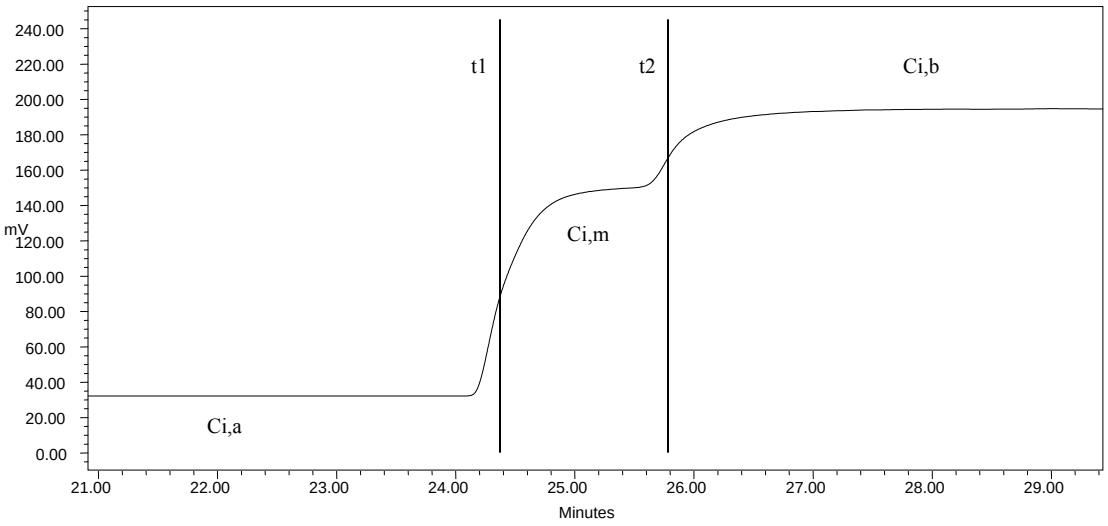


Figure 27: Example of Breakthrough Curve for a Binary Mixture showing Points of Measurement for Determination of the Isotherm

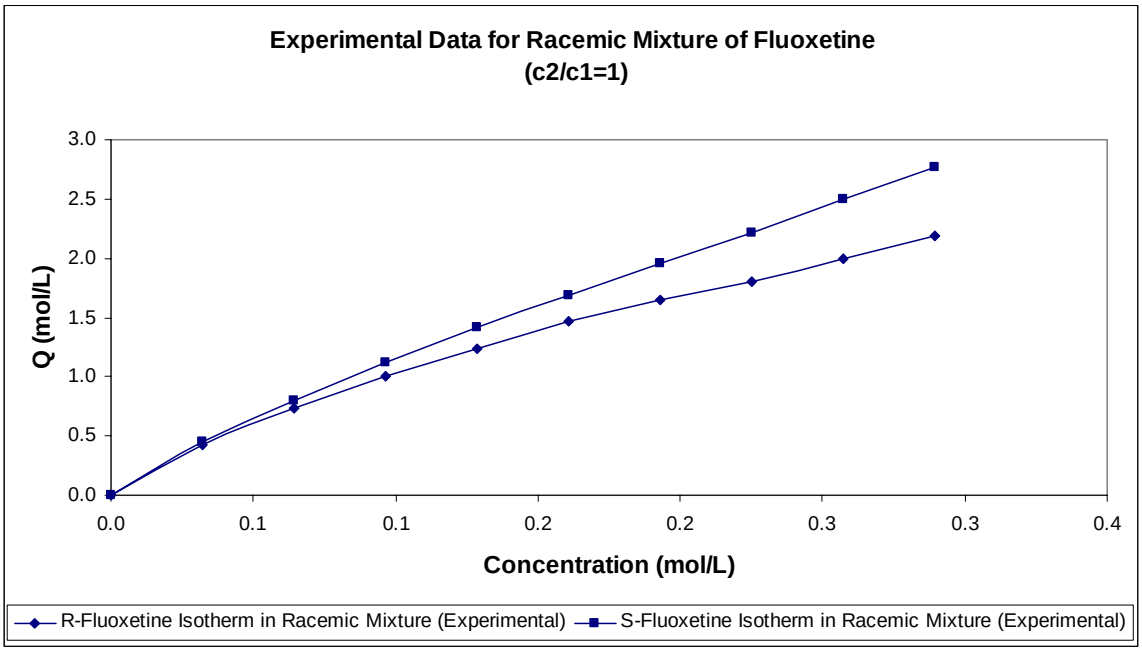


Figure 28: Experimental Data for Multi-Component Isotherm of R- and S-Fluoxetine in the Racemic Mixture

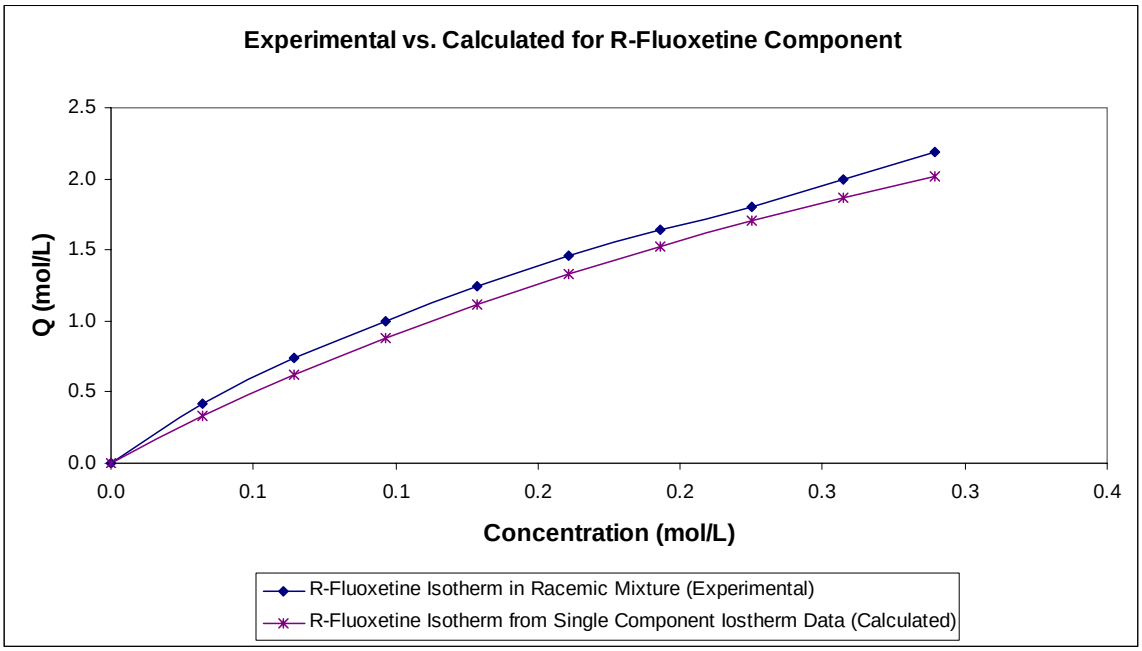


Figure 29: Experimental Competitive Isotherm Data compared to Competitive Langmuir Isotherm Data calculated from the Langmuir Parameters ($a_i$ and $b_i$) of the Single Component Isotherm of R-Fluoxetine

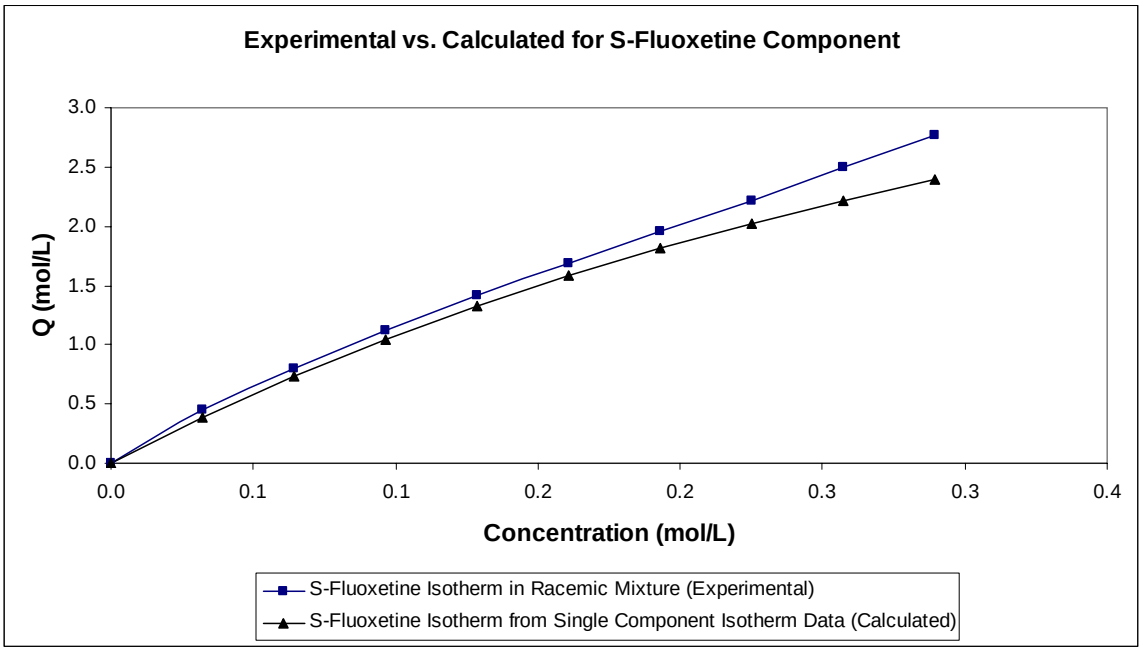


Figure 30: Experimental Competitive Isotherm Data compared to Competitive Langmuir Isotherm Data calculated from the Langmuir Parameters ($a_i$ and $b_i$) of the Single Component Isotherm of S-Fluoxetine

single component isotherms can be determined easily either using frontal analysis in the general case or the simplest and easiest method, the Retention Time method (assuming that the isotherm is Langmurian). As can be seen from Figure 29 and 30, the multi-component isotherm of the chiral compound Fluoxetine can be predicted fairly well from the isotherms of each single component. The experimental competitive isotherm data closely matches with the competitive isotherm data predicted from the single component isotherms.

## CONCLUSION

The need for large scale separation methods for pharmaceutical components is evident. Optimizing these methods experimentally can be time consuming and a waste of large amounts of materials and it is impossible due to the complex nature of multi-component separation without understanding and implementing the theory of non-linear chromatography. A mathematical model for determining the retention properties and behavior of each compound in a mixture and then using that information in the practical realm is a great help to both pharmaceutical and biotechnology companies. The competitive equilibrium isotherm of each component in the mixture is necessary to feed in the mathematical model to optimize the separation and determine the optimum operating conditions for preparative separation. The equilibrium isotherms of both enantiomers of Fluoxetine were determined experimentally by four different methods and then fitted to one of the most common isotherm models, the Langmuir Isotherm Model. The single component isotherms of the enantiomers did not fit the model of the Langmuir Isotherm in all cases. The Frontal Analysis method fit the Langmuir model; however the FACP and ECP methods did not. This could be due to a kinetic effect at low concentrations or to the stationary phase having two sites with different energy levels. The competitive equilibrium isotherm of each enantiomer in the racemic mixture was also determined

using the Method of Mass Balance (MMB) from the experimental data of binary frontal analysis. The experimental values obtained from determining the competitive binary isotherm compared favorably to the theoretical competitive Langmuir isotherm values predicted from the single isotherm data. This is most likely due to the fact that the single component isotherm of each enantiomer follows the Langmuir model and also the column saturation capacity of each enantiomer are similar due to similar physiochemical properties of isomers. This important conclusion may be generalized for all chiral separation when the single component isotherm follows the Langmuir model although several more experimental verifications are needed to confirm this generalization. This shows that for this chiral compound, the multi-component isotherm may be predicted from the data obtained in determining the single component isotherm. The ability to predict the multi-component isotherm, without having to go through the tedious, cumbersome, time consuming and difficult process of determining it experimentally, could potentially save a company large amounts of time and money.

# REFERENCES


Cavazzini, A., Kaczmarski, K., Szabelski, P., Zhou, D., Liu, X., Guiochon, G., *Modeling of the Separation of the Enantiomers of 1-Phenyl-1-propanol on Cellulose Tribenzoate, Analytical Chemistry.* 2001 (73) 5704-5715.

Chiral Technologies, Inc., *Operations Guide for DAICEL Chiral Columns*, Second Edition.

Feng, J., May, S. W., *High-performance liquid chromatographic enantiomeric separation of an enzyme inhibitor which possesses both a chiral center and tautomeric moieties. Journal of Chromatography A.* 2001 (905) 103-109.

Gatti, G., Bonomi, I., Marchiselli, R., Fattore, C., Spina, E., Scorde, G., Pacifici, R., Perucca, E., *Improved enantioselective assay for the determination of fluoxetine and norfluoxetine enantiomers in human plasma by liquid chromatography. Journal of Chromatography B.* 2003 (784) 375-383

Golshan-Shirazi, S., Huang, J., Guichon, G. A., *Comparison of an Experimental Competitive Isotherm and the Levan-Vermeulen Model and Prediction of Band Profiles in a Case of Selectivity Reversal. Analytical Chemistry*. 1991 (91) 1147-1154.

Gritti, F., Guiochon, G., *Influence of a buffered solution on the adsorption isotherm and overload band profiles of an ionizable compound. Journal of Chromatography A.* 2003 (1028) 197-210.

Guiochon, G., Felinger, A., Shirazi, D., Katti, A. M., *Fundamentals of Preparative and Nonlinear Chromatography*. Elsevier Amsterdam, The Netherlands, 2006.

Jacobson, J. M., Frenz, J. H., Horvath, C., *Measurement of competitive adsorption isotherms by frontal chromatography. Ind. Eng. Chem. Res*. 1987 (26) 43-50.

Jacobson, S., Shirazi, D., Guiochon, G., *Chromatographic band profiles and band separation of enantiomers at high concentration. Journal of the American Chemical Society.* 1990 (112) 6492-6498.

Matthijs, N., Perrin, C., Maftouh, M., Massart, D. L., Vander Heyden, Y., *Definition and system implementation of strategies for method development of chiral separations in normal- or reversed-phase liquid chromatography using polysaccharide-based stationary phases. Journal of Chromatography A.* 2004 (1041) 119-133.

Migliorini, C., Mazzotti, M., *Modeling Chromatographic Chiral Separation under Nonlinear Competitive Conditions. AIChE Journal.* 2000 (46) 1530-1539.

Milbachler, K., Kaczmarske, K., Seidel-Morgenster, A., Guiochon, G., *Measurement and modeling of the equilibrium behavior of the Troger's base enantiomenrs on an amylase-based chiral stationary phase. Journal of Chromatography A.* 2002 (955) 35-52.

Miller, L., Weyker, C., *Analytical and preparative resolution of enantiomers of prostaglandin precursors and prostaglandins by liquid chromatography on derivatized cellulose chiral stationary phases. Journal of Chromatography B.* 1990 (511) 97-107.

Nix, R., M., *An Introduction to Surface Chemistry.* Queen Mary University of London. 2003. retrieved on November 27, 2007 from http://www.chem.qmul.ac.uk/surfaces/scc/scat3_1.htm.

Olsen, B. A., Wirth, D. D., Larew, J. S., *Determination of fluoxetine hydrochloride enantiomeric excess using high-performance liquid chromatography with chiral stationary phases. Journal of Pharmaceutical and Biomedical Analysis.* 1998 (17) 623-630.

Perrin, C., Vu, V. A., Matthijs, N., Maftouh, M., Massart, D. L., Vander Heyden, Y., *Screening approach for chiral separation of pharmaceuticals. Journal of Chromatography A.* 2002 (947) 69-83.

Seidel-Morgenster, A., *Experimental determination of single solute and competitive adsorption isotherm. Journal of Chromatography A*. 2004 (1037) 255-272.

Stinson, S., *Chiral Pharmaceuticals, Chemical & Engineering News,* 2001 (79) 79-97.

Stinson, S., *Counting on Chiral Drugs, Chemical & Engineering News*, 1998 (76) 1-19.

Wainer, I. W., *A Practical Guide to the Selection and Use of HPLC Chiral Stationary Phases.* JT Baker 1988.

Wang, T., Wenslow, R.M., *Effects of alcohol mobile phase modifiers on the structure and chiral selectivity of amylase tris(3,2-dimethylphenylcarbamate) chiral stationary phase. Journal of Chromatography A*. 2003 (1015) 99-110.

Zhou, D., Kaczmarski, K., Guiochon, G., *Comparison of the binary equilibrium isotherms of the 1-indanol enantiomers on three high-performance liquid chromatography columns of different sizes. Journal of Chromtography A*. 2003 (1015) 73-87.