INVESTIGATING THE ADSORPTION BEHAVIOR OF SIMPLE BENZENE DERIVATIVES ON CROSS-LINKED DEXTRAN POLYMER

Lappeenranta–Lahti University of Technology LUT

Chemical Engineering Master`s Degree

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Aysu Gamze Cansu

Examiner(s): Professor Tuomo Sainio,

D.Sc. Tiina Rissanen.

ABSTRACT

Lappeenranta–Lahti University of Technology LUT
LUT School of Engineering Science
Chemical Engineering

Aysu Gamze CANSU

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Master's thesis literature review
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Keywords: Sephadex, phytochemicals, adsorption, batch adsorption, column chromatography, adsorption isotherms

Phytochemicals are compounds found in plants that are known for their anti-inflammatory, antioxidant, and anticancer properties, making them important for human health. While these chemicals can be synthesized, research has shown that patients tolerate natural extracts, such as those from willow bark, better. However, purifying these substances from plant matrices is complicated due to their low concentration, expensive scale-up process and complexity of the plant matrix. In this thesis, the aim was to explore the behavior of phytochemicals on Sephadex-G gel. Previous studies have shown unexpected behavior of this gel, and understanding this behavior could enhance phytochemical separation. Model compounds were used with varying chemical properties and worked with column and batch adsorption. Based on the results presented in this thesis, it can be concluded that the Sephadex G-25 matrix has different affinities

toward different organic compounds. The retention time of phenol was observed to be the longest, followed by toluene and benzene, indicating that phenol has the strongest interaction with the Sephadex G-25 matrix. However, further research is needed to determine the exact nature of the interaction between phenol and the Sephadex G-25 matrix, as the data obtained in this study is not sufficient to conclude that hydrogen bonding is the only factor contributing to this interaction. More research is needed to further understand the source of the interaction between Sephadex gel and phenolic compounds.

SYMBOLS AND ABBREVIATIONS

SEC	size exclusion chromatography
IS	International System of Units
$t_{R'}$	adjusted retention time
t_0	dead time
t_R	retention time
HPLC	High-Performance Liquid Chromatography

UV	ultraviolet
k	retention factor or capacity factor or mass-distribution ratio
V_R	retention volume
V_M	volume of the mobile phase
V_S	volume of the stationary phase
KD	thermodynamic equilibrium constant
K	partition coefficient
φ	phase ratio
ε	column porosity
u_s	superficial velocity
u	interstitial velocity
Q	volumetric flow rate
A_{col}	cross-sectional area of the column
r	column radius
V_{void}	empty volume of column
GPC	Gel Permeation Chromatography
IUPAC	International Union of Pure and Applied Chemists
KSEC	SEC partition coefficient
RI	refraftive index

R	gas constant (8.314 J/mol K)
Ce or C	solute concentration in mobile phase
dC/dt	rate of change of solute concentration in mobile phase
dC/dz	rate of change of solute concentration with respect to column length
dq/dt	rate of change of solute concentration in stationary phase
q or qe	solute concentration in stationary phase in equilibrium
z	column length
t	time
$q_{max:}$	maximum adsorption capacity
K_L	Langmuir constant, also known as the equilibrium constant
K_F	Freundlich isotherm constant
n	Freundlich isotherm constant
b	Temkin constant related to sorption heat
K_m	Temkin isotherm constant

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1 Introduction

This study aims to comprehend the separation mechanism of Sephadex gel by investigating the adsorption behaviors of phenol, benzene, and toluene on cross-linked Sephadex dextran gel. The investigation of these behaviors aims to understanding of phytochemicals separation from natural resources by using Sephadex gel. To achieve this objective, column and batch experiments were designed, considering various parameters such as flow rate and concentration. However, it is crucial to acknowledge the limitations inherent in this study, as they have an impact on the obtained results and the overall thesis. Firstly, the employed simplified model compounds and assumptions may not capture the complexity of real-world systems. Secondly, the analytical techniques used to measure adsorption behavior and quantify separation efficiency also possess inherent limitations. Factors such as precision, accuracy, and detection limits of these techniques should be acknowledged due to their potential influence on the results. Lastly, resource limitations, including time, budget, and the availability of specific equipment or resources, may have constrained the capacity and scope of the thesis. Recognizing these limitations ensures a transparent and responsible approach to the research. Despite these constraints, the study contributes understanding of adsorption behaviors of phenol, benzene, and toluene on cross-linked dextran polymer.

Phytochemicals are non-nutritive bioactive compounds present in plants, and they play an essential role in the promotion of human health. They are primarily responsible for producing antioxidants to capture harmful radicals following oxidative stress, which is the root cause of most chronic illnesses (Mohamed et al. 2021). Phytochemicals have also been shown to have anti-inflammatory, anticancer, and antidiabetic properties (Manach, et al. 2005), making them valuable in the prevention and treatment of diseases. We can produce phytochemicals synthetically, but based on the literature, willow barks preparations were better tolerated by patients than synthetic ones (Schmid et al. 2001) (Durak and Gawlik-Dziki, 2014). As a result, individuals began to revert to using natural resources to obtain phytochemicals. The separation procedure is still difficult because of the intricacy of plant chemicals and their comparable polarity. We can improve the manufacturing process of phytochemicals by understanding the mechanism. A better, more

practical, and more affordable way to separate these compounds would benefit the pharmaceutical sector since they are valuable and crucial to the synthesis of medicines.

Before delving into the literature review, it is essential to provide a detailed problem description. The selection of Sephadex gel as the adsorption material was motivated by its debated nature in the existing literature and its relevance to my supervisor's project.

In the previous study, Sephadex G-10 is used, therefore, the focus will be on this gel. Sephadex is a hydrophilic size exclusion column that is dextran based and commonly used in the separation of proteins. Jinze Dou et al. (2021) studied certain phytochemicals which are salicin, picein, triandrin, and (+)-catechin in the Sephadex column and observed surprising results. The studied phytochemicals are shown in Figure 1.

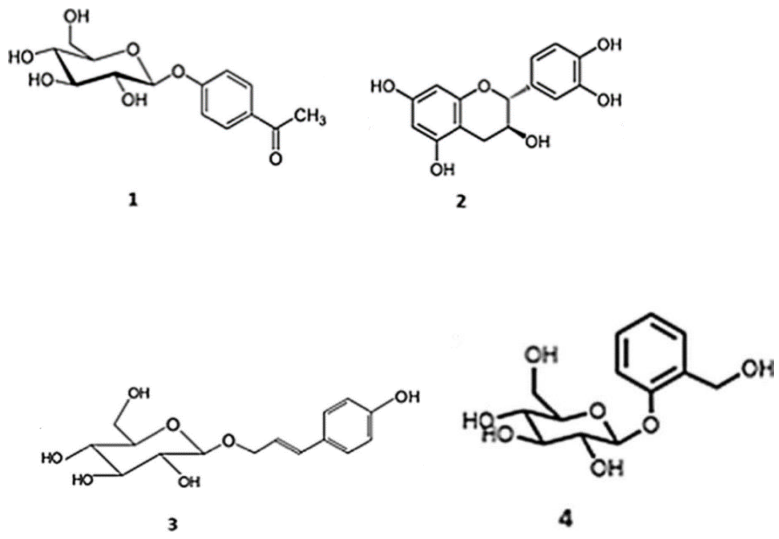


Figure 1. Chemical structure of phytochemicals: **1** picein (298.29 g/ mol), **2** (+) – catechin (290.26 g/mol), **3** triandrin (312.31g / mol), **4** salicin (286.28 g/mol)

Based on the results shown in Figure 2, the separation mechanism of the Sephadex G-10 column was not only based on size exclusion but also involved other interactions, such as hydrogen bonding. This was suggested because the glycidyl moieties in the column might influence hydrogen bonding formation. However, the retention time of some molecules was found to be rather high and could not be attributed to hydrogen bonds or any other process. The smallest and most hydrophilic molecules, such as monosaccharides, were eluted first, which was puzzling.

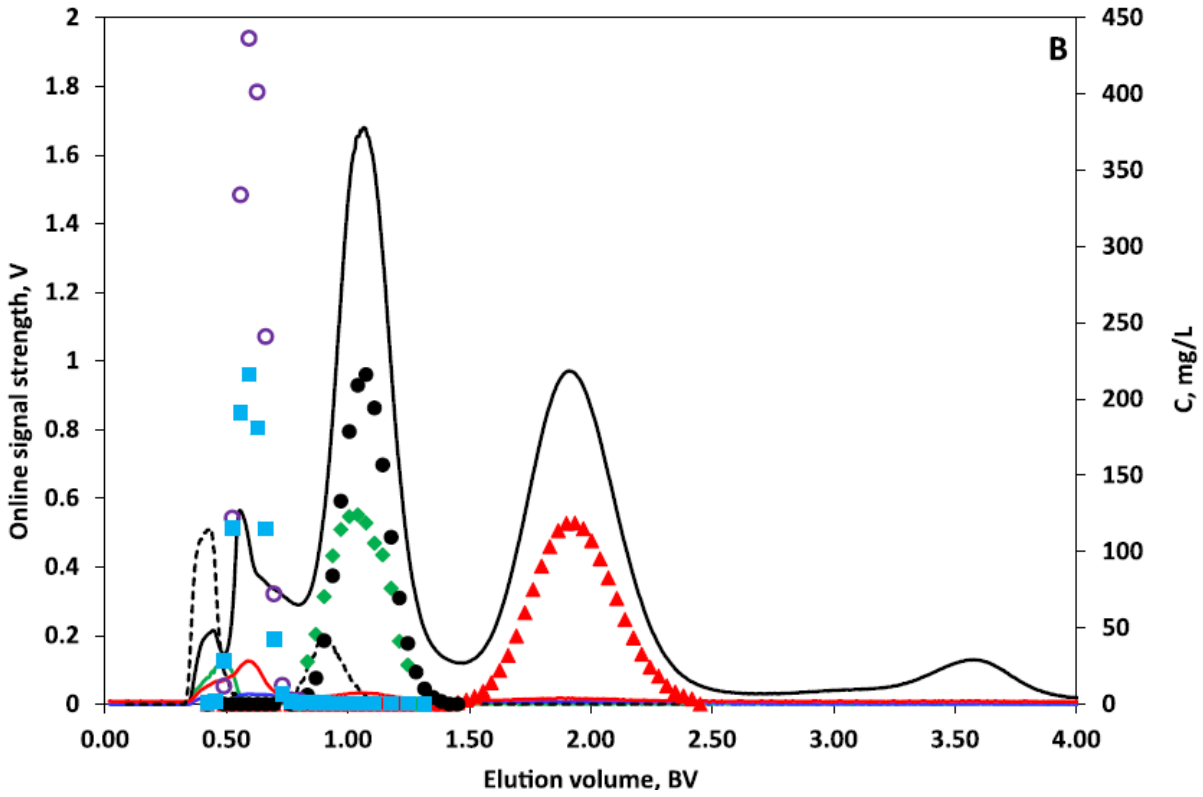


Figure 2. Chromatographic fractionation of willow bark water extract. Gel is Sephadex G-10 and as an eluent pure water was used. Symbols: black solid filled circle = picein; red solid filled triangle = triandrin; green diamond = salicin-like compounds; purple open circle = fructose; light blue square = glucose (Dou et al. 2021)

In summary, this study aims to provide a comprehensive understanding of adsorption mechanisms, including batch and column adsorption, as well as the fundamentals of chromatography. While the topic is extensive, the essential parameters and methods applied in this thesis will be outlined.

Additionally, an experimental procedure will be detailed, followed by a conclusion that relates to the theoretical framework.

2 Adsorption and Chromatography: Techniques and Applications

In this chapter, the fascinating world of batch adsorption column chromatography and column adsorption is delved into, exploring the principles, applications, and techniques involved.

2.1 Batch Adsorption

In the batch adsorption process, batch treatment, such as continuous fixed beds, continuous fluidized beds, and pulsed beds are all used to bring adsorbent and adsorbent into contact with one another (Patel, 2022). Batch adsorption is frequently employed in laboratory settings to create isotherm data models, thermodynamic studies, and kinetic parameters that are crucial for forecasting and contrasting adsorption performance (Saltalı et al. 2007).

In this thesis, the main aim of using the batch adsorption process is to determine the adsorption isotherms to help us to understand the behavior's of different molecules. The isotherms will be explained in more detail in the following chapters.

2.2 Column Chromatography

Column chromatography. It is a separation technique that separates the component based on their interaction/distribution between the stationary and mobile phases. On the other hand, column adsorption describes the procedure of adhering or holding particular molecules or species to the surface or pores of a solid material inside the column. Adsorption is one of the methods through which separation takes place in the setting of chromatography. The analyte molecules contact with the surface of the stationary phase, such as Sephadex gel, in column chromatography, which causes them to be retained or eluted. Based on the method used the mobile phase can be liquid or gas and the stationary phase can be solid or liquid (Keller. and Giddings 2016). In this thesis, we will use the solid stationary phase and liquid mobile phase.

Chromatography allows the separation of molecules based on various characteristics, including their molecular size, specific binding, charges, hydrophobicity, and hydrophilicity. While certain mechanisms such as affinity chromatography may be used alone, mixed mechanisms can also be employed depending on the specific separation requirements.

The main aims of column adsorption are determining breakthrough curves, breakthrough parameters and adsorption parameters. These parameters will be explained in terms of mathematics in the following chapters.

Chromatography can be broadly categorized into two sections based on their purpose: linear and nonlinear (Kumar et al., 2020). Linear scale chromatography, also called analytical chromatography, is used to analyze the constituents of a mixture on a qualitative and quantitative level. The "linear" aspect of this method refers to the analyte concentration being low enough to result in a linear isotherm shape, where the equilibrium concentrations of a component in the stationary and mobile phases are proportional (Lovas, 2022; Edström, 2014). On the other hand, preparative chromatography, also known as nonlinear scale chromatography, is a method that separates the components of a mixture based on their distribution between a stationary and mobile phase. This technique is often used to isolate high-value compounds or optimize preparative separations (Edström, 2014). In nonlinear chromatography, the phase equilibrium isotherms are not linear, and the concentration of a component in the stationary phase is not proportional to that in the mobile phase at equilibrium (Kumar et al., 2020). Understanding the distinction between these two chromatographic sections is essential for developing effective separation strategies and optimizing separation processes.

3. Fundamentals of liquid chromatography

Liquid chromatography is a widely used separation technique in various fields of science, and it is important to understand its fundamental principles to optimize and effectively use this technique. In the previous section, the column chromatography was introduced, which is a type of liquid chromatography. In this chapter, a deeper delving will be done into liquid chromatography, although it is acknowledged that it is an extremely broad topic that cannot be covered in its entirety

in one chapter or even one book. Instead, the focus will be on the specific method used in this thesis. The chapter will primarily discuss linear elution chromatography, which is a widely used.

3.1 Elution chromatography

As it is already explained previously that the substances separated from each other based on their distribution between solid and mobile phase. The separation efficiency is determined by the two factors: Purity and recovery yields. The degree of purity or impureness of a certain component or compound following separation is referred to as purity. It gives the proportion of the requested component to the sum of all the components found in the sample. A higher purity value denotes a higher degree of impurity separation and elimination. On the other side, recovery yield quantifies the amount of the targeted component that is successfully recovered or retained in order to assess the effectiveness of the separation procedure. The ratio between the quantity of the target component that was obtained after separation and the quantity that was initially present in the sample is given as a percentage. A higher recovery yield indicates a higher efficiency in the extraction or separation of the desired component. In this thesis the aim is not separating the substances from each other so, no need to go deeper explanation of these factors.

In chromatography, the distribution of two components between the stationary and mobile phases is controlled by a proportionality constant, K, which is measured by the relative magnitude of the two equilibrium constants. When K equals 1, we can say that the analyte is equally distributed. K is usually considered to be independent of concentration but can be altered by numerous factors such as temperature. The magnitude of K is determined by the thermodynamics of the chromatographic system (Heftmann, 2004).

3.2 The chromatogram

The liquid chromatography system comprises several main components that are essential, regardless of the objective of the study. These components are illustrated in Figure 3 and include a pump to maintain a constant flow rate, a detector to identify substances in the column outlet, and a computer to receive signals from the detector and convert them into a chromatogram. The

detector used can vary depending on the properties and purpose of the study, with options including ultraviolet, pH meter, refractive index, and fluorescence detectors.

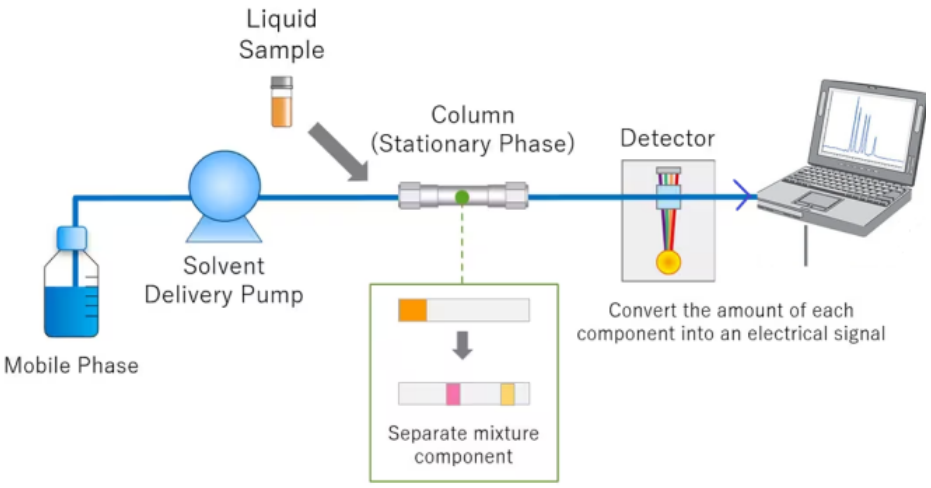


Figure 3. Demonstration of liquid chromatography system (Shimadzu.com).

A chromatogram is a visual representation of the results of chromatographic separation (Hamilton and Sewell, 1982). Although it is not a part of the chromatographic system, it provides crucial information about the system. It is a graph that plots the signal intensity (usually measured in absorbance) of the detector as a function of time or volume. Each peak on the chromatogram corresponds to a separated component of the mixture, and the peak area represents the amount of that component present. The chromatogram can be analyzed to determine various parameters, including retention time, peak area, peak width, and peak height, which all have the potential to offer useful details regarding each component of the studied mixture.

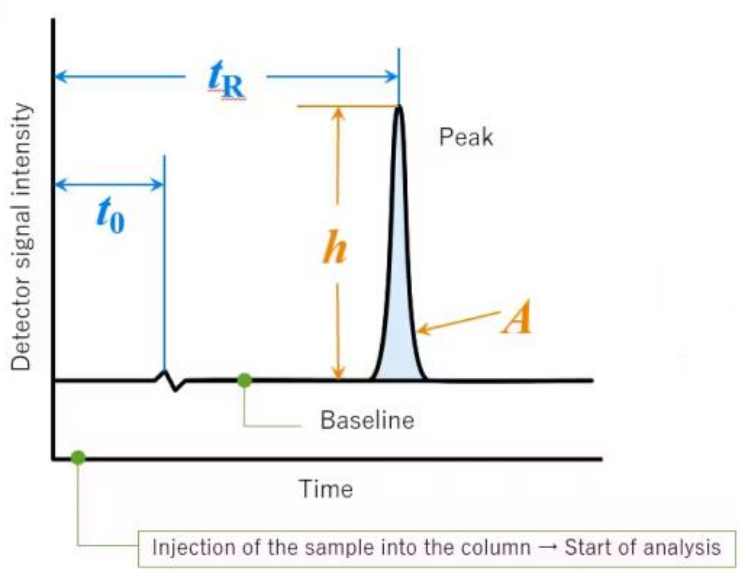


Figure 4. An example chromatogram (Shimadzu.com)

Figure 4 depicts several important terms in liquid chromatography. The first term, t_0 or dead time, represents the time an unretained substance (usually an inert compound) remains in the liquid phase before elution. The retention time (t_R) is described as the duration the analyte remains in the column before elution. It is the most crucial parameter that provides information on the solute's interaction with the column. We can determine the time solute spend in the stationary phase which is also known adjusted retention time by Equation 1.

$$t_R{}' = t_R - t_0 \quad (1)$$

Nonetheless, the column dead time does not solely originate from t_0, the time spent in the mobile phase. It can also arise from other sources, including the design of the system and device connections, which need to be taken into account (Heftmann, 2004).

In Figure 4, a Gaussian-shaped peak is observed, which is typical for linear systems. However, at higher concentrations, the peak shape becomes non-symmetric due to the nonlinearity of the sorption isotherm. In such cases, one can extrapolate the retention time (t_R) or use the first moment of the peak to obtain the corrected retention time ($t_{R'}$) (Cazes, 2004).

3.3 Column porosity

Column porosity is a critical parameter in chromatography, which refers to the amount of empty space within a chromatography column used for separating different chemical components in a mixture. The porosity of a column affects the efficiency, speed, and resolution of a separation. The average size of the void spaces between the solid packing material and the total volume of those void spaces are essential characteristics that determine the column's porosity (Waters Corporation, 2005). The interaction between the liquid and stationary phases relies on the availability of surface area, which is determined in large part by porosity. Understanding the column porosity is necessary when choosing an appropriate separation method for a given application. Porosity can be calculated by the Equation 2.

$$\varepsilon = \frac{V_{void}}{V_{total}} \tag{2}$$

Where, ε is the porosity, V_{void} volume of empty column and V_{total} is the total volume of the column. V_{total} can be calculated by Equation 3.

$$V_{total} = \pi r^2 L \tag{3}$$

Where, r (cm) is the radius of the column and L (cm) is the length of the column.

Last, we need to determine void volume of the column. For this, an inert substance is used to determine the retention time and by Equation 4 it can be calculated.

$$V_{void} = Q t_R \quad (4)$$

Where, Q is the volumetric flow rate in ml/min and t_R is the retention factor.

3.4 Retention factor

The retention factor is an additional significant parameter that can be determined from the chromatogram, similar to the retention time. It provides insight into the chemical interactions taking place within the chromatographic system. The retention factor is dimensionless and can be calculated using Equation 5 (Hage, 2018).

$$k = \frac{t_r - t_0}{t_0} \quad (5)$$

Where, k is the retention factor also known as the capacity factor. It has many meanings, and it also gives partition or distribution ratio by Equation 5a, so it is also known as mass-distribution ratio.

$$k = \frac{amount\ of\ solute\ in\ stationary\ phase}{amount\ of\ solute\ in\ mobile\ phase} \quad (5a)$$

As previously mentioned, the retention factor is a crucial value that can be correlated with other system parameters, such as the retention volume. Equation 6 can describe this relationship:

$$V_R = V_M(1 + k) \quad (6)$$

Equation 6 is equivalent to the fundamental equation of chromatography, which is Equation 7.

$$V_R = V_M + KDV_s \quad (7)$$

Where, V_R represents the retention volume, V_M is the volume of the mobile phase, V_S is the volume of the stationary phase, and KD is the thermodynamic equilibrium constant of the given chromatographic system it is also known as partition coefficient. The natural logarithm of the retention factor has a particular thermodynamic significance; it represents the free energy change at a particular temperature.

Finally, Equation 7 can be used to determine the phase ratio of the column, which is an important parameter for characterizing the chromatographic system and Equation 8 can be obtained. This equation relates the volume of the stationary phase (V_S) to the total column volume (V_M), which includes both the stationary and mobile phases. Then, the phase ratio can be connected to the retention factor by using Equation 8a.

$$\frac{V_S}{V_M} = \varphi = \frac{1-\varepsilon}{\varepsilon} \quad (8)$$

$$k = \varphi KD \quad (8a)$$

Where, φ is the phase ratio. ε is the column porosity.

3.5 Flow velocity

Flow velocity describes the distance traveled per unit of liquid in m/s (Duderstadt and Martin, 1979). In the chromatographic system, constant velocity is one of the important operational parameters. In the column system, there are two different velocities. The first one is the superficial velocity, which we assume the column is empty. The superficial velocity can be written as Equation 9.

$$u_s = \frac{Q}{A_{col}} \quad (9)$$

Where, u_s (m/s) is the superficial velocity, Q (m^3/s) is the volumetric flow rate, and $A_{col\ (m^2)}$ is cross sectional area of column equal to πr^2.

The superficial velocity, which is defined as the distance traveled by a unit of liquid per unit of time assuming that the column is empty. However, for packed columns, the superficial velocity cannot be used as a measure of flow velocity. Instead, interstitial velocity will be used. In packed column chromatography, the interstitial velocity represents the average velocity of the mobile phase through the void spaces between the particles in the column packing (Quinn, 2014).

The interstitial velocity is defined as the average linear velocity of the mobile phase through the interstitial spaces between the stationary phase particles in a packed column. The interstitial velocity can be calculated using the porosity of the packed column and the volumetric flow rate of the mobile phase, as shown in Equation 10.

$$u = \frac{Q}{A_{col}\varepsilon} \tag{10}$$

Where, u (m/s) is the interstitial velocity, A_{col} is the cross-sectional area of the column in m^2, and epsilon is the porosity of the packed column.

The term mobile phase velocity and plate height also need to be explored in detail since they have a direct effect on the separation of peaks. Plate height, also known as H, is a measure of the effectiveness of a chromatographic column. It quantifies the distance a solute need to travel through the column and is inversely related to the resolution of the separation. In simple terms, plate height assesses the efficiency of a column in separating different components of a mixture. A smaller plate height indicates greater efficiency and better separation, while a larger plate height suggests lower efficiency and reduced separation capabilities (Doran, 1995). There is a complex relationship between plate height and the mobile phase velocity. At lower phase velocities, column efficiency is limited by longitudinal diffusion, and at higher velocities, plate height is limited by the two-mass transfer terms (Guiochon and Felinger, 2002). According to the Van Deemter equation in Equation 11, if the mobile phase flow rate is set too low, the longitudinal factor (b/u) will increase and so will the plate height (Snyder and Kirkland, 1979).

Therefore, it is important to find the optimal flow rate for a particular column to achieve the best separation and resolution of peaks (Guiochon and Felinger, 2002).

In addition to the velocities described above, other velocity terms are also employed in chromatographic systems, including the concentration wave velocity, which is the speed at which a solute concentration moves through the column, and the mobile phase velocity, which is the speed of the mobile phase passing through the column. These additional velocity terms will be further explored in subsequent chapters.

3.6 Mobile phase

The liquid that flows through the chromatography column and conveys solute, known as the mobile phase, is essential to the separation procedure. Water is frequently utilized as the mobile phase in Sephadex gel chromatography because of its capacity to interact with the hydrophilic functional groups on the gel matrix (Guo, 2015). The mobile phase's composition can be changed depending on the sample's properties and the desired separation. Organic solvents may be added to water to improve separation efficiency in some circumstances, but water alone is generally adequate. To optimize separation, the flow rate, pressure, and temperature of the mobile phase can all be varied. On Sephadex gel, water plays a crucial role, which we will see in the hydrophobic interaction section.

4. Size exclusion chromatography and adsorbents

As mentioned in the introduction, the previous study utilized Sephadex G series as the adsorbent material. Sephadex G is primarily designed for size exclusion chromatography, a technique that separates molecules based on their size and shape. However, it also displays some adsorption effects. To fully grasp the significance of the previous study, it is essential to have a clear understanding of the underlying principles of size exclusion chromatography, including how it incorporates both exclusion and adsorption mechanisms to achieve molecule separation.

Furthermore, it is valuable to provide a general overview of commonly used adsorbents in chromatography.

4.1 Size exclusion chromatography

Gel permeation chromatography (GPC) and size exclusion chromatography (SEC) are two interchangeable terms that refer to the same chromatographic process. The International Union of Pure and Applied Chemists (IUPAC) prefers the term SEC, although GPC is still commonly used (Agilent, 2014). SEC is a separation technique that sorts molecules in solution based on their size, and in some cases, molecular weight. This method is particularly useful for separating large molecules or macromolecular complexes, such as proteins and commercial polymers (Garrett and Grisham, 2013)

In SEC, molecules in a solution are separated based on their size and, in certain cases, molecular weight. During size exclusion chromatography, molecules are sorted based on their ability to enter the pores of the stationary phase. Smaller molecules spend more time within these pores, while larger molecules pass by them more easily. While the mobile phase flow through the column, smaller particles enter the pores, leading to their retention. In contrast, larger molecules continue to move through the column (Paul-Dauphin, 2007*)*. Consequently, larger molecules are eluted from the column more rapidly than smaller molecules. This results in smaller molecules having a longer retention time compared to larger ones.

The elution of small molecules is often described as a linear relationship between the logarithm of molecular weight (M) of the solute and the elution volume from the chromatographic column (Porath and Flodin, 1959). The molecular size of proteins is typically related to their molecular weight, making this relationship a useful tool for their separation by SEC. However, for polysaccharides and other random coil polymers, a simple, general relationship between size and molecular weight is not possible (Dubin, 1988). As a result, relating their molecular weight to their elution volume in SEC has proven to be challenging.

Size exclusion chromatography (SEC) has several advantages over other chromatographic techniques, including low elution volume, easy separation of small molecules from larger ones,

preservation of the biological activity of the particles to be separated, and, in some cases, easy determination of molecular weight (Garrett and Grisham, 2013). However, SEC is not without its drawbacks, including unexpected interactions between solute and stationary phase, broadening of the bands, and band overlapping, which can negatively impact the resolution of the separation (Polymer Science Learning Center, 2005).

4.2 Size exclusion chromatography adsorbents (Stationary phases)

There are several types and brands of columns used in size exclusion chromatography. However, these columns can be classified into three categories based on their bed material, and the separation size limit. The classification of columns is typically based on the pore size, which determines the size of molecules that can pass through the column. Table 1 shows the classification of size exclusion chromatography columns based on their bed material and separation size limit.

Table 1. The classification of size exclusion chromatography columns (Priyamstudycentre.com, 2022)

Material	**Trade name**	**Molecular mass limit (Da)**
Dextran	Sephadex G-10	0 to 700
	Sephadex G-25	1000 to 5000
	Sephadex G-50	1500 to 30000
	Sephadex G-75	3000 to 70000
	Sephadex G-100	4000 to 150000
	Sephadex G-150	5000 to 300000
	Sephadex G-200	5000 to 800000
Polyacrylamide	Bio-gel P-2	100 to 1800
	Bio-gel P-6	1000 to 6000
	Bio-gel P-60	3000 to 60000

	Bio-gel P-150	15000 to 150000
	Bio-gel P-300	16000 to 400000
Agarose	Sepharose 2B	2×10^6 to 25×10^6
	Sepharose 4B	3×10^5 to 3×10^6
	Sepharose 6B	10^4 to 20×10^6

Since our focus area will be dextran based Sephadex columns, it will be detailed explained.

4.2.1 Dextran based adsorbents: Sephadex

Dextran is a complex branched glucan, a type of polysaccharide derived from the condensation of glucose. Sephadex, a type of column obtained by crosslinked dextran with epichlorohydrin, is primarily used in SEC and occasionally in ion exchange chromatography as well (Porath, and Flodin, 1959). Since the discovery of Sephadex in the 1950s, it has been widely used for the separation of water-soluble biopolymers, including proteins, polysaccharides, peptides, oligosaccharides, simple sugars, and poly macromolecular complexes. Figure 5 illustrates the general structure of a Sephadex column.

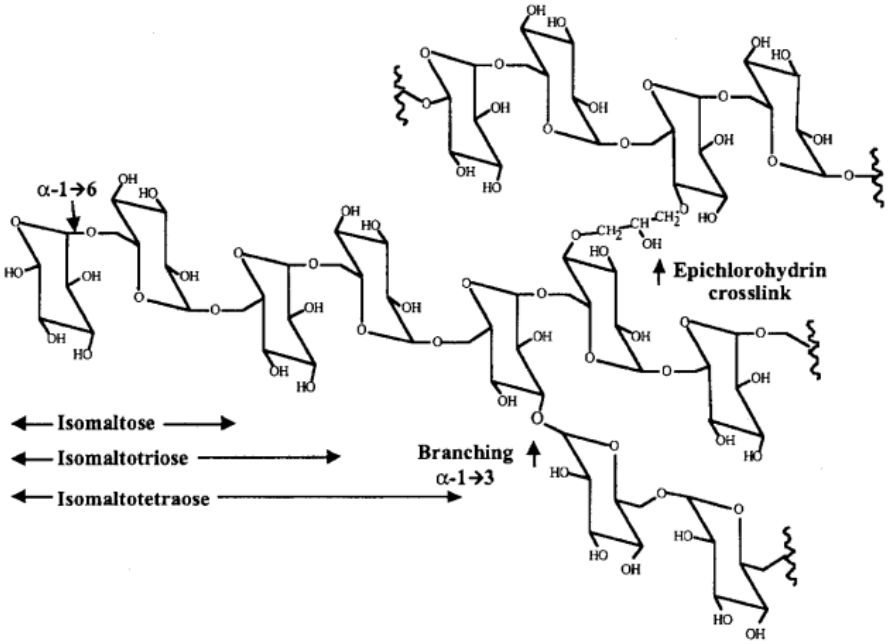


Figure 5. Sephadex structural components (Srisawat, 2001)

Sephadex is a type of column used in size exclusion chromatography that is obtained by crosslinking dextran with epichlorohydrin. It has been used since the 1950s to analyze water-soluble biopolymers, including proteins, polysaccharides, peptides, oligosaccharides, simple sugars, and macromolecular complexes. Sephadex G series columns are based on different dextran molecular weight ranges and have varying degrees of cross-linking. A higher degree of cross-linking results in a lower G-value and a narrower molecular weight fractionation range, with reduced medium swelling. These hydrophilic columns can be used with water or inorganic polar compounds. On the other hand, Sephadex LH series is a lipophilic column made from cross-linked Sephadex G-25 dextran beads that have been hydroxypropylated to yield both hydrophilic and lipophilic characteristics. This column is specifically designed for the gel filtration of natural products, such as steroids, lipids, and low molecular weight peptides, in organic solvents. (Ellingboeet al. 1970)

4.2.2 Polyacrylamide based adsorbents, Bio gel

Acrylamide monomers and the cross-linker N, N-methlylenebisacrylamide are polymerized to form polyacrylamide gel. It is inert, synthetic hydrogels most used for protein separation in gel electrophoresis (Vincent and Engler, 2017)

4.2.3Agarose based gel adsorbents, Sepharose

Agarose-based gel adsorbents are widely used in chromatography for the separation and purification of biomolecules. Sepharose is a popular brand of agarose-based gel adsorbents, produced by cross-linking agarose with epichlorohydrin. The cross-linking creates a three-dimensional network of pores that allows biomolecules to be separated based on their size, shape, and charge. Sepharose has a high binding capacity for a variety of biomolecules, including proteins, peptides, and nucleic acids (Arunima and Bhattacharjee, 2020). It is commonly used in protein purification, DNA/RNA isolation, and immunoprecipitation (Pharmacia, 1998). Sepharose is available in different forms, such as Sepharose 4B, Sepharose CL-4B, and Sepharose CL-6B, with different pore sizes and surface chemistries.

5. Separation mechanism of Sephadex gel

In this chapter, a comprehensive explanation of the separation mechanism of Sephadex gel is provided, encompassing the principles of gel filtration chromatography and the physicochemical properties of Sephadex gel. Based on the technique's name, size exclusion chromatography should sort solutes by their size, but Sephadex gels are often reported to have undesirable interactions with solutes in the literature. This thesis does not aim to separate substances by size, but rather to identify the interactions that hinder the column's size exclusion separation. Mechanisms related to this topic are complex and sometimes contradictory, with combined mechanisms being responsible for undesired interactions. Through a merging of theory and literature, this section will provide insight into the column dynamics and aid in the experimental portion of the study.

5.1 Hydrophilic Interaction

The literature provides substantial evidence that hydrophilic Sephadex gels have a high affinity for retaining phenolic compounds, as supported by extensive research. The retention mechanism in hydrophilic interaction chromatography is intricate and multifaceted, with a thorough

understanding necessitating an explanation of the various mechanisms at play, such as hydrogen bonding, electrostatic interactions, and hydrophobic effects.

5.1.1 Hydrogen Bond

One of the primary reasons for the interactions between Sephadex gel and aromatic solutes can be attributed to the formation of hydrogen bonds between them. This leads to two crucial questions: First, which part of the gel structure does the solute interact with? Second, what mechanism occurs when the interaction takes place? Determan and Walter (1968) found that phenolic compounds did not exhibit any affinity towards a dextran mixture (without an ether linkage) but had high affinity towards polyethylene glycol (with an ether linkage). Consequently, they concluded that the interaction between the gel and aromatic solutes is likely to be due to hydroxy group interaction with ether bridges. Additionally, the binding mechanism of hydrogen may differ at different pH values (Brook and Housley, 1969). Figure 6 illustrates the suggested binding mechanism at different pH values.

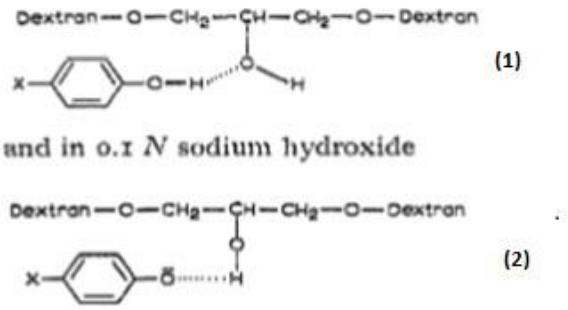


Figure 6. Suggested hydrogen binding mechanism at two different pH values (Brook and Housley, 1969).

Number one corresponds to pH 4, and number two corresponds to pH 9. Brook and Housley, (1969) It was concluded that at higher pH values, the size exclusion effect begins to dominate the adsorption process. Sephadex gel has different functional groups, the ionization state of these functional groups on the Sephadex gel is influenced by the pH of the solution. For instance, a

functional group will be protonated and carry a positive charge if the pH is lower than its pKa value. The functional group, on the other hand, will be deprotonated and have a negative charge if the pH is higher than the pKa. Additionally, the pH can have an impact on the gel's general hydrophilicity or hydrophobicity. The water structure around the gel particles may change as a result of pH changes, which may have an effect on the solutes' accessibility to the gel matrix and the potency of their interactions (Harsa, 1990) (Lin and Castell, 1978).

However, for the second question which is what mechanism occurs when the interaction takes place? There is still no definitive answer, and further research is needed to address this issue. It is also possible that interactions between Sephadex gel and aromatic solutes may be a combination of different mechanisms. Nevertheless, this chapter will focus on the hydrogen bond mechanism as the primary explanation for these interactions.

Hydrogen bonding is a type of intermolecular interaction that occurs due to the physical interactions between hydrogen and the atoms of oxygen, fluorine, and nitrogen. These interactions are also known as noncovalent interactions. Sometimes if two monomers joined together more than one hydrogen bond may form, and hydrogen bonds can even occur within a single molecule (Szalewicz, 2003). Usually, when two molecules that contain hydrogen and an electronegative atom are a sufficient distance apart, they may be attracted to each other through hydrogen bonding. However, if the distance is shortened beyond a certain extent, the attraction between them may become stronger. It is important to note that if the distance becomes too small, they may react chemically instead (Szalewicz, 2003).

Intermolecular forces are produced through a well-known physical mechanism. While electrostatic forces shape the hydrogen bond significantly, three other factors also impact it: induction, dispersion, and exchange energies, which together make up the total interaction energy. Hydrogen bonds can mainly be classified based on their bond energy. Typically, hydrogen bonds range from 2 to 15 kJ/mol, but weaker bonds with 0.5 kJ/mol also exist in the literature. Some hydrogen bonds, like the H-F interaction energy at 40 kJ/mol, are relatively strong. Weak hydrogen bonds involve X-H groups with lower polarity, such as C-H or less polar acceptors like N2. When X-H is attached to a π bond on the acceptor, it's also considered a weak hydrogen bond which is demonstrated in Figure 7.

Figure 7. Example of hydrogen bonds. X-H group attaches to a π bond. (a) Complex of chloroform with benzene. (b) Complex of hydrogen chloride with ethylene (Szalewicz, 2003)

Hydrogen bonding in liquids is more complex compared to gas or solid phase. Liquids with hydrogen bonding exhibit anomalous characteristics that differ from basic liquids. Understanding hydrogen bonding is important for understanding interactions between water molecules, hydrogen bonds between aromatic groups, and Sephadex gel. The key parameter in these interactions is the nature of the hydrogen bond. The hydrogen bond between gel and phenolic groups can be classified as a hydrophilic interaction. However, the hydrogen bond in water molecules can be a reason for hydrophobic interaction in a hydrophilic column. Thus, understanding the anomalous structure of water is crucial and will be explained in detail in the Hydrophobic Interaction chapter.

Studies have shown that hydrogen bonding is a significant force in the interaction between Sephadex gel and phenolic compounds. Streuli (1970) conducted a study that provided evidence for a hydrogen bond interaction between phenolic compounds and the gel. Streuli (1970) observed that molecules containing either oxygen or nitrogen atoms had high adsorption values, indicating the formation of stronger hydrogen bonds.

Another study by Cleland and Cushman (1969) demonstrated the importance of hydrogen bonding in the interaction between Sephadex gel and catechol derivatives. The authors found that the adsorption of these derivatives increased as the pH decreased due to increased protonation of the phenolic group, which enhanced the hydrogen bonding with the gel.

Similarly, a study by Ghosh et al. (2012) investigated the interaction between Sephadex gel and various phenolic compounds, including catechol and resorcinol. The study found that the interaction was due to the formation of hydrogen bonds between the hydroxyl groups of the phenolic compounds and the hydroxyl and carboxylic acid groups of Sephadex gel. The study used techniques such as FTIR spectroscopy and surface tension measurements to confirm the hydrogen bond interaction.

Overall, these studies suggest that hydrogen bonding plays a crucial role in the interaction between Sephadex gel and phenolic compounds, highlighting the importance of this type of interaction in chromatographic separations.

5.1.2 π electron cloud interaction

π electron cloud interaction happens between π electrons of the molecules. It is a type of non-covalent interaction. This interaction involves the interaction of an electron-rich system, such as a metal (cationic or neutral), an anion, a different molecule, or another system, with an electron-deficient system, like the electrostatic interaction that occurs between a negatively charged region and a positively charged one. In Sephadex, this interaction is suggested to occur between the π electron of phenolic groups and unpaired electrons of Sephadex's ether bridge oxygen.

Streuli (1970) suggested that the adsorption of phenolic compounds on Sephadex cannot be solely due to hydrogen bonding and suggested that π electron cloud systems are also involved in the interaction. His study found a linear relationship between adsorption and resonance energy values, which are a measure of the π electron cloud density of hydrocarbons. This relationship suggests that adsorption takes place through π electron cloud interactions between hydrocarbons and the gel. Eaker and Porath (1975) also supported the role of π electron cloud interactions, but only for colinear molecules.

However, the literature is contradictory on this topic. For example, Johnels et al. (1982) observed that increasing the proton-donating ability of phenols resulted in a larger retention volume, indicating that if the interaction was due to π bonding, lower retention volumes would be expected. Similarly, A.J.W. and Housley (1969) found that halogen-substituted phenols showed high adsorption on Sephadex, which could be due to adsorption occurring over the halogen group instead of the hydroxyl group.

De Ligny (1978) suggested a unique perspective and proposed that when the adsorption is not due to -OH moieties, it must be the phenyl ring's π electrons that act as an electron donor towards the -OH groups of the Sephadex, causing an interaction between the gel and solute.

In conclusion, while the literature on the topic is contradictory, several studies suggest that π electron cloud interaction plays a role in the interaction between Sephadex gel and phenolic compounds, along with hydrogen bonding. The exact mechanisms and factors affecting this interaction, however, require further investigation.

5.1.3 Electrostatic Interaction

Electrostatic interaction is a well-known phenomenon between ionic solutes and SEC stationary phases. Sephadex, a widely used SEC gel, contains carboxylic acid groups (4 μeq/ g for gel for Sephadex G-10, and 45 μeq/ g for Sephadex G-25) that are primarily responsible for the ionic exclusion effect below pH 10. This interaction can affect the behavior of solutes during chromatography, particularly those with aromatic structures such as phenolic compounds (Birdi, 1985)

The electrostatic interaction between Sephadex and phenolic compounds has also been studied by Kim and Lee (1995), who examined the adsorption of phenol and its derivatives onto Sephadex LH-20. They observed that the adsorption capacity of the gel increased with increasing hydrophobicity of the phenols, indicating the presence of hydrophobic interactions. The authors also proposed that the adsorption of phenols onto Sephadex LH-20 was due to both electrostatic and hydrophobic interactions.

Chen et al. (2020) used Sephadex LH-20 gel to separate and purify phenolic compounds from the leaves of a plant species. The results showed that the gel exhibited high selectivity for the phenolic compounds, which was attributed to the electrostatic interaction between the carboxylic acid groups on the gel and the phenolic compounds.

In conclusion, the electrostatic interaction between Sephadex and phenolic compounds is a complex phenomenon that involves both electrostatic and hydrophobic interactions. Further studies are needed to fully understand the mechanisms involved in this interaction and to optimize chromatographic separation of phenolic compounds using Sephadex gels.

5.2 Hydrophobic Interaction

Hydrophobic interaction is a significant phenomenon observed in Sephadex gel, albeit often overlooked due to its hydrophilic nature. In order to utilize "hydrophobic interaction chromatography," three criteria must be met according to the literature: (1) identification of hydrophobic sites on the stationary phase, (2) tighter binding of the solute at higher temperatures, which is entropy-driven, and (3) binding at relatively high salt concentrations and elution at lower salt concentrations (Morris, 1977). Before starting to discuss the first criterion, we will look into the water structure and its relationship with the hydrophobic side in the gel. Despite its mass, water is liquid at room temperature, whereas many organic molecules without hydrogen bonds are gases at the same temperature (Lipshutzet al., 2018). In water, nonpolar and some polar substances aggregate, a process termed hydrophobic interaction. This aggregation has intriguing thermodynamic characteristics because in some circumstances, the change in energy caused by the aggregation is positive. Thus, the process's primary driving force is the entropic change, rather than the energetic change (Sinanoglu, 1980). When nonpolar solute particles are dispersed, the water molecules tend to align themselves in an ordered manner around the solute molecules. The formation of aggregates results in a lack of order, which is also referred to as the hydration theory or solvation theory in the literature. There are various suggested water molecule structures around hydrophobic solutes, some of which are illustrated in Figure 8.

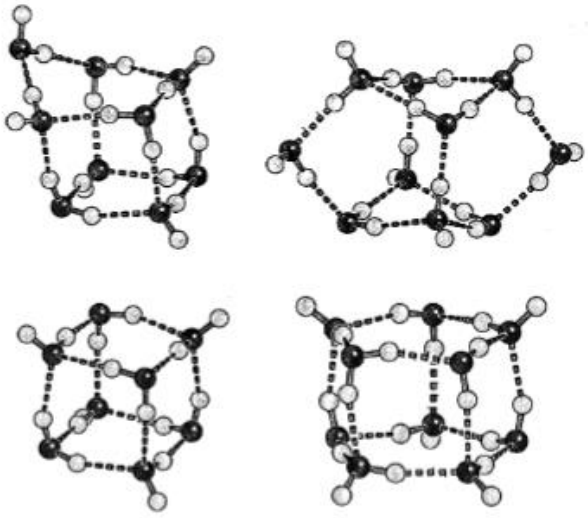


Figure 8. Spectra of water clusters from octamer to decamer (Buck et al., 1998).

The structure of water around nonpolar solutes is a debated topic. Some researchers propose that water molecules form structures known as clathrate cages around the solutes, while others suggest the existence of a more disordered network of water molecules. Additionally, some authors believe that cyclic hexamers are the dominant structures shown in various molecular simulations.

After understanding the structure of water around hydrophobic molecules, we can explain the first criterion related to the hydrophobic nature of the hydrophilic gel. It is worth noting that many hydrophilic gels exhibit hydrophobicity under certain conditions, which can affect their separation performance. (Ping et al. 2019) (Dubin, 1988). The hydrophobic nature of the sugars found in polysaccharide gels can be one reason for the hydrophobicity of the gel. Although sugars are hydrophilic due to their solubility in water, their -CH groups confer hydrophobicity, which is masked by the overall hydrophilicity of the sugar molecule. There is convincing evidence to support the hydrophobic nature of sugar molecules, including their intramolecular hydrophobicity, which results in a strong affinity for polystyrene gel in aqueous media and the cosolvent effect of sugars, which increases the solubility of aromatic hydrocarbons in aqueous systems. Furthermore, the solubility of aromatic rings increases with the number of glucose residuals (Janado and Yano, 1985).

The interaction between sugar and hydrocarbon molecules requires the exclusion of water molecules in between, allowing for closer proximity. The hydration water-sheaths surrounding the sugar molecules must be relaxed to permit access of hydrocarbon solutes to the specific interaction site of sugar with higher -CH density relative to -OH (Dubin, 1988). This suggests the presence of structure-breaking sides in the sugar-aromatic ring interaction. The classical and nonclassical hydrophobic effects in media of water is demonstrated in Figure 9.

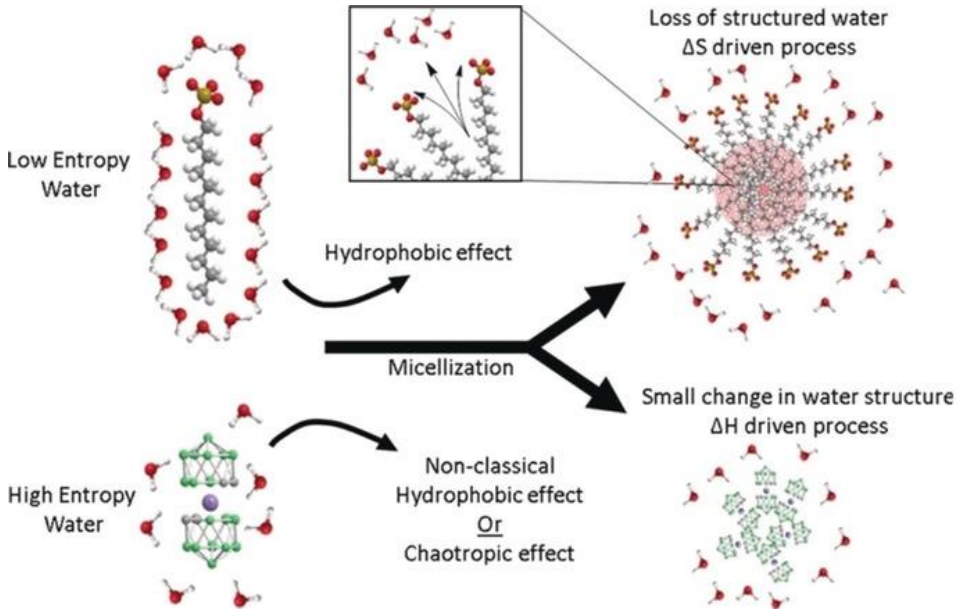


Figure 9. Molecular picture of micellization driven by classical and nonclassical hydrophobic effects (Fernandez-Alvarez et al., 2017).

The affinity of nonpolar solutes towards gels has been attributed to both the ether linkage and the hydroxyl of the diether-linked glycerol crosslinks. However, this affinity was understood to arise from the anomalous nature of water in tightly crosslinked gels. Direct interaction of nonpolar groups with matrix surfaces has also been suggested to make an important contribution to nonpolar affinity. Bywater and Marsden (1983) suggested that the interior of tightly crosslinked Sephadex gels with high ether oxygen/hydroxy ratios are assumed to possess an ether-like property.

Further evidence for the hydrophobic nature of Sephadex gels has been found in various studies. For example, Zhou et al. (2004) investigated the interaction between Sephadex G-25 and nonpolar solutes and found that the gel exhibited hydrophobic interaction. In another study, Kim and Lim (2006) reported that Sephadex G-25 and Sephadex G-50 exhibited hydrophobic interaction with polycyclic aromatic hydrocarbons. Additionally, Ambade and Han (2019) reported that Sephadex G-100 exhibited hydrophobic interaction with n-alkyl alcohols.

These findings suggest that the hydrophobic nature of Sephadex gels can impact their separation performance, particularly in the case of nonpolar solutes. The hydrophobic interaction between Sephadex gels and nonpolar solutes can be attributed to the -CH groups in the vicinity of the ether oxygen atom of the cross-links, as well as the -CH3 groups of the hydroxypropyl group in Sephadex LH-20 (Determann and Lampert, 1972). Moreover, polysaccharide gels such as Sephadex, cellulose, and Sepharose are typically made up of an equal number of -CH and -OH groups, and it is known that under certain conditions they exhibit hydrophobicity (Gong et al., 2019; Dubin, 1988).

Overall, these studies provide strong evidence for the hydrophobic nature of Sephadex gels, which is important to consider when designing and optimizing separation protocols involving nonpolar solutes.

6 Adsorption Isotherms

An adsorption isotherm is a graphical representation of the relationship between the concentration of a solute in the mobile phase (x-axis) and the concentration of the solute in the stationary phase (y-axis) at a fixed temperature (Guiochon et al. 2006).

The application of chromatographic techniques has been in use for over 100 years, yet questions remain regarding the retention mechanism. It has been suggested that the retention is controlled by a combination of partitioning and adsorption processes (Dorsey & Dill, 1986). Determination of adsorption isotherms can aid in the understanding of the adsorption mechanism. Each isotherm provides valuable information about the retention mechanism of the solute on the adsorbent. A

better understanding of the retention mechanism can lead to more efficient separation and ultimately save time and money.

The isotherm can exhibit various shapes depending on the mechanism of the compound's adsorption. Different isotherm shapes have been proposed and characterized for gas-solid equilibria, but they can also be applied to liquid-solid equilibrium (Brunauer et al., 1940) (Sing, 1985). Isotherms can be classified based on their shape, parameter number, or physical meaning. However, each classification has its limitations (Wang and Guo, 2020). Therefore, the following section will provide a general overview of the most common isotherms.

6.1 Linear adsorption model

The linear model of adsorption is widely applied due to its mathematical simplicity. The linear adsorption model can be explained by Henry's law, which states that at low concentrations, the amount of solute adsorbed onto the adsorbent is proportional to the concentration of the solute in the solution (Ghosh, 2018). This model assumes that only a single layer of molecules is adsorbed onto the surface of the adsorbent and that there is no interaction between the adsorbed molecules. While the linear model is limited in its ability to describe the complex adsorption behavior of many systems, it remains a valuable tool for the initial characterization of adsorption processes. Henry's law is in Equation 11.

$$q_e = KC_e \tag{11}$$

Where, K is the partition coefficient, also known as the Henry constant, it is the slope of the isotherm. The partition coefficient is a measure of a component's affinity for the stationary phase compared to the mobile phase. A high partition coefficient indicates a greater affinity for the stationary phase, while a low partition coefficient indicates a greater affinity for the mobile phase.

Therefore, the partition coefficient can be used in chromatography to evaluate a component's retention on the column and its affinity towards the stationary phase (Mandala and Nandi, 2021).

The linear model represents the scenario of low adsorption site coverage ratio. Thus, at low initial adsorbate concentrations, the linear model simulates monolayer adsorption (Khan et al., 2019; Wang & Guo, 2020). It is important to note that all isotherm models trend towards linearity at low concentrations. The linear and nonlinear forms of adsorption equations will be explained in the next chapter to avoid repetition of the same parameters.

6. 2 Adsorption Isotherm models

As we discussed in the previous sections there are many different isotherms models. Some isotherms have two and some of them has three different parameters. Due to complexity of the three parameters isotherms, they will not be explained in this thesis.

Langmuir model: The Langmuir isotherm is one of the most widely used models to describe the adsorption of solutes onto surfaces. Originally derived from gas-solid adsorption, the Langmuir equation can be written in both linear and nonlinear forms. In this section, we will explore the basic principles of the Langmuir model and its applications in various fields. The linear and nonlinear form of the Langmuir can be written by Equations 12 and 13.

$$\frac{C_e}{q_e} = \frac{C_e}{q_{max}} + \frac{1}{q_{max} K_l} \tag{12}$$

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \tag{13}$$

Where, q_e (mg. g^{-1}) is the adsorbed amount of solute on adsorbent, C_e (mg. L^{-1}) is the equilibrium concentration in liquid, q_{max} (mg. g^{-1}) is the maximum adsorption capacity and K_L ($L.mg^{-1}$) is The Langmuir constant, also known as the equilibrium constant, is a measure of the interaction between an adsorbate and a surface. A high value of K_L suggests a strong interaction, while a low value

suggests a weak interaction. This relationship between K_L and adsorbent-adsorbate interaction is fundamental to the Langmuir isotherm, which is a widely used model for describing adsorption behavior (Langmuir, 1918). The Langmuir isotherm model is predicated on several assumptions, including monolayer coverage, constant energy of adsorption, and negligible interactions between solute molecules. In this model, the number of available adsorption sites is limited, and the rate of sorption is determined by the ratio of the concentration of molecules in contact with the solid phase to the number of unoccupied sites. (Ferus-Comelo, 2011).

Freundlich model: The Freundlich adsorption model is generally more suitable for describing non-ideal adsorption behavior on heterogeneous surfaces. This model proposes that multiple diverse sites, each with a distinct free energy of sorption, are active concurrently (Mu, T.-H. and Sun, H.-N. 2019). Equations 14 and 15 represent the linear and nonlinear forms of the Freundlich model, respectively.

$$\ln(q_e) = \frac{1}{n} C_e + ln\left(K_f\right) \tag{14}$$

$$q_e = K_F C_e^{\frac{1}{n}} \tag{15}$$

Where, q_e (mg. g^{-1}) is the adsorbed amount of solute on adsorbent, C_e (mg. L^{-1}) is the equilibrium concentration in liquid, K_F ($L^{1/n}.mg^{1-1/n}.\ g^{-1}$) and n are the Freundlich isotherm constants. The n value is generally between 0.7 and 1. If n=1 the Freundlich model will be linear (Xing and Pignatello, 2005) (Freundlich, 1906). The Freundlich isotherm has some limitations; The constant, K_F and n values change with temperature, it is a purely empirical model with no theorical basis, and if concentration of adsorbate is too high the isotherm deviates (Singh, 2016).

Temkin model: The Temkin isotherm model is used to describe multilayer adsorption between an adsorbent and adsorbate, considering their interaction. It assumes a uniform distribution of binding energies up to a maximum binding energy and that the adsorption heat of all molecules decreases linearly with increasing coverage of the adsorbent surface. However, the model ignores extremely

low or high concentration values (Patil, 2021). Equations 16 and 17 represent the linear and nonlinear forms of the Temkin isotherm model.

$$q_e = \frac{RT}{b} ln(C_e) + \frac{RT}{b} ln(K_m) \quad (16)$$

$$q_e = \frac{RT}{b} ln(K_m C_e) \quad (17)$$

Where, R (J/ (mol K)) is the universal gas constant, T (Kelvin) is the temperature, b (J/mol) is the Temkin constant related to sorption heat, and K_m (L/g) is the Temkin isotherm constant.

7. Mathematical equations in batch and column system

In this chapter, the mass balance equations for batch and column chromatography will be explored. In addition, propagation velocity for the column chromatography will be discussed.

7.1 Mass balance equation of batch adsorption

Like other processes to explain batch adsorption process first we will need a mass balance equation and an equilibrium relation such as Langmuir, Freundlich isotherms which will be discuss later. The batch process is shown in Figure 10.

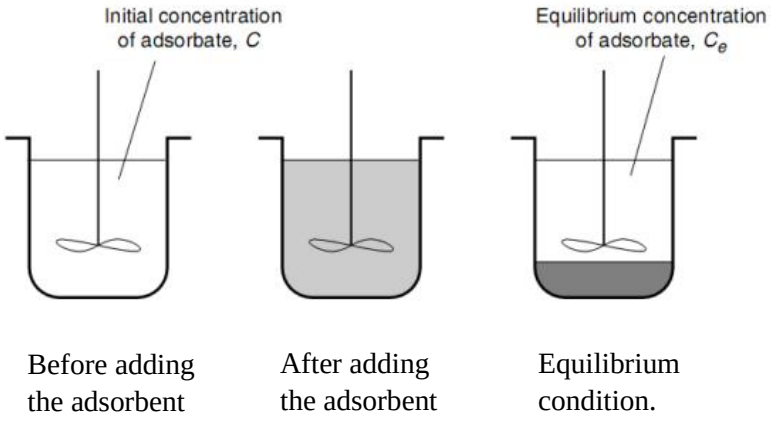


Figure 10. The batch adsorption process (Zhang and Smoczynski. 2019)

The mass balance equation for batch process can be written by Equation 18:

$$qM + CV = q_eM + C_eV \tag{18}$$

The parameter q represents the initial adsorbed amount of solute on the adsorbent, which is typically assumed to be zero. M represents the mass of the adsorbent in grams, while C denotes the initial concentration of the feed in grams per liter. V is the volume of the feed in liters, qe represents the equilibrium concentration on the adsorbent in grams per gram or milligrams per gram, and Ce represents the equilibrium concentration in the liquid phase (Geankoplis, 1978).

7.2 Mass Balance Equation of Column Chromatography

The separation outcome relies on fluid dynamics, mass transfer events, and equilibrium thermodynamics (Smith, 2008). The importance of mass transfer kinetics and phase equilibria thermodynamics varies depending on the experimental conditions. However, the mass of each component of the injected mixture remains constant throughout the chromatographic separation process.

According to the differential mass balance in the mobile phase in Figure 11, the amount accumulated in the slice is equivalent to the difference between the quantity of component C that enters a slice of a column with a thickness of z during time t and the quantity of the same component that exits this slice during the same period. This is shown in Equation 19 (Jones, 2010).

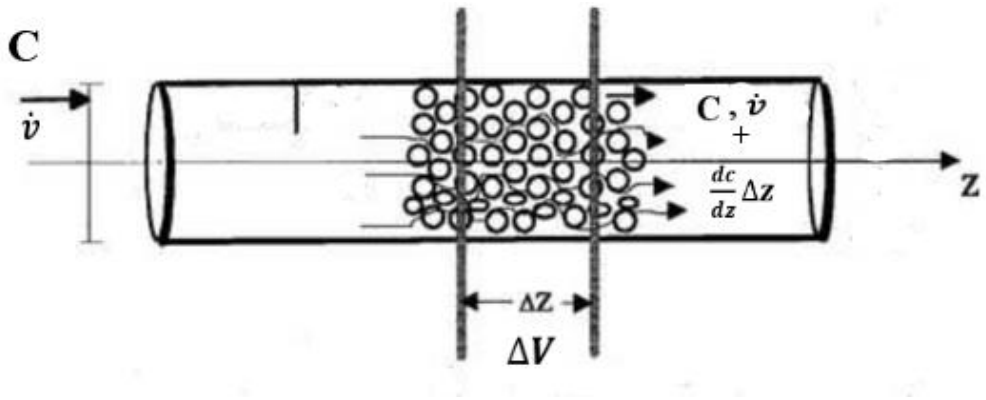


Figure 11. Differential mass balance in a column slice (Guiochon, et al. 2006)

$$Accumulative\ mass\ = Mass\ enters\ the\ silice\ - Mass\ exits\ the\ silice$$

The above formula is valid if we assume that the system is radially homogeneous and isothermal. Based on this, the Equation 20 can be written.

$$\frac{dC}{dt}\Delta V^L + \frac{dq}{dt}\Delta V^S = CQ - \left(C + \frac{dC}{dz}\Delta z\right)Q \qquad (20)$$

Where, q is the solute concentration in stationary phase in equilibrium, C is the solute concentration in mobile phase, Q is the local average mobile phase flux (m^3/s), $\Delta V^L = A_{col}\Delta z\varepsilon$, $\Delta V^S = A_{col}\Delta z(1-\varepsilon)$

The mass balance equations have two independent variables, the time t and the column length z.

A_{col} is column cross sectional area equal to πr^2 and ε is the porosity of the surface. If we supplement the ΔV^L and ΔV^S, we will obtain the following Equation 21.

$$\frac{dC}{dt}A_{col}\Delta z\varepsilon + \frac{dq}{dt}A_{col}\Delta z(1-\varepsilon) = CQ - \left(C + \frac{dC}{dz}\Delta z\right)Q \tag{21}$$

The equation is reorganized, and the following Equation 22 can be obtained.

$$\frac{dC}{dz}Q = A_{col}\left(\frac{dC}{dt}\varepsilon + \frac{dq}{dt}(1-\varepsilon)\right) \tag{22}$$

After converting volumetric flow rate to the velocity, it can be merged to the Equation 22, and we can obtain Equation 22 a.

$$u\frac{dC}{dz} = \frac{dC}{dt} + \frac{(1-\varepsilon)}{\varepsilon}\frac{dq}{dt} \tag{22a}$$

The equation 22a includes two functions, namely C and q, which necessitate an additional equation or relationship between them to be solved. To solve this equation, other factors such as a mass balance in the stationary phase, a kinetic equation, or a lumped mass transfer kinetic equation, as well as an adsorption isotherm, may accompany equation 22a, depending on the chromatographic model being used. In the current study, an adsorption isotherm was used to construct the relationship between C and q.

In the case of a single dependent variable, the relationship is expressed as the derivative of the adsorption isotherm which is dq(c)/dt. Based on this we can apply the chain rule and obtain Equation 23.

$$\frac{dq(c)}{dt} = \frac{dq}{dt}\frac{dc}{dt} \tag{23}$$

Equation 23 is supplemented to the Equation 22a and the final Equation 22b is obtained.

$$\frac{dc}{dt}\left(1 + \frac{(1-\varepsilon)}{\varepsilon}\frac{dq}{dc}\right) + u\frac{dc}{dz} = \tag{22b}$$

7.3 Concentration wave velocity

The rate at which a specific concentration value spreads across the system is called the concentration wave velocity. We can observe that a component that is adsorbing or desorbing has a lower concentration velocity than a component that is not interacting with the solid phase. To express the concentration velocity in Equation 23, we can convert the concentration into the terms of independent variables and calculate the total differential (Hankins et al. 2010)

$$\frac{dc}{dt} + \frac{dc}{dz}\frac{dz}{dt} = 0 \tag{23}$$

The equation 22b can be substituted for dc/dt So, the Equation 24 can be expressed.

$$-\frac{u\frac{dC}{dz}}{\left(1 + \frac{(1-\varepsilon)}{\varepsilon}\frac{dq}{dc}\right)} = -\frac{dc}{dz}\frac{dz}{dt} \tag{24}$$

Where, $dz/dt=u_c$ is concentration velocity. If the equation is reorganized, Equation 24a can be obtained.

$$u_c = \frac{u}{\left(1 + \frac{(1-\varepsilon)}{\varepsilon}\frac{dq}{dc}\right)} \tag{24a}$$

7.3.1 Relation between retention time and concentration velocity

One of the other important parameters for us to connect concentration velocity and Henry constant with the retention time. In the column length L, retention time t_R can be written as the Equation 25 and can connect with the Equation (24a).

$$u_c = \frac{u}{\left(1 + \frac{(1-\varepsilon)}{\varepsilon}\frac{dq}{dc}\right)} = \frac{L}{t_R} \tag{25}$$

Since the aim main aim is connection adsorption isotherms with the retention time, the Equation 25 can be reorganized, and Equation (25a) can be obtained.

$$\frac{dq}{dc} = \left(\frac{t_R u}{L} - 1\right)\frac{\varepsilon}{1-\varepsilon} \tag{25a}$$

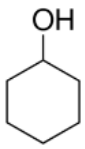
8. Experimental procedure and results

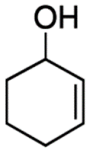
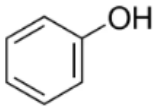
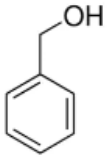
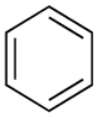
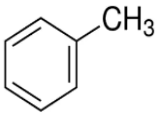
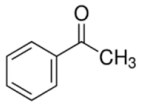
In this chapter, the experimental design and results are presented in detail. The experimental procedure consisted of several steps, including sample preparation, instrumental work, and data acquisition.

8.1 Experimental Design

Following an extensive literature review, we found that there are various mechanisms involved in the adsorption process of Sephadex gels. Previous studies have utilized different chemical compounds to investigate these mechanisms. In this study, we adopt a similar approach and apply it to our research. To begin, we chose to work with simpler compounds instead of the main compounds such as salicin and catechin. Specifically, we selected Cyclohexanol, 2-cyclohexen-1-ol, phenol benzene, toluene, acetophenone, and benzyl alcohol based on their molecular structure and physical properties. However, due to limited time and availability, we were unable to obtain all of the selected compounds. Table 2 displays the properties of these potential model compounds but, we only worked with benzene, toluene and phenol. The selection of these compounds enables us to obtain a more comprehensive understanding of the adsorption mechanisms involved in Sephadex gels and to make a direct comparison between our results and those from previous studies.

Table 2. Structure and physical properties of potential model compounds.

Chemicals names and structures	Molecular weight (g/mol)	Density (g/ml)	Solubility, in water, 20 °C	Vapor Pressure, 20 °C (Pa)	Dipole moment (D)
	100.15	0.96	3.60 g/ 100 mL	133	1.85

Cyclohexanol					
 2-cyclohexen-1-ol	98.14	1	-	482	-
 Phenol	94.11	1.07	8.3 g/ 100 ml	53.32	1.53
 Benzyl alcohol	108.14	1.04	4.29 g/ 100 mL	179.98	1.67
 Benzene	78.114	0.87	1.82 g/ L	9999.17	0
 Toluene	92.14	0.87	0.52 g/L	2933.10	0.36
 Acetophenone	120.15	1.02	6.30 mg/ L	60	3.05

The selection of model compounds for this study was based on a systematic approach aimed at altering their chemical properties. During the selection process, solubility served as a limiting factor. As previously mentioned in the discussion of Sephadex gel's mechanism, several mechanisms were of interest and needed to be tested. The first approach involved testing the interaction of π electron bonds with the gel. Cyclohexanol and 2-cyclohexen-1-ol were chosen as suitable candidates for this purpose. One of these compounds lacked π electrons, allowing us to observe whether the presence of π electrons influences retention. The second mechanism under investigation aimed to determine whether the gel exhibits hydrophobic affinity towards the -CH group. To explore this, phenol and benzyl alcohol were selected as appropriate candidates. Benzyl alcohol contains the -CH group between benzene ring and -OH group, which may impact the adsorption process. Lastly, we sought to examine the effect of the -CH3 group by varying its location. Additionally, we aimed to investigate whether the unpaired electron of oxygen has any influence on adsorption. However, it is important to note that due to the similarities in the properties of the selected model compounds, the obtained results would not provide clear and conclusive findings. The close resemblance of these compounds could potentially hinder our ability to draw definitive conclusions.

8.2 Materials

The experimental materials used in this study were chosen based on their ability to meet the required specifications for the proposed research. These materials were sourced from reputable suppliers and were of the highest available purity. All materials were carefully handled and stored according to the manufacturer's instructions to ensure their quality and consistency throughout the experiment. Safety considerations were of paramount importance throughout the experiment. Appropriate protective measures were taken to ensure that all experimenters were able to work safely with the materials. These measures included the use of personal protective equipment, as well as the implementation of standard laboratory protocols. Table 3 lists all the devices and products used in this study.

Table 3. The devices and materials used in the study.

Materials	Properties, brand
Glass column	Length: 67,7 cm Radius: 0.75 cm
Pumps	Knauer HPLC pumps P 4. 1 S
Degasser	Knauer degasser
Detectors	Waters 2487 UV detector and RI Detector
UV	UV- 6300PC Double Beam Spectrometer
Gel	Sephadex- G25

8.2.1 Column Chromatography

After careful consideration, the model compounds were selected and determined that chromatographic analysis would be the optimal experimental technique to investigate their adsorption properties on the Sephadex gel matrix. The chromatographic system, as illustrated in Figure 12, comprised two pumps for the eluent and mobile phase, a degasser unit, UV and IR spectroscopy detectors, and a packed glass column. The mobile phase used in the chromatographic system consisted of pure water. The solutes analyzed in the system were phenol, toluene, and benzene, each at different concentrations.

The initial step involved packing the glass column with a slurry of Sephadex G-25. The column had a length of 67.7 cm and a radius of 0.75 cm. As the gel was in a slurry form, the exact water content or swelling degree was not determined.

To ensure equilibrium conditions, the column was equilibrated with pure water as the mobile phase. The goal was to evaluate the adsorption behavior of the selected compounds, and chromatographic analysis allowed us to accurately measure their interactions with the Sephadex gel matrix.

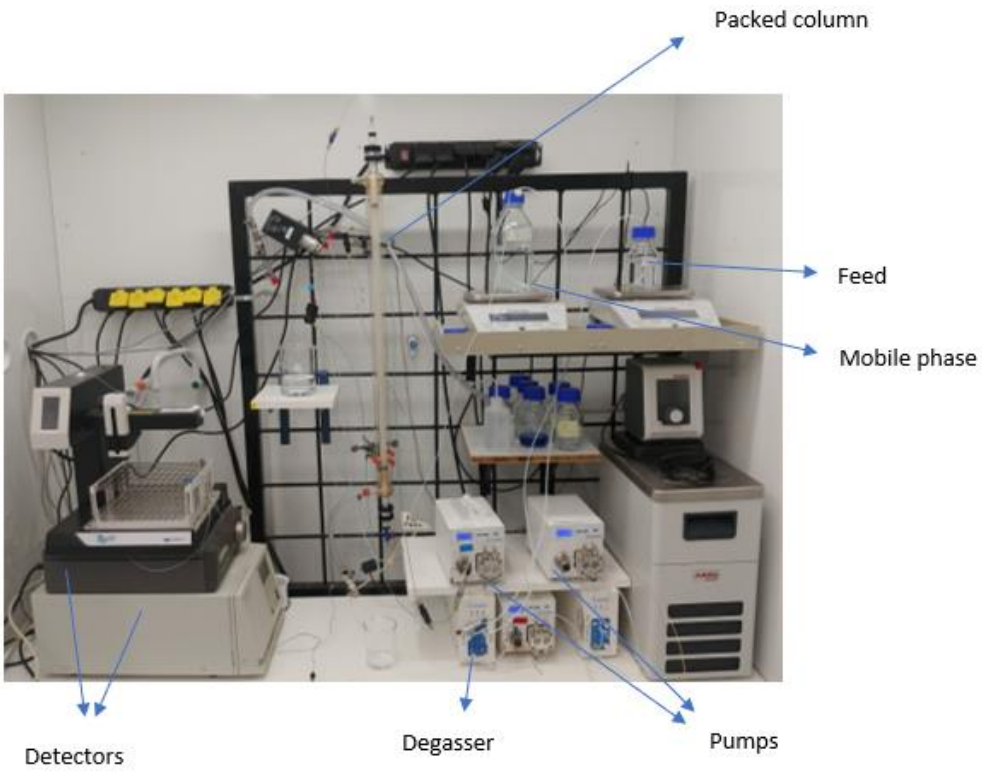


Figure 12. The chromatographic system used in the thesis.

The experiments were conducted at 20°C. Before running the solutes on the column, the column porosity was determined by using Equation 2, which yielded a value of 0.41. To determine the void volume of the column 1g/ L blue dextran was used. One bead volume was measured as 118 ml. Detailed calculations and the chromatogram of blue dextran are provided in Appendix 1.

Various solutions of the model compounds (benzene, toluene, phenol) were prepared, and their concentrations were varied. These solutions were then passed through the column, and UV and RI detectors were used to detect their behavior. In addition, the flow rate, concentration, and feed volume of the solutions were manipulated to observe their effects on the Sephadex gel. Rather than attempting to separate the compounds, our focus was on understanding their interactions with the gel.

8.2.2 Batch adsorption

As previously mentioned, due to limitations in time and equipment for determining adsorption isotherms, the chromatographic system was changed to batch adsorption system, as shown in Figure 13.

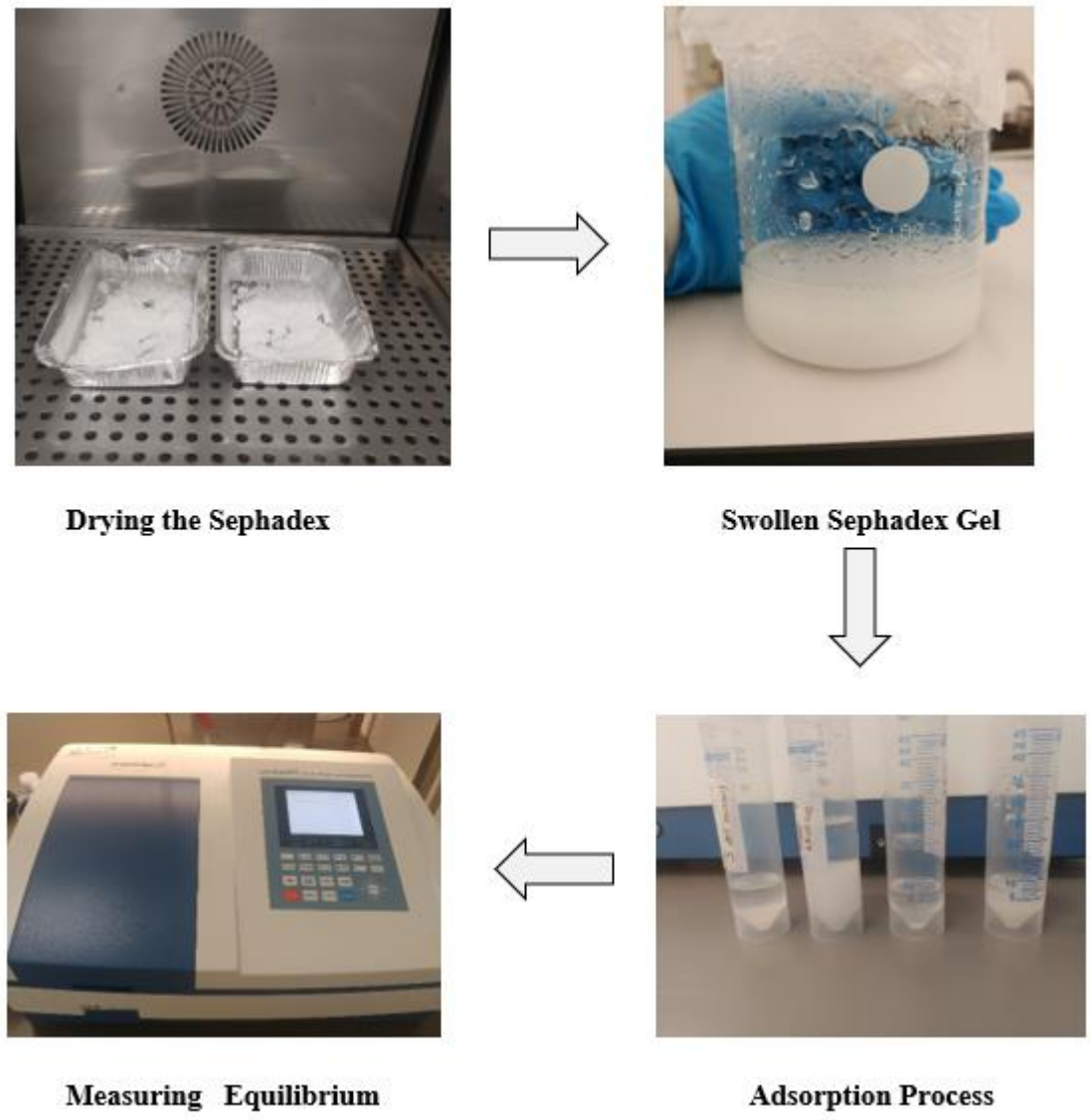


Figure 13. Batch adsorption experiments system

Figure 13 illustrates the batch adsorption experimental system was used to investigate the adsorption behavior of phenol, benzene, and toluene on the Sephadex G-25 gel matrix. Prior to

conducting the experiments, calibration curves were prepared by using UV-vis measurements. The calibration curves are represented in Appendix 1.

In the batch adsorption process, first the slurry Sephadex G-25 gel was dried in an oven at 105°C until there was no further mass change. According to the literature, 1 g of dry Sephadex gel can absorb between 4-5 ml of water, so the gel was allowed to swell for 24 hours to determine the exact amount of free water. A volumetric cylinder was used to measure the volume of water above the particles after the gel had swelled. Additionally, the free water between the particles was also counted and considered in the concentration calculations. This was important because the free water had a significant dilution effect on the initial concentration, which needed to be accounted for to obtain accurate results.

For the batch process, the initial concentrations varied with the constant amount of adsorbent and let them to mix for 24 hours at 450 rpm. The details are represented in Table 4.

Table 4. The variables in the batch adsorption experiments.

Molecule	**Concentrations (g/L)**	**Slurry adsorbent (g)**
Benzene	1.5	15.6
	1	
	0.5	
	0.25	
Phenol	0.2	15.6
	0.1	
	0.05	
	0.025	
Toluene	0.3	15.6
	0.15	
	0.075	

8.2.3 UV-vis measurements

UV- 6300PC Double Beam Spectrometer was utilized for measuring the equilibrium concentrations and calibration curves. For each compound, the liquid portion was separated and analyzed using UV-Vis. As wavelength of 270 nm for phenol, 225 nm for benzene, and 260 nm for toluene were used. The absorbance corresponding to the concentration value was noted and the equilibrium concentrations were determined with the help of calibration charts. The experiments were repeated for various concentrations of the model compounds to generate adsorption isotherms.

8.3 Column chromatography results and discussion

In this section, the results of the column experiments are discussed, and the adsorption behavior of phenol, benzene, and toluene on the Sephadex G-25 gel matrix is analyzed.

After determining the porosity of the Sephadex G-25 gel matrix, the experimental procedure started with phenol. The primary objective of the initial experiments was to investigate the adsorption behavior of phenol on the Sephadex G-25 gel matrix. The result clearly demonstrates the significant adsorption of phenol on the Sephadex G-25 gel matrix, as evidenced by its prolonged retention time compared to other molecules such as toluene and benzene. Figure 14 provides a visual representation of this adsorption behavior. Notably, the chromatogram shows a nonlinear response for phenol, with distinct characteristics observed in the peak front and tailing regions. Specifically, the asymmetry in the peak shape indicates that phenol interacts with the gel matrix in a complex manner.

By progressively diluting the phenol solution, it was discovered that concentrations below 0.0016 g/L phenol exhibited linear adsorption behavior on the matrix. This finding can be important as it provides crucial information for the design and implementation of future experiments involving phenol adsorption onto the Sephadex G-25 matrix, specifically within the linear range of concentrations.

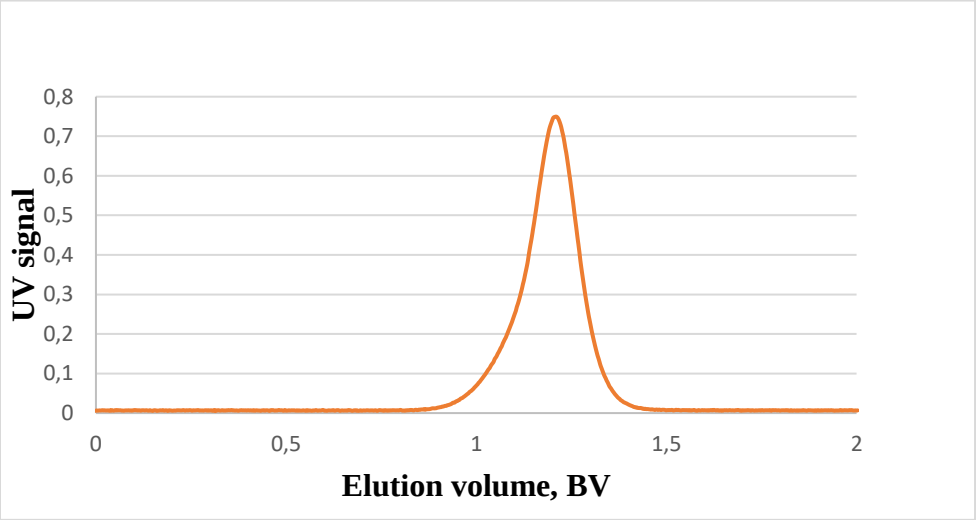


Figure 14. Phenol chromatogram (Concentration, 1g/L; feed amount, 3 ml; Bead volume, 118 ml).

After obtaining our initial results with a specific flow rate, it was decided to explore the effect of varying the flow rate on the adsorption behavior of phenol on the Sephadex G-25 gel matrix. Upon changing the flow rate, it was observed that a broadening of the phenol peak, indicating increased dispersion and changes in the chromatographic behavior of the compound on the gel matrix in Figure 15. This broadening is commonly associated with higher diffusion and dispersion effects, which can influence the efficiency and resolution of the chromatographic separation.

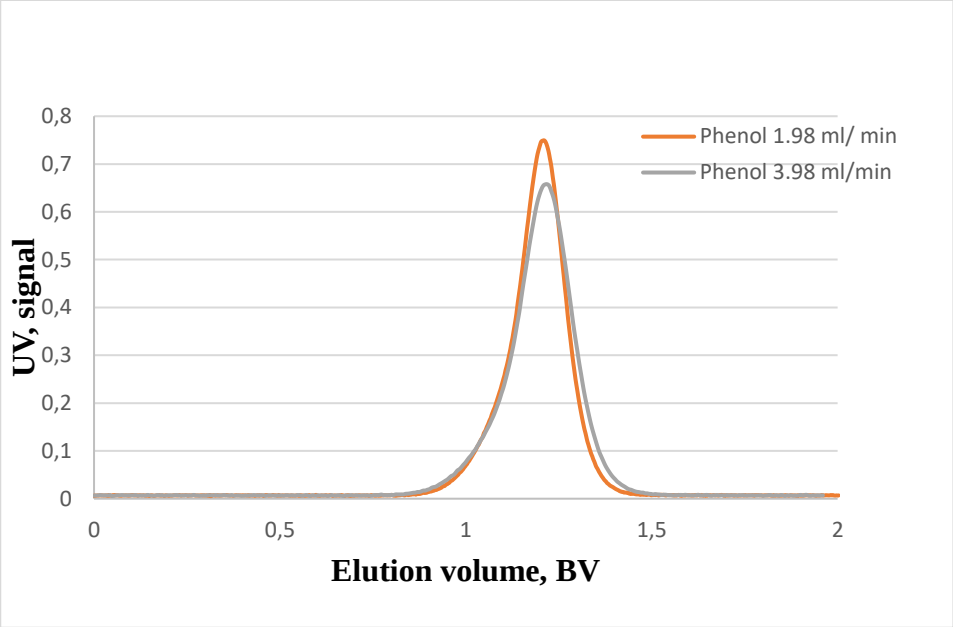


Figure 15. Phenol chromatogram at different flow rate (Concentration, 1g/L; feed amount, 3 ml; Bead volume, 118 ml).

To further explore the interaction between phenol and the Sephadex G-25 matrix, the impact of feed volume on the retention time was investigated. At a constant flow rate, solutions of varying concentrations and feed volumes were injected into the column. As anticipated, it was observed that the retention time of phenol shifted with increasing feed volume, indicating the non-linear adsorption behavior of the compound on the Sephadex G-25 matrix. As the feed volume increased, the retention time of phenol also increased.

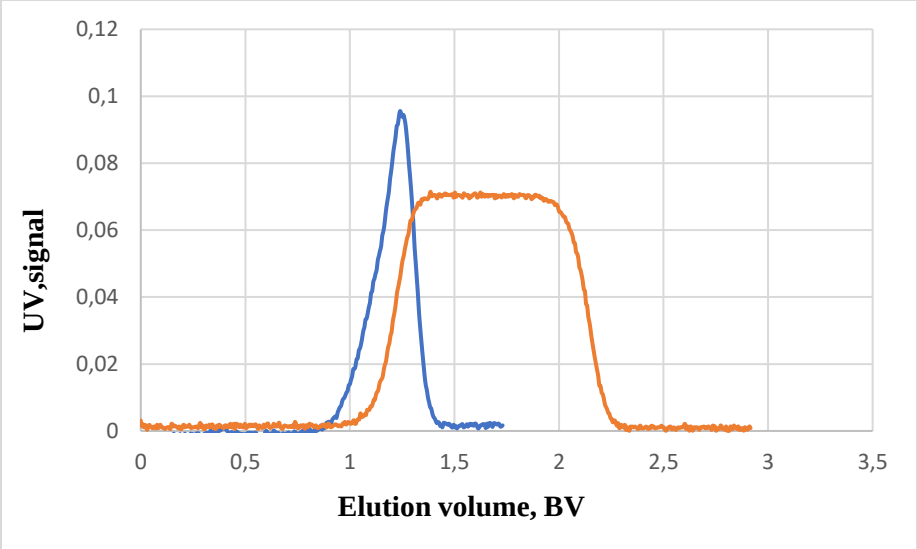


Figure 16. Phenol chromatogram at different feed and concentration. (Blue peak: %1 of BV as feed; concentration, 0.008 g/ L. Orange peak: %100 of BV as feed; concentration 0.0016 g/ L)

In order to evaluate the impact of the changed parameters, the Henry constant for phenol experiments was calculated. The results are presented in Table 5 and were calculated using Equation 25a, which is detailed in Appendix 1.

Table 5. Henry constants for phenol at different parameters.

Compound	Feed amount(mL)	Concetration g/L	Flux ml/min	Retention time	Henry Constants
Phenol	1,19	1	1,98	76,55	1,32
Phenol	3,57	1	1,98	73,64	1,28
Phenol	3,57	1	1,98	73,61	1,28
Phenol	3,57	1	3,98	38,36	1,39
Phenol	3,57	1	3,98	38,22	1,38
Phenol	3,57	0,2	1,98	70,22	1,19
Phenol	3,57	0,2	1,98	70,2	1,19
Phenol	3,57	0,2	3,98	35,28	1,22
Phenol	3,57	0,2	3,98	35,17	1,22
Phenol	3,53	0,04	3,92	35,66	1,22

Phenol	3,53	0,008	3,92	43,94	1,66
Phenol	3,53	0,0016	3,92	64,48	2,75
Phenol	117	0,008	3,92	43,47	1,63

The Henry constant is a measure of the affinity of a solute for the stationary phase in chromatography. A higher Henry constant indicates a stronger interaction between the solute and the stationary phase, while a lower Henry constant indicates a weaker interaction. Looking at the table, we can see that the Henry constant for phenol varied depending on the experimental conditions. For example, when the feed amount was increased from 1.19 mL to 3.57 mL, the Henry constant decreased from 1.32 to 1.28. This indicates that the interaction between phenol and the Sephadex G-25 matrix is weaker at higher feed amounts. This could be due to factors such as decreased availability of binding sites. Flow rate effect on Henry constant was also intended to explain but, there was no comparable data for this.

Overall, these results suggest that the affinity of phenol for the Sephadex G-25 matrix is influenced by a variety of factors, including feed amount, concentration, and flux rate. The variation in the Henry constant values shows that the interaction between the phenol and the Sephadex G-25 matrix can be tuned by adjusting these parameters, which can be useful in optimizing the chromatography separation process.

Next, we performed experiments with toluene and benzene under the same conditions. Due to some technical issues, we were unable to test all the model compounds from Table 2.

Figure 17 and Figure 18 show the peaks obtained for toluene and benzene, respectively. As expected, both compounds exhibited linear behavior and did not show a high affinity towards Sephadex G-25. The linear behavior of the peaks indicates that the adsorption of these compounds on the matrix is not as strong as that of phenol.

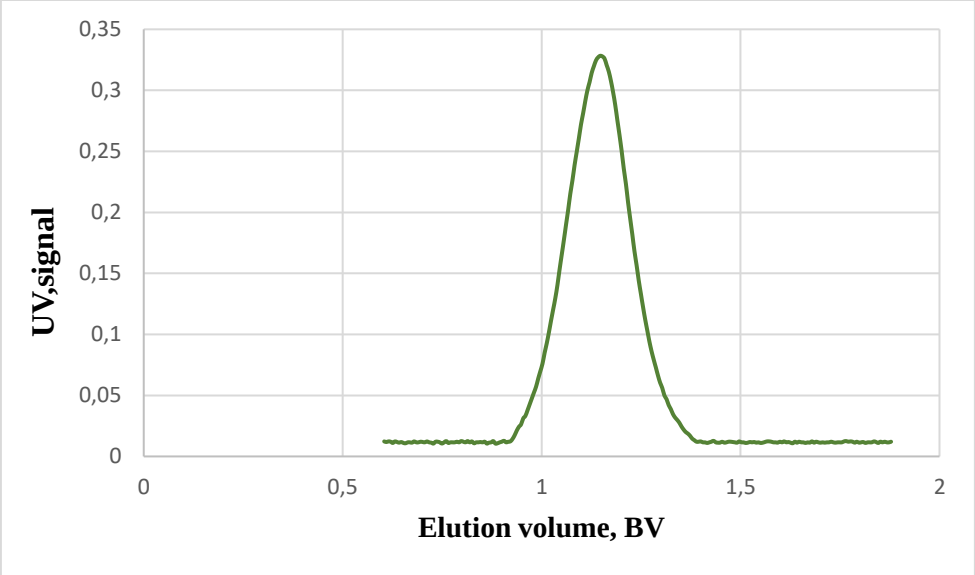


Figure 17. Toluene chromatogram (Concentration, 1g/L; feed amount, 3 ml; Bead volume, 118 ml).

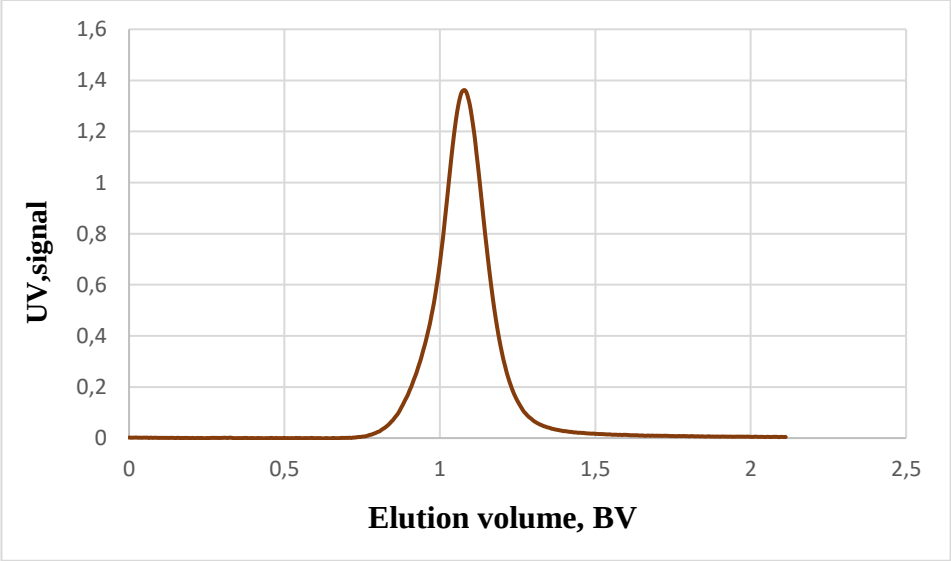


Figure 18. Benzene chromatogram (Concentration, 1g/L; feed amount, 3 ml; Bead volume, 118 ml).

The only parameter changed for benzene and toluene was the flow rate. The Henry constants for both compounds were then calculated and presented in Table 6.

Table 6. Henry constant of benzene and toluene at different flow rates.

Compound	Feed amount(ml)	Concetration (g/L)	Flux (ml/min)	Retention time (min)	Henry Constant
Benzene	3.57	1	1.89	66	1.00
Benzene	3.57	1	3.83	44	1.14
Toluene	3.57	1	1.89	66	1.10
Toluene	3.57	1	3.83	45	1.74

Based on the data presented in Table 6, we can see that for both benzene and toluene, the Henry constant increased as the flow rate decreased. This indicates that the interaction between these compounds and the Sephadex G-25 matrix is stronger at lower flow rates. It's also interesting to note that the Henry constant for toluene is generally higher than that of benzene, indicating a stronger interaction with the Sephadex G-25 matrix. This could be due to differences in the molecular structure of these compounds, which can affect their affinity for certain matrices. Overall, these results suggest that the Sephadex G-25 matrix may be more effective in adsorbing toluene compared to benzene at low flow rates.

All together we compared these three components' retention times and are demonstrated in Figure 19.

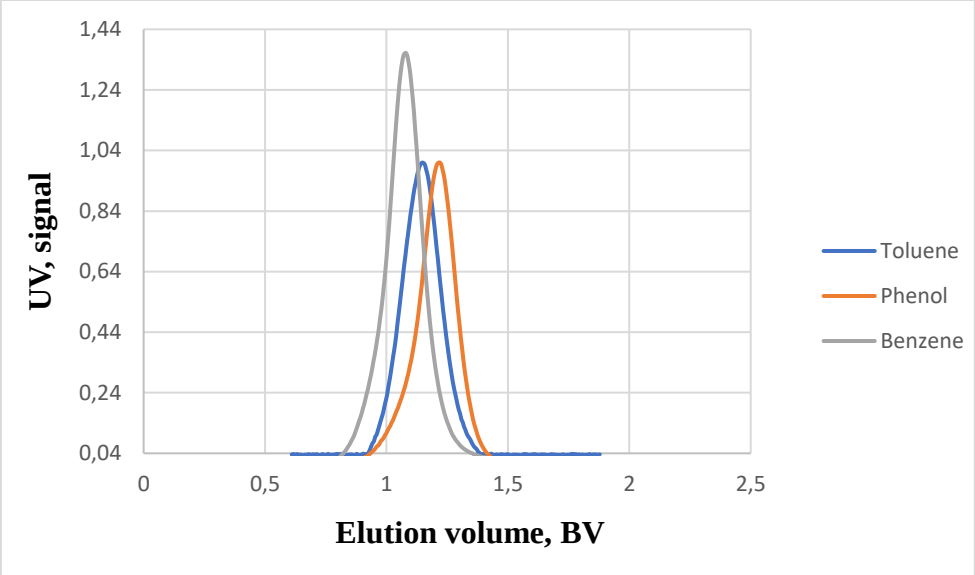


Figure 19. Chromatogram of benzene, toluene, and phenol (Concentration, 1g/L; feed amount, 3 ml; Bead volume, 118 ml).

Based on the given data, it can be concluded that the retention time order for these three compounds is phenol > toluene > benzene. This means that phenol takes the longest time to travel through the Sephadex G-25 matrix, while benzene has the shortest retention time.

The higher retention time of phenol compared to benzene and toluene on Sephadex G-25 can be attributed to the differences in their chemical properties. Phenol has a hydroxyl group (-OH) attached to a benzene ring, which makes it more polar and more likely to interact with the hydrophilic Sephadex matrix through hydrogen bonding and dipole-dipole interactions. In contrast, benzene and toluene are nonpolar compounds without any functional groups, and therefore have weaker interactions with the Sephadex matrix. While hydrogen bonding is one possible explanation for the observed retention behavior, other factors such as polarizability, molecular size, and shape could also play a role. Therefore, further experiments and analysis would be needed to confirm or refute the hypothesis that hydrogen bonding is the main interaction mechanism between phenol and Sephadex G-25. In addition to the hydrogen bond interactions between phenol and the Sephadex G-25 matrix, the π electron cloud of the phenol molecule can also contribute to the observed retention behavior. The phenol molecule has a benzene ring with a hydroxyl (-OH) group attached to it. The -OH group is an electron-donating group, which increases the electron density of the benzene ring, making it more polarizable. As

the phenol molecule passes through the Sephadex G-25 matrix, the π electron cloud of the benzene ring can interact with the matrix through π-π stacking interactions. The Sephadex G-25 matrix contains a network of hydrophilic and hydrophobic regions, and the hydrophobic regions may have aromatic rings that can interact with the phenol molecule through π-π stacking. The combination of the hydrogen bond and π-π stacking interactions may lead to a stronger retention of phenol in the Sephadex G-25 matrix compared to benzene and toluene, which lack the polar -OH group and do not have the same degree of electron density in their aromatic rings. The methyl (-CH3) group in toluene is also an electron-donating group, which can interact with the electron-deficient sites on the Sephadex G-25 matrix. However, the difference in the degree of electron donation between the -OH group in phenol and the -CH3 group in toluene can explain why phenol has a higher affinity for the Sephadex G-25 matrix than toluene. The -OH group in phenol is a stronger electron donor than the -CH3 group in toluene, which leads to a stronger interaction with the electron-deficient sites on the Sephadex G-25 matrix.

8.4 Batch adsorption results and discussion

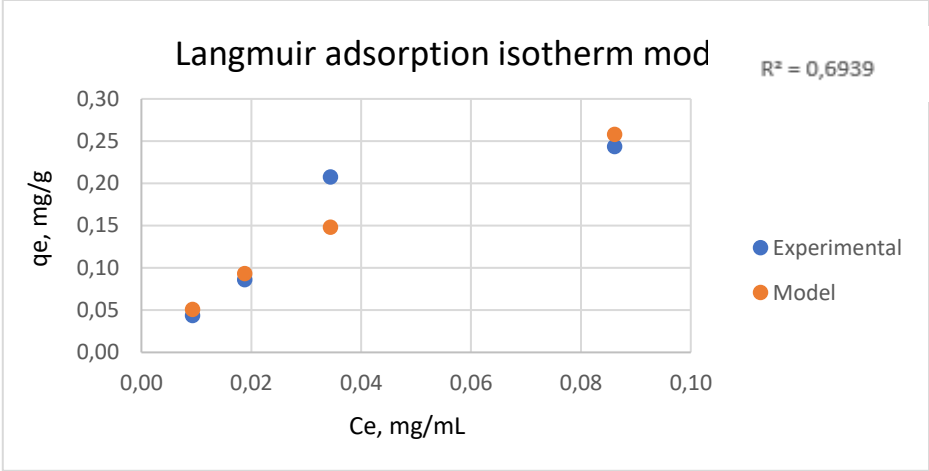
The batch adsorption experiments were conducted to further investigate the adsorption behavior of the adsorbent material. In this section, the results of the batch experiments are presented and analyzed and compared with the column experiments previously discussed. The experimental equilibrium concentration is calculated by Equation 18. For the model calculations, Equation 12 for Langmuir and Equation 14 for Freundlich were used. The results are demonstrated in Table 7. In the case of toluene batch experiments, UV measurements were attempted to determine the adsorption isotherm, but due to the low absorbance of the samples, the resulting concentrations were negative, rendering the data invalid. Therefore, it was not possible to obtain the adsorption isotherm data for toluene.

Table 7. Experimental and model values of the batch adsorption.

	Initial Concentration (mg/mL)	Amount of adsorbent (g)	Volume of Solution (mL)	Experimental		Langmuir model	Freundlich model
				Equilibrium concentration in liquid, Ce (mg/mL)	Equilibrium concentration on solid, qe (mg/g)	qe (mg/g)	qe (mg/g)
Phenol	0.12	2.2	10	0.09	0.24	0.258	0.30
	0.06	2.0	10	0.03	0.21	0.148	0.14
	0.03	2.1	10	0.02	0.09	0.093	0.09
	0.01	2.1	10	0.01	0.04	0.051	0.05
Benzene	1.50	2	10	0.36	9.68	11.622	11.82
	1.00	2.01	10	0.18	6.92	4.101	4.73
	0.50	2.01	10	0.11	3.31	2.167	2.36
	0.25	2.0	10	0.10	1.25	1.974	2.11

Langmuir, Freundlich and Temkin models were applied. For the Temkin model the values were quite bad so, it was not demonstrated in the thesis. Based on the Figure 20, it can be said that phenol has the highest R^2 in Freundlich model, which is 0.89. Langmuir model has 0.69 R^2 which was not surprise. Because in the column experiments anti-Langmuir behavior was observed. Since Freundlich model fitting is not also perfect, it is possible that the adsorption behavior of phenol on Sephadex G-25 gel is more complex and cannot be adequately described by a single isotherm model.

The analysis of benzene adsorption using the Langmuir and Freundlich models resulted in an R2 value of 0.85 for the Langmuir model, indicating a relatively better fit compared to the Freundlich model in Figure 21. However, it is important to note that the interpretation of this result should be approached with caution due to the limitations of the experimental data.



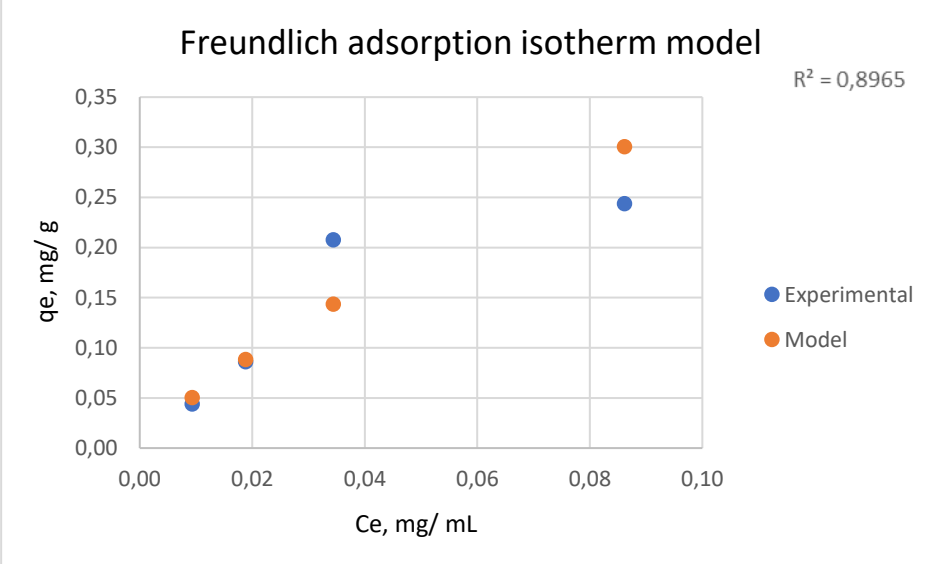
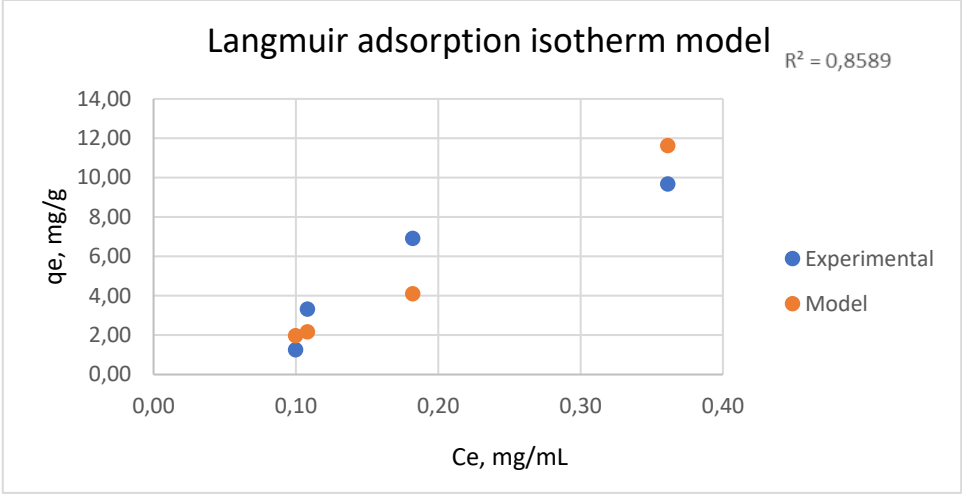


Figure 20. Langmuir and Freundlich adsorption isotherm models fitting for phenol.



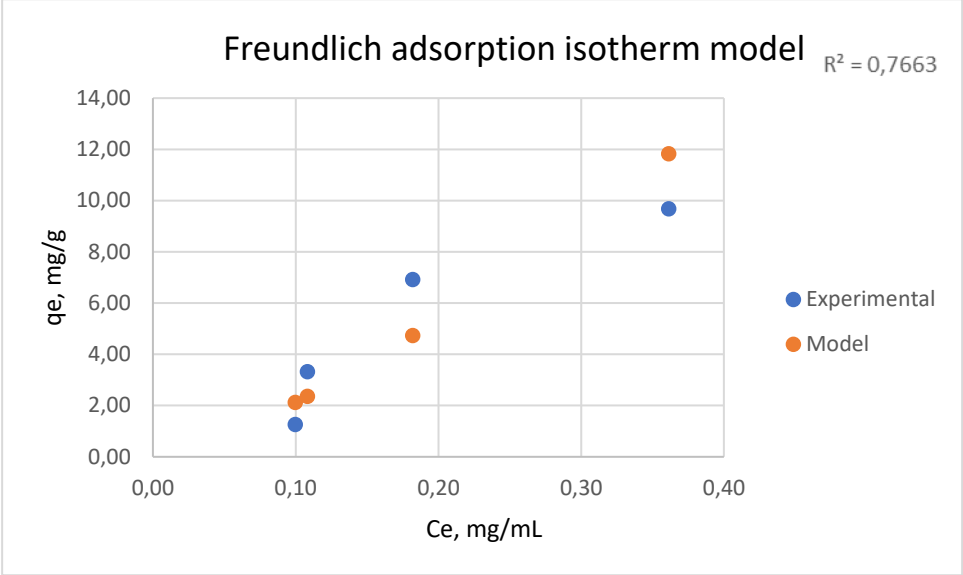


Figure 21. Langmuir and Freundlich adsorption isotherm models fitting for benzene.

8.5 Conclusion

Based on the results presented in this thesis, it can be concluded that the Sephadex G-25 matrix has different affinities towards different organic compounds. The retention time of phenol was observed to be the longest, followed by toluene and benzene, indicating that phenol had the strongest interaction with the Sephadex G-25 matrix. The Henry constant of phenol was also found to vary depending on the experimental conditions, such as feed amount, concentration, and flux rate. However, further research is needed to determine the exact nature of the interaction

between phenol and the Sephadex G-25 matrix, as the data obtained in this study is not sufficient to conclude that hydrogen bonding is the only factor contributing to this interaction. The study also showed that toluene and benzene did not exhibit strong affinity towards the Sephadex G-25 matrix, as expected compared to phenol.

The results of the batch adsorption experiments were limited in providing meaningful data due to the challenges associated with the lack of repeatable and reliable experiments. The constraints of time and budget prevented us from conducting a sufficient number of replicates and ensuring consistent experimental conditions. The Langmuir and Freundlich models were applied to analyze the adsorption behavior of phenol and benzene on Sephadex gel, with the best fit obtained for phenol Freundlich model and for the benzene Langmuir model, respectively. However, the adsorption behavior of toluene could not be adequately determined due to technical limitations.

Further research is indeed necessary to enhance our understanding of the adsorption behavior of simple molecules on Sephadex gel. By gaining more insights into the interactions and mechanisms involved in the adsorption process, we can lay a solid foundation for future experiments involving more complex molecules, such as phytochemicals.

Appendix 1

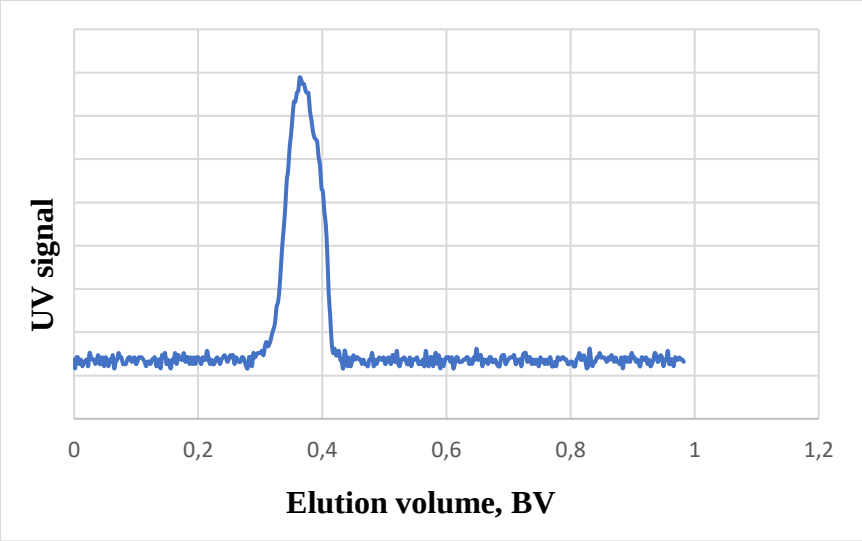


Figure 22. 1g/ L Blue Dextran`s chromatogram on Sephadex- G25 (Eluent: Water, Column: Sephadex-G25, Temperature: 20 C°, Flow rate: 1.74 ml/min, Wavelength: 620 nm, Feed amount: 3.57 ml 1 BV; 117,7 ml)

Porosity calculation:

$$Volume\ of\ empty\ column = 3.16\ \times 0.075m^2 \times 0.66m = 0{,}000118\ L = 118\ ml$$

$$Void\ volume\ of\ the\ column = 28.10\ min \times 0.014 \frac{BV}{min} = 0.41\ BV$$

$$Porosity = \frac{0.41\ BV}{1\ BV} = 0.41$$

Henry constant calculation: An example calculation is demonstrated, and it applied to all components.

$$Henry\ constant = \left(\frac{5.31 \frac{cm}{min} \times 64.48\ min}{66.6\ cm} - 1 \right) \times \left(\frac{0.41}{1 - 0.41} \right) = 2.75$$

Calibration curves:

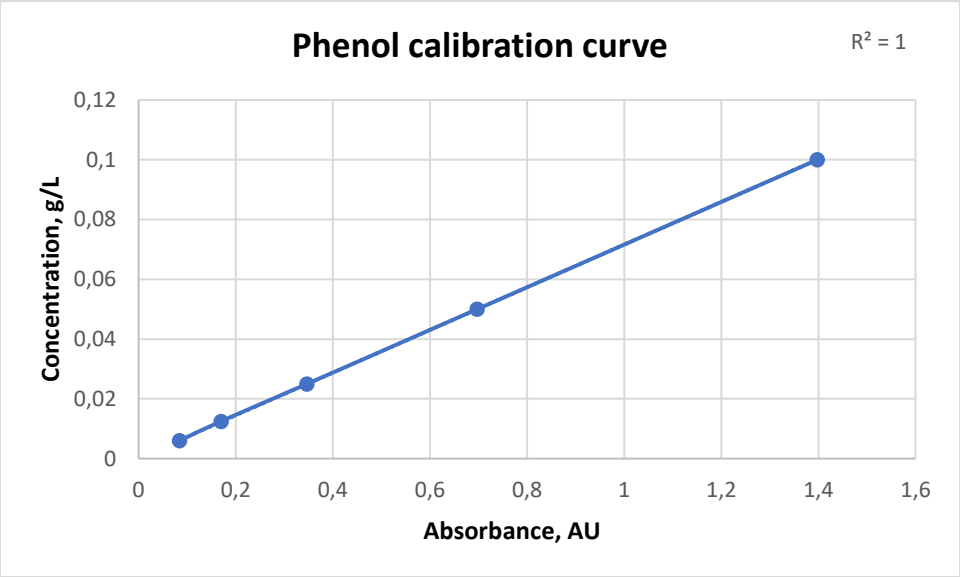


Figure 23. Calibration curve of phenol.

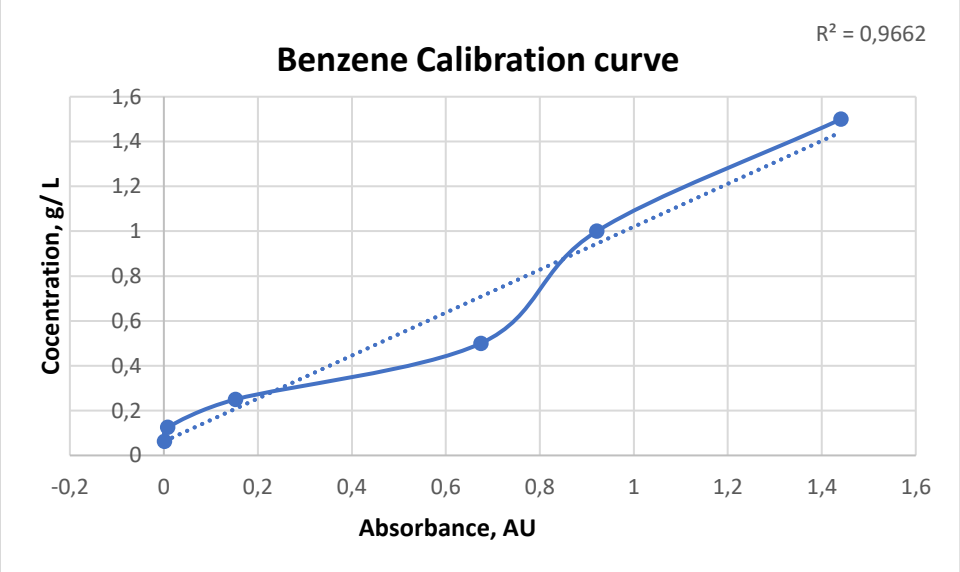


Figure 24. Benzene calibration curve.

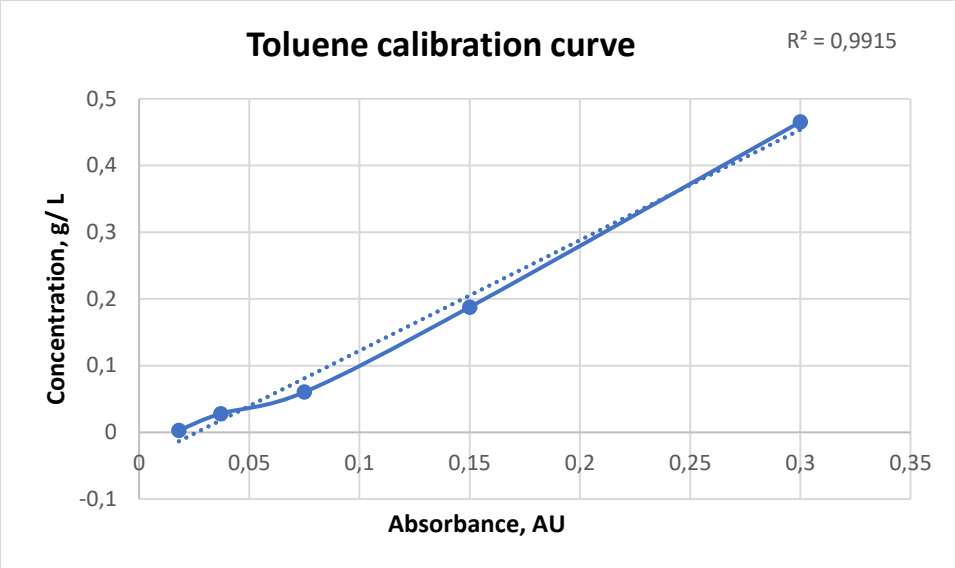


Figure 25. Toluene calibration curve.

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