Characterization of Molecular Adsorption Using Liquid Chromatography and Mass Spectrometry

TORGNY UNDIN
Abstract


Molecular adsorption is a key feature in several disciplines of chemistry, covering as diverse fields as chromatographic separation to biomaterial development. This thesis aims at developing methods and techniques for the characterization of molecular adsorption at the liquid-solid interface. Two different experimental models were used, small molecular interaction characterization using liquid chromatography and complex protein adsorption on polymeric materials possible for biological sampling. Holistic approaches, where both detailed molecular interactions and identifications of trends, could improve the fundamental understanding of adsorption systems, were invariably part of the scientific process.

The characterization of small molecular interactions on liquid chromatography stationary phases via adsorption isotherm determination used combined data from physical phase parameters i.e. carbon loading, linear-, and nonlinear-characterization methods. These experiments were conducted on high performance liquid chromatography systems, using both ordinary reversed phase stationary phases, and hybrid phases. The expansion of the improved elution by characteristic point (ECP) for adsorption isotherm determination, led to that previous impossible isotherm types, having inflexion points, now could be determined by the method. It also reduced errors in isotherm parameters due to the elimination of inaccurate determined retention times where the mobile phase concentration was zero.

The characterization of protein adsorption where performed in an unbiased way. Adsorbed proteins on different surfaces were identified using mass spectrometry (MS) and data dependent acquisition or a targeted method. Prior MS, an improved on surface enzymatic digestion (oSED) method was used to enable identification and quantitation of adsorbed protein originating from ventricular cerebrospinal fluid (vCSF). oSED was found to be able to experimentally determine large variations in protein adsorption characteristics between native and coated polycarbonate surfaces in contact with vCSF. The method was also confirmed being mechanistic in favor of enzymatic digestion of the proteins adsorbed on a surface, rather than a prior desorption into solution before digestion.

An improvement of the overall understanding of adsorption systems was not only achieved with the oSED method as a promising tool for characterization of protein adsorption on arbitrary surfaces, but also the use of linear and nonlinear approaches in stationary phase characterization that strengthened drawn conclusions.

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Till Familjen
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.
The author’s contribution to the papers:

Paper I: Took part in the planning, did the experiments and wrote parts of the paper.

Paper II: Took part in the planning and writing of the paper.

Paper III: Took part in the planning, performed the major part of the laboratory experiments and wrote parts of the paper.

Paper IV: Took part in the planning, performed all of the laboratory experiments and wrote the major part of the paper.

Paper V: Took part in the planning, performed the major part of the laboratory experiments and wrote parts of the paper.

Paper VI: Took part in the planning, performed the laboratory experiments and wrote parts of the paper.

Manuscript not included in the thesis:

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### Abbreviations and Definitions

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<tr>
<td>Adsorbate</td>
<td>Solid or liquid upon which a adsorbent will adsorb</td>
</tr>
<tr>
<td>Adsorbent</td>
<td>Atom or molecule which adsorb onto a adsorbate</td>
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<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
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<tr>
<td>ECP</td>
<td>Elution by Characteristic Points</td>
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<tr>
<td>ESI</td>
<td>Electro Spray Ionization</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSM</td>
<td>Hydrophobic Subtraction Method</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>ODS</td>
<td>OctaDecylSilyl (C&lt;sub&gt;18&lt;/sub&gt;)</td>
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1 Introduction

What is the similarity between a Gecko Lizard and a big pharmaceutical company? The Gecko lizard can use adsorption [1,2] to reach new all-time heights on walls, and the pharmaceutical companies can use adsorption to get an all-time high on Wall Street…

In a variety of fields adsorptions of molecules can be both the greatest asset as well as the cause of (many) problems. Applications that heavily rely on adsorption mechanisms in our daily life around us are: catalysts, filters (water treatment, and air treatment), lubrications, coatings, separation of liquids out of gases, chemical purification and separation (chromatography), to mention a few common ones.

One important process in the industry, used both for preparation and analysis of substances, is the aforementioned chromatographic separation. Both small molecules, e.g. Active Pharmaceutical Ingredient (API), and larger proteins and protein complexes can be separated via different chromatographic methods, which use the differences in adsorption behavior among molecules in order to separate them. This adsorption mechanism can be more or less controlled, optimized and studied in a detailed way due to the in vitro nature of the process.

In the opposite case, in an in vivo system, when adsorption cannot be controlled or directly measured, the unspecific adsorption of different proteins is the most pronounced threat to a successful integration of a biomaterial with native tissues. This will cause problems and increase costs for not only for the healthcare system, but also for the individual suffering from the problems [3]. The challenges is to be able to measure and to characterize the adsorption in terms of gaining a deeper understanding, and at the same time also benefit fields that not solely focusing on specific adsorption interaction, but rather seek to a slight control over the phenomenon. Even if the many adsorption processes have been under the scrutiny of researchers for a long time there are still a lot of challenges ahead regarding the complete picture of adsorption. To create a specific adsorption model systems with the aim to gain deeper knowledge of the adsorption process, requires long term dedication in terms of research, investors, and many techniques [4]. In one way or another chemical research conducted on daily basis in the field of life science always consists of compounds in solutions. In this thesis, one major factor will change during the different experimental paths: the complexity of the studied adsorption systems. The complexity in the adsorption systems
ranges from small, rigid, single, and well defined molecules in Reversed Phase Liquid Chromatography (RPLC) columns (Paper I-III), to biological samples where proteins in human ventricular Cerebrospinal fluid (vCSF) adsorb onto filter membranes (Paper IV-VI). As will be described later, the well-defined nature of the “none”-complex chromatographic adsorption system is actually quite heterogenic, making it very difficult to be able to fully determine even rather simple interactions. Due to the difficulties in determining the specific interactions involved in the adsorption process of only one or two substances in a well-defined absorption system, it is at this stage impossible to discriminate between all possible interactions in a biological sample, that lead to the event of adsorption onto a surface. Instead the focus of that part of the thesis will be to find methods that identify which molecules (proteins) and to what extent those adsorb at a certain point of time. This might reveal whether some properties among the adsorbing molecules are shared and can be linked to surface and solution properties of the adsorption system that governs specific adsorption.

![Figure 1](image_url). A visualization of differences between a complex *in vivo* adsorption system, and an almost ideal adsorption system. Notable is that the ideal system is truly a part of the more complex system; the trick is to make the volume small enough. Image courtesy of Andreas Dahlin.
2 Background - adsorption

Adsorption is the event when an atom or molecule (the adsorbate), adsorbs to a surface (the adsorbent) without the creation of a chemical bond. This can occur in gas-solid, gas-liquid, and liquid-solid interfaces. In this thesis all experiments have been conducted in a liquid-solid interface and therefore the adsorbate will often be named solute, since the molecules of interest are dissolved in a solution. And for convenience the adsorbent will mainly be referred to as the surface. The IUPAC definition of adsorption is: “An increase in the concentration of a dissolved substance at the interface of a condensed and a liquid phase due to the operation of surface forces. Adsorption can also occur at the interface of a condensed and a gaseous phase.” [5].

The adsorption process of atoms and molecules can be described via an adsorption isotherm. This describes the amount of solute adsorbed on the surface as a function of the solute concentration in the liquid. The IUPAC definition for adsorption isotherms in chromatography (also applies for general liquid-solid adsorption) is: “Isotherm describing adsorption of the sample component on the surface of the stationary phase from the mobile phase” [6]. In other words, under constant temperature, “the sample” (solute) can be either on the surface or not. This is a simplification, but will do as reference point.

Interactions

Instead of stronger chemical bonds, such as: ion-, covalent-, and metal-bonds, the interactions that govern the adsorption mechanism comprise of much weaker surface forces such as: hydrogen bonds and van der Waal’s interactions. These weaker bonds, or intermolecular forces, can act over longer distances without inflicting any greater rearrangement of the electron density for the substrate and adsorbent atoms. As a consequence they cannot be linked in a similar way as covalent bonds that are capable of forming close and strong interactions.

As previously mentioned, there are different types of chemical bonds and surface forces. Small- and macro-molecular adsorption comprise of dipole-dipole interactions (hydrogen bonding and ion-dipoles) and van der Waal interactions (permanent and induced dipoles, and the combination of both).
The interactions have longer effective distances than chemical bonds and will contribute to both aligning molecules to the surface, as well as acting as an attractive (and also repulsive) force. Compared to the bond strength for covalent and ionic bonds which are in the range of 50-4000 kJ/mol, the intermolecular forces are in the range of 1-50 kJ/mol, for hydrogen bonds and different van der Waal’s interactions. These weaker interactions are also relatively easy to brake and therefore often a reversible event. Adsorption per se does not require desorption (the opposite of adsorption), but is often in equilibrium with the latter event, making the two events act as parts of a dependent system which is useful when it comes to determining the adsorption. Another driving force is the gain in entropy when nonpolar molecules interact with a nonpolar surface and releases structured water on the surface, known as hydrophobic effect. This effect is naturally higher for larger and more nonpolar molecules. This thesis encompasses both single solute and complex solutions it needs to be highlighted that interactions also occur between molecules which contribute to the total strength of adsorbing forces.

The increase of knowledge and computational power has made it possible to make more and more accurate molecular dynamics simulations of molecular interactions during adsorption with increased complexity. Simulations will probably be an increasingly useful tool in future research concerning fundamental understanding of adsorption interactions, especially when comparing simulations to even more precise experimental adsorption data [7].

Small molecular adsorption

One important process in the industry, used both for preparation and analysis of substances, is the chromatographic separation mentioned above. Both small molecules, e.g. API, and larger proteins and protein complexes worth more than $100 billion each year [8] are separated via different chromatographic methods. These different methods can also be used in different scales, divided into two main categories: Nonlinear and Linear.

Nonlinear chromatographic processes aim at retrieving the actual substances of interest via a separation mechanism. Larger amounts (volumes and/or concentrations) of different substances, mainly pharmaceutical compounds, are often the target for this separation process. The purification often conducted in industrial-scale, where different production modes (steady state recycling (SSR) and simulated moving beds (SMB)) are developed to maximize the output. The demand to determine the thermo dynamical properties of the adsorption via the adsorption isotherm is due to the need to optimize the entire separation process, which is the main reason to do research in the field of nonlinear and preparative chromatography. The adsorption isotherm model must be capable of relating $q$ to $C$, but the model does however not have to be physical-chemical valid in terms of describing
the actual interactions. The characterization of the separation system includes: the equilibrium isotherms and the competitive isotherm for the participating solutes, mass transfer kinetics, column efficiency, etc. And if only high concentrations are used in the separation, low concentration data can be discarded. These parameters are then used in mathematical descriptions of the column which is called a column model and are used to predict the elution profile of the separation.

The second reason to having a nonlinear focus in chromatography is the need of a fundamental understanding of the separation process and the thermodynamic properties of the physical-chemical interactions governing the adsorption-desorption process. Here an accurate (in terms of relating to physical interactions) adsorption isotherm is of utmost importance to be able to measure the actual interaction parameters in a thermodynamic context. This is going to be further explained in section 3.

Analytical chromatography on the other hand mainly seeks to analyze what kind of substance is present in a sample-mixture as well as to quantify how much of each substance is present, e.g. pharmaceuticals in blood samples, toxins in food, qualitative and quantitative measurements of materials, etc. Here, the thermodynamical properties of the system “only” govern the retention time. The true thermodynamic properties of different interactions are on the other hand almost impossible to retrieve since the only output parameter is: the retention time. Under linear conditions, small changes in mobile phase concentration neither affect the retention time nor the peak shape. Instead, differences between peak shapes are an effect of different kinetic effects. In the Figure 2 slope \( H \) near the origin describes the relationship between the two states of the solute \( q \) as function of \( C \), which is also named distribution coefficient at equilibrium.

![Figure 2. A type-I adsorption isotherm: The concentration of adsorbed solute \( q \) plotted as function of the concentration in the mobile phase \( C \). The slope of the black dotted line is the Henry constant \( H \). The initial linear part (black ellipse) often referred to as “analytical” and the later nonlinear later part (dark grey nonlinear slope) as “preparative” in terms of categorizing the research field](image-url)
Macromolecular adsorption

In the previous chapters, the aim has been to decipher in what way adsorbent and adsorbate interact in order to give a defined adsorption pattern. One of the conclusions made clear is: Only a slight increase of the complexity of an adsorption system makes the determination of the physical interaction forces very difficult, and furthermore, the parameters of the adsorption isotherm will most likely lack physical meaning. The question is, how should adsorption be determined in complex biological applications that have a complex environment in terms of molecules and surface parameters. Materials that are brought in contact with biological matrices \textit{in vivo}, e.g. blood, plasma, serum, cerebrospinal fluid (CSF), are often referred to as biomaterials. The immediate response, when a foreign material is implanted is the formation of a protein layer on the material surface [9,10]. Protein adsorption may initiate a cascade of processes that lead to biofouling [9] that finally can form tissue scar, i.e. encapsulation [11,12], which will increase the risk of graft rejection. The complex and dynamic adsorption mechanism depends on many parameters. These include, first, the chemical and physical properties of the surface, e.g. charge, hydrophobicity, roughness and heterogeneity. Secondly, parameters such as the composition, complexity and concentration of the protein sample all influence the adsorption mechanism together with the chemical properties of the liquid in which the proteins are present, for example, pH, ion strength and salt components. Lastly, the physical properties of the complete system, which include sample temperature and different convective fluxes, also affect adsorption [13]. Fundamental issues behind protein adsorption are; the reversibility/irreversibility of protein adsorption, the so-called Vroman effect [14], capacity of multilayer adsorptions [15], energetics [16], and applicability of thermodynamic/computational models [17] on the characteristics of the adsorption process.

This more complex adsorption environment can be slightly reduced and then more accurate describe the adsorption systems in different steps of protein purification. This is an important filed of process chromatography, but will fall somewhat in between the high complexity of macro molecular adsorption system and the less complex small molecular adsorption system, and will thus not be investigated in this thesis.

Adsorption systems

In order to study adsorption in a more specific way, a system comprising of suitable surfaces and solutes needs to be created. To gain detailed information about the interactions leading to adsorption, both characteristics about the surface, the solvent, and the solute need to be known in detail. Since the molecules are small and the interactions weak, a system having a large area compared to the solution is preferred. Ideally, the absorption sys-
tem should be linked to a detector that in real time is capable of measuring even the slightest change in adsorbed molecules.

Packed RPLC columns were selected as model system for the molecular interaction part. The stationary phase consists of small (in this thesis 5 μm) porous silica spheres which have a large surface area (Figure 3). Even if the strength of the adsorption of the solutes onto the stationary phase is fairly weak, the great surface area and the low liquid to stationary phase ratio, are able to give different impact to the retention time (time when the solute is coming out of the column) of different solutes.

![Figure 3. Close up photos of the stationary phase (bottom image), the porous silica particle (upper left image) and a model picture of the covalently bonded C\textsubscript{18} molecules (upper right image). Reprinted with permission from Elsevier.](image)

The silica surface can be tailor-made to interact with specific solutes. And the most common modification is covalent attachment of alkyl chains of different lengths (often OctaDecylSilyl (ODS) or more referred to as C18) in order to give the surface the hydrophobic property. The surface is then capable of interacting with solutes via solvophobic effects [18] due to the presence of an organic modifier in the mobile phase which is utilized during the experiments in Paper I and Paper III. Older ODS stationary phases often had different metallic impurities in the silica which could lead to acidic silanol groups adjacent to the metallic atoms. One method to reduce these adverse effects that could lead to severe tailing of charged solutes is by “end-capping”, which substitutes some of the silanols on the surface with a methyl group. As we can see here our ambition to create a molecular uniform system is already starting to degrade, which is evident in Paper III.
Figure 4. Schematic image over an adsorption system containing poly carbonate filter membranes as a model surface and vCSF as model protein solution. The system was kept under constant temperature and mild rocking.

In the case of analyzing interactions among small molecules a well-defined system of solutes and buffer components on a well-studied and homogenous surface was used. In the studies of protein adsorption mechanisms, almost the opposite approach was used. The adsorption system comprised of poly carbonate filter membranes while the protein solution was native vCSF (Paper IV and V) (Figure 4). Micro dialysis tubing made of polyurethane was adsorption template for a mixture of the six most abundant proteins in vCSF Paper VI.

As a final reminder one must notice that, if any of the three components in the system (Solute, Surface, and Solution) is altered in some way, the adsorption characteristics will change [4].
3 Determination of interactions – targeted adsorption characterization

The word *isotherm* originating from the two geek words: *isos* = equal and *thermós* = heat, indicates that constant temperature is required. The relationship with dependence of the amount of the adsorbing molecules in close vicinity to the surface, which corresponds to the partial pressure of a substance in gaseous phase and the concentration if the adsorbent is in a solution. To determine the “right” isotherm and assign the correct parametric values need to be done stepwise to ensure a physical interpretation of the adsorption isotherm. There are a variety of practical methods used to determine the adsorption isotherm of a separation system. Dynamic methods are methods that are often performed on a chromatic system and it is what has been used in this thesis (Paper I-Paper III). To find the “true” adsorption isotherm, obstacles will stand in the way. Low solubility of the solute, changes in viscosity, altered buffer capacity in the sample liquid, and saturated UV-detector response, are all factors that have to be taken into account when designing the experiments, especially when wanting to maximize the concentration range of the determined adsorption isotherm. When these problems are minimized, the next step is to select a method that can be used to determine the adsorption isotherm. They are all different in terms of practical application, amount of work, amount of substance and solvents used, and finally precision. The following two sections are explanations of the method to determine a valid adsorption isotherm.

Isotherms

Only fitting the adsorption data point to a random model will not give any interpretation of the actual interaction mechanism. Instead there are some general steps that have to be taken in order to reduce the number of possible adsorption isotherm models being valid for the separation system characterized. An adsorption process is mostly described as an equilibrium between an adsorbed state and a free state. Under constant temperature, the relation between the adsorbed concentration on the surface ($q$) and the concentration ($C$) of the solute in the mobile phase [19] is called an adsorption isotherm. In
Figure 5. Adsorption isotherm types (I-V) including brief sketches to visualize the adsorption mechanisms of a single solute onto a surface.

Some of these adsorption isotherms are usually not physically realistic for a wider concentration range in RPLC, e.g. the continuous increasing fraction of solute in the stationary phase described of type-III and type-II. More valid types are IV and V that becomes saturated at higher concentrations i.e. reaches a maximum in stationary phase concentration (monolayer saturation capacity \(q_s\)). This behavior is the most anticipated even in complex adsorption systems if not some kind of chemical binding or biological growth would take place, and form curves of type-II and -III. But that on the other hand would not be adsorption... Each adsorption isotherm type can be described with different adsorption isotherm models depending on the separation system, e.g. what molecules participate in the adsorption process, the topology of the surface. One of the most known models is the Langmuir adsorption isotherm which in its simplest form defines a limited amount \(q_s\) of identical interaction sites (identical \(K\)) are presented in the Eq. 1 on the next page:
\[ q = \sum_i \frac{q_{s,i}K_iC}{1+K_iC} \quad (1) \]

Eq. 1 can describe the adsorption isotherm in Figure 5 top left when \( i = 1 \). For a type-I isotherm the Tóth model is also possible. Under linear conditions in Eq. 1 \((i = 1)\) the equilibrium constant can be described with \( H = H_i = q_{s,i}K_i \) for the Langmuir adsorption isotherm model. The Langmuir model can be further expanded to a number of different adsorption sites \((i)\) having equal association equilibrium constants \((K_i)\) with a finite monolayer saturation capacity for each interaction site \((q_{s,i})\). However, these individual values of \( q_{s,i} \) and \( K_i \), contributing to \( H \), cannot be retrieved with linear methods. As for \( i = 1 \), this assumes that the adsorbed molecules do not affect surrounding sites and no solute-solute interactions (both on the surface and between surface and mobile phase) are possible. When molecules actually interact the BET isotherm might be suitable. This model is an expansion of the Langmuir model describing formations of new layers on top of each other according to:

\[ q = \frac{q_sKC}{(1-bC)(1+(K-b)C)} \quad (2) \]

When \( b < \frac{K}{2} \) it is a type-III, but if \( b \geq \frac{K}{2} \) a type-II isotherm. A type-II adsorption isotherm (Figure 5) has an inflection point which will make the operational line not uniform through the entire adsorption and desorption (Figure 6) when the injected plateau concentration \((C)\) is above the inflection point concentration. An operational line is the description of the thermodynamic constraints governing the possible shape of the elution profile.
One model that is able to describe a type-V isotherm is the Moreau model which has been used to describe the interaction of propranolol on a Kromasil-C<sub>18</sub> column [20]: It can be written as:

\[ q = q_s \frac{KC + IK^2C^2}{1 + 2KC + IK^2C^2} \]  \hspace{1cm} (3)

Here \( I \) is the solute-solute interaction parameter for adjacent molecules adsorbed onto the stationary phase. Other adsorption isotherms curvatures that are responsible for a complex nature of the elution profiles are previously investigated [21], having more than one inflection point are type-IV. These models are often simplifications of the actual process, but sometimes good enough to be used in other applications e.g. the before mentioned elution profile prediction. Next part will present methods for experimental determination of the adsorption isotherm.

**Experimental adsorption isotherm determination**

Frontal analysis (FA) is regarded as one of the most accurate methods for determination of single component adsorption isotherms. FA is often performed in a “staircase mode” in order to speed up the experiment and reduce solvent and solutes [4]. The experiment is most easily performed on a binary pump system where one pump governs the pure mobile phase whilst the other pump distributes the mobile phase containing the solute in a concentration as near the maximum solubility as possible.

Elution by characteristic points (ECP) uses the diffuse part of the elution profile to measure the adsorption isotherm. Both methods have proven fast and fairly reliable under some given conditions when investigating adsorp-
tion isotherms of type-I. The theory which ECP is based on, states infinite column efficiency and a rectangular injection profile in order to produce an accurate adsorption isotherm. The first assumption cannot be eliminate in real experiments, but a sufficient amount of plates $N > 2000$, when using Langmuir as model, can reduced errors to less than 3 % [22]. If bi-Langmuir is the model, a minimum plate count of 5000 for error smaller than 5 % is required. The second assumption regarding the rectangular injection profile induces great errors, due to the often large injection volumes that get a dispersed tail. This error can however be reduced with a modified CUT-injection method [23]. The standard method to retrieve raw adsorption data is the integration of the concentration from 0 to $C$ according to:

$$q(C) = \frac{1}{V_a} \int_0^C (V_R(C) - V_{inj} - V_0) dC$$

(4)

This equation is visualized in Figure 7 where three interaction steps between 0 and different $C$ provide data points in the corresponding adsorption isotherm.

**Figure 7.** Left plot: Elution profile from an overloaded injection with dead volume ($V_0$) and injection volume ($V_{inj}$) and calculations of Eq.4 gives the corresponding adsorption isotherm in the right plot.

Since there is need for a continuous diffuse part for the whole concentration range, small disturbances can be devastating for the errors. There are also difficulties in trying to establish the “true” $V_R$ for the zero concentration of the diffuse rear part. Rearrangement of equation 4 gives:
\[
\frac{dq(C)}{dC} = \frac{V_r(C) - V_{\text{inj}} - V_0}{V_a}
\]  
(5)

The use of slope data instead of integrated raw concentration data also opens up for the determination of adsorption isotherms other than of type-I. To further evaluate if the most appropriate adsorption isotherm is selected, the next sections contain a set of evaluation methods fit for that purpose.

**Scatchard plots**

Some of the main characters of different adsorption isotherms can be visualized in a Scatchard plot, where the adsorption isotherm data \( \frac{q}{C} \) is plotted against \( q \). The different curvatures of the Scatchard plot in Figure 8 can give useful indications of possible adsorption models and a number of interaction sites for the solute on the stationary phase. Straight lines indicate single-site models, and curved lines indicate more than one interaction site (heterogeneous models) e.g. bi-Langmuir and Töth (both type-I), while slopes containing maximum and minimum are adsorption isotherms having inflection points (type-II and type-V) in the Scatchard plots.

![Figure 8. Adsorption isotherm models plotted as Scatchard plots.](image)

In addition to this, the slope of a Langmuir model is the corresponding \( K \)-value and intersect with the \( q/C \) axis gives the \( q_sK \) value. However using slope data (\( dq/dC \)) in a Scatchard plot cannot be made without first integrating the raw data.
Adsorption Energy Distribution (AED)

The step calculating the Adsorption Energy Distribution (AED) is a powerful tool to further eliminate possible adsorption isotherm models. This method is capable of answering whether the adsorption models are energetically heterogeneous or not. If the adsorption processes have more than one interaction site between the solute and the stationary phase, this will be visualized in the AED plot (Figure 9). This relative parameter values on the local independent interaction sites with different relative association equilibrium constant ($K$) and mono saturation capacity ($q_s$) (Figure 9).

\[
q(C) = \int_{K_{\text{min}}}^{K_{\text{max}}} f(\ln K)\theta(C,K)d\ln K
\]

$\theta(C,K)$ is the local adsorption isotherm model (in this thesis Langmuir was used), and $K_{\text{max}}$ and $K_{\text{min}}$ are related to the inverse maximum and minimum concentration ($C$) of the solute in the mobile phase for which the adsorption isotherm was determined. In this study a slightly larger concentration span ($0.1/C_{\text{max}}$ to $10/C_{\text{min}}$) was used in order to aid the conversion to adsorption sites in proximities of the integration limits. The AED integral was solved with an iterative algorithm (expectation-maximization method) [24]. The AED is also capable of using raw adsorption isotherm data as well as raw slope data [25]. These relative values can be compared to the later determined values of $K$ and $q_s$ from next part.
Model prediction and fitting

Previous sections have described different methods to acquire adsorption isotherm data and also methods that gain additional knowledge of some of the main characteristics of the measured raw adsorption data (three first steps in Figure 10). The steps from previous sections can, in the following order act as a guide on how to reduce the probable model candidates:

![Diagram showing the steps: Determine raw adsorption data, Make a Scatchard plot (Integrate slope data), Calculation of (AED) Adsorption Energy Distribution, Fit models and evaluate parameters, Predict elution profiles.]

*Figure 10. The route of determine an adsorption isotherm physical valid for the separation process*

In step four (*Figure 10*) fitting the actual data from the raw isotherm to the different adsorption isotherm models (e.g. Langmuir, bi-Langmuir, etc.) are made. A vast number of initial parameter values and the Marquardt-Levenberg algorithm were used for the fitting step in this thesis. These initial guesses were randomized over all possible solutions in order to eliminate a false global optimum (based on a local optimum) for the set of parameters. This will lead to parameter estimation rather than an adsorption isotherm fitting, since we already assumed suitable models rather than “true” ones. As a test of the hypothesis that compared to other models, the selected adsorption isotherm model actually are the one most suitable to describe the adsorption process in the column, a Fisher test is conducted (Eq. 7). The optimal parameters for each suitable adsorption isotherm, previously found during the fitting process, are compared to the experimental adsorption isotherm data.
Here $q_{\text{fit}}$ is the predicted stationary phase concentration of the selected model and $\bar{q}$ is the average stationary phase concentration value. $p$ is the actual number of parameters that can be changed in the model and $n$ are the number of data points in the adsorption isotherm, which in the case of using ECP-slope are numerous. A critical F-test is then used in order to evaluate if there is a significant difference between the Fisher parameters from the compared models. The residual sums of squares need to be normal distributed if the compared models contain nested variables.

The final test will of course be to check if the calculated parameters in the selected adsorption isotherm have a rational meaning and can account for any physical interpretation.

**Linear characterization**

Analytical chemists often want direction to speed up selection of a substitute to their now discontinued favorite column. Linear characterization methods aim at narrowing down the number of possible columns with similar separation properties under linear conditions. In the case with orthogonal two dimensional HPLC the methods also can suggest the most orthogonal column compared to the one selected [26,27]. It has also been suggested that linear characterization can be used as a qualitative control of batch to batch reproducibility in stationary phase manufacturing [28]. The linear methods are based on the measurement of the retention time of a set of solutes.

There are a couple of different methods available e.g. the “Tanaka-test” [29], “Engelhardt” [30] and the Hydrophobic Subtraction Model (HSM) [31]. The major advantage with the linear characterization is of course the fast laboratory work and the straightforward interpretation of the results which it is intended to give. The disadvantages are the lack of absolute values of the physical and chemical interactions, and the very limited insights of the behavior of the separation system as the concentrations and volumes of the injected solutes rises. In order to bring some kind of systematic characterization of the vast possible interaction between the solute and stationary phase, some main types of interactions are defined. Here the HSM-method general descriptors are presented (*Figure 11*), which are very similar to the Tanaka method. One should note that this descriptors aim at explaining the most common interactions in this “simple” and almost “ideal” adsorption system. In HSM, five stationary phase parameters are defined to characterize the selectivity and the properties of a ODS stationary phase [32]; hydro-
phobicity (H), steric selectivity (S*), hydrogen-bond acidity (A) and hydrogen-bond basicity (B), and cat ion-exchange capacity (C).

\[ \log \left( \frac{k}{k_{EB}} \right) = \eta'H - \sigma'S^* + \beta'A + \alpha'B + \kappa'C(2.8) \]  \hspace{1cm} (8)

The logarithm of the retention factor of a compound (\( k \)), relative to ethylbenzene (\( k_{EB} \)) and independent so-called column parameters (H, S*, A, B and C) associated with the corresponding properties of the solute molecule: Hydrophobicity (\( \eta' \)), bulkiness (\( \sigma' \)), hydrogen-bond basicity (\( \alpha' \)), hydrogen-bond acidity (\( \beta' \)), and effective ionic charge (\( \kappa' \)).

Figure 11. Description of the interaction between stationary phase and solute (grey shape) in an ODS-column: hydrophobicity (\( \eta'H \)), bulkiness (\( \sigma'S^* \)), hydrogen-bond basicity (\( \beta'A \)), hydrogen-bond acidity (\( \alpha'B \)), and effective ionic charge (\( \kappa'C \)).

These coefficients are relative to values for ethylbenzene, the reference compound for which all the coefficients are zero, and the values of each column parameter (H, S*, A, B and C) are relative to a hypothetical, average type-B (high purity silica) C18 column. Any column which behaves identically to this average column will have H equal to 1, and all the other column parameters equal to zero. These interaction parameters are visualized in Figure 11. So far over five hundred columns have been characterized with this method. The database containing all column data has become a suitable starting place for statistical approaches on solute-stationary phase interactions based on linear characterization parameters. This makes it possible to screen for the possible performance of new stationary phases.
Contribution to the field

The general idea to characterize an adsorption event with a Langmuir adsorption isotherm seems by most scientists as totally valid in almost any case. When the adsorption isotherm is used as one a part of an objective function to optimize revenue, it can be described by an arbitrary (but best fitting) polynomial totally lacking any physical meaning. But when the opposite is of interest, trying to find and calculate what interactions that are related to e.g. a physical surface parameter, then it is of uppermost concern that the parameters of the selected adsorption isotherm actually describes the physical interactions. My work has mainly been focusing in trying to combine nonlinear adsorption isotherm determination methods for single molecular species and linear characterization methods to see if they can detect differences in surface chemistry (Paper III). Due to the extensive comparison of retention time shift depending on molecular parameters in relationship to an ODS surface during the development of the HSM, a subset of the most protruding interaction sites resolved by nonlinear adsorption isotherm determination will most likely correspond to each other. Also was the aim to determine if deeper knowledge about hybrid stationary phases can be gained by the combined characterization. The overall conclusions here is that even if many methods generates specific numbers, a holistic view taking many factors into account are more likely to give a “true” interactions even if they in the end look imprecise. Linked to the task of determine adsorption isotherms with ECP, I performed experimental validation of an improved adsorption isotherm determination method ECP-slope (Paper I). Comparison with simulated chromatograms having adsorption isotherm parameters similar to the experimental, did confirm faster AED conversion and less sensitivity against inaccurate determined \( V_r \) where \( C = 0 \) compared to classical integration ECP method. On additional note on a more brief level is that a rearrangement of Equation 4 to Equation 5 could have such impact, by only changing the way you calculate the same thing. There are a vast number of functions contributing to adsorption parameters…
As an even further evolvement of the improved ECP-slope method, some guidelines and prerequisites where defined for the determination of different adsorption isotherm having inflection points. Here simulated elution profile was used to evaluate and determine the optimal experimental settings. The adsorption isotherm data is hard to determine in the proximity of the inflexion point but if the follow up experiment, on the other side of the inflexion point is wisely constructed, then there will only be needed one more experiment than there are inflexion point in the adsorption Isotherm (Paper II). This paper also gives some insights in how useful the solving of a column model such as the Equilibrium-Dispersive model, to retrieve a simulated injection profile can be: Quit much faster than do empirical tests in the lab.

Figure 12. Visualization of the relationship between different part of an overloaded cut-injection elution profile and the slope of the adsorption isotherm using ECP-slope data.
4 Molecular identification – unbiased adsorption characterization

In the previous chapters the aim has been to decipher in what way adsorbent and adsorbate interact in order to give a defined adsorption pattern. One of the overall conclusions that can be made clear: Only slight increases of the complexity of an adsorption system make the determination of the physical interaction forces very difficult. It will most likely result in an adsorption isotherm having parameters most likely lack physical meaning. Now in the case with a non-ideal system, an adsorption isotherm lacks any meaning due to the complexity. Instead the measurable values are identified proteins and their amount, to give information about the adsorption process.

![Graph showing the number of identified proteins adsorbed to a polycarbonated surface as function of time.](image)

*Figure 13.* The number of identified proteins adsorbed to a polycarbonated surface as function of time.
General methods for protein adsorption detection

There are many different tools and approaches that can be used to detect and validate adsorption characteristics of molecules. To only mention a few, the focus will be on those that are primarily used or particular useful in the field of adsorption characterization in liquid-solid interfaces, and among the ones with focus on protein adsorption measurements.

The in situ-group, consisting of: Quartz Crystal Microbalance with Dissipative monitoring (QCM-D) \cite{33} which can measure small mass changes adsorbed to flat and fixed surfaces by measuring the change in frequency across a quartz crystal, Surface Plasmon Resonance (SPR) \cite{34,35,36} capable of quantitate proteins and kinetic studies in the proximity to gold and silver surfaces, Ellipsometry \cite{37} measures the thickness of protein layers if the optical parameters are known, which also can be used in combination with QCM, giving both mass and thickness in real-time, Attenuated Total Internal Reflection Fourier Transform Infra-Red (ATR-FTIR and FTIR) measures absorbance of wavelength related to different chemical bonds in molecules \cite{38,39} which was tried in relation to Paper IV (data not shown), but due to the properties of the surface it was not further used, Circular Dichroism (CD) \cite{40,41} can be used to determine changes in secondary structures in proteins upon adsorption, Atomic Force Microscopy (AFM) \cite{42,43} is capable of both measuring surface topology and forces when manipulating the surface with the cantilever.

The ex situ-group can be very useful even if the requirement of removal of proteins from the surface or the alteration the absorption system in a fundamental way, to be able to perform the measurement. Consisting of: X-ray Photoelectron Spectroscopy (XPS) \cite{44} that measures composition of surface atoms by the energy of emitted electrons after excitation, Surface Electron Microscopy (SEM) \cite{45} gives the surface topology after treatment with metals. These techniques also can contribute to detailed surface characterization measurements prior adsorption events.

As additions to the mentioned interfacial methods, methods measuring the protein concentration in the solution can be tools to determine the depletion of proteins with in the liquid phase. Colorimetric methods such as Bradford \cite{46}, Lorey \cite{47}, Bicinchoninic Acid (BCA) \cite{48}, and a new method, Dot-it-Spot-it \cite{49}, are often used to determine the absolute protein concentration in solutions of unknown protein samples. The dynamic measurement of the solute concentration can be conducted with common LC detectors such as Diode Array Detector (DAD), Refractive Index (RI), and Ultra Violet (UV). DAD was used in Paper I-III for small molecular dynamic adsorption determination. Many of the techniques stated above are complementary and will contribute to different insights of the surface or the solute, to the overall picture of the adsorption system.
Methods for protein identification

There are basically only two methods that are possible to measure the content of unique proteins in a complex sample. The widely used Enzyme-Linked Immunosorbent Assay (ELISA) \[50\], and Mass Spectrometry (MS).

ELISA uses the specificity of antibodies which are coupled to enzymes capable of transforming substrates into visible colors to permit detection. This method requires a priori knowledge about what protein to detect and a specific antibody to each protein of interest. The method is sensitive and does not require expensive equipment or heavy computational work, but often more manual work and antibodies which can be quite rare and expensive. However, the extensive use of ELISA has in research and clinical application led to miniaturization, increased sensitivity, decreased cost for reagents, and more automated procedures which reduces variations due to human errors \[51\]. In addition to ELISA, Western blot and Proximity Ligation Assay (PLA) also utilize antibodies for detection.

Mass spectrometry on the other hand requires expensive equipment and different software capable of retrieve and analyze instrument specific raw data, as well as use databases for result comparison before the actual identification of proteins is possible. Since the first discoveries were made \[52\], almost 100 years of continuously development and improvement has rewarded the field of mass spectrometry with several Noble prizes in both physics and chemistry \[53-56\]. For the use of MS in protein identification there are mainly two main principles used, both having numerous of sub-approaches to utilize equipment more efficient.

Top-down: which aims at detect full sized proteins by the weight and their specific isotope pattern.

Bottom-up: also named shotgun proteomics \[57,58\], analyzes enzymatically cleaved fragments of proteins. The method mainly utilizes the fragmentation pattern of the peptide (precursor ion), and the exact mass in combination with e.g. the retention time for a previously identified peptide. Bottom up is the most used method, both in the quest of identifying proteins and quantifying them \[59\]. This is the approach I have used for all experiments in Paper IV-VI, due to the nature of the on Surface Enzymatic Digestion (oSED) method (see next section).

In the field of biomaterial research combination with protein adsorption two major MS ionization methods are used. First: Matrix Assisted Laser Desorption Ionization (MALDI) \[60\] acting on proteins and peptides on a surface. The second: Electrospray ionization (ESI) \[61\], is the most used ionization method when analyzing proteins in solution \[62\]. Both are more deeply described under the chapter: Mass spectrometry
On Surface Enzymatic Digestion (oSED)

The formation of tryptic peptides is a mandatory step in sample preparation for bottom-up proteomics. Proteins adsorbed onto a surface are required to desorb in one way or another to get into the MS. Could these two steps be combined in order to act as an enzymatic razor, shaving of peptides of adsorbed proteins on an arbitrary surface, to screen for their identity? For this purpose the on surface enzymatic digestion (oSED) is a versatile tool (Paper IV-VI).

The use of tryptic digestion of the “adsorbed” proteins was first reported by Aebersold et.al.[63], when producing pure peptides from low amounts of N-terminally blocked proteins electroblotted onto nitrocellulose after polyacrylamide gel electrophoresis. Aguilar et.al. [64] identified specific sites on Cyt c interacting with the stationary phase on a Reversed Phase (RP) columns. Further studies on both Cyt c and the digestion properties of trypsin has also given insights in the dependence of a MS-system capable of detecting all peptides of interest [65]. Methods similar to oSED has been used in order to concentrate diluted samples, as well as clean the samples from different salts and additives [66-69]. Often the opposite procedure is more common in order to produce higher concentrations of tryptic peptides, namely the immobilization and adsorption of trypsin on the surface [70-73] before the addition of a protein containing sample.

Figure 14. Schematic view of the creation of tryptic peptides from adsorbed proteins via the oSED method. Image courtesy of Andreas Dahlin.

oSED is a development of a combination of older methods, and recent developed on filter digestion [74] methods for bottom-up proteomics, the addition of Dithiothreitol (DTT) [75] and Iodoacetamide (IAA) [76] was made to the adsorbed proteins prior trypsination according to Figure 14. The first step breaks the cysteine bridges and the second step then alkylates them to reduce reactivity that could create new unwanted disulfide bonds by oxidation. In the final step the addition of trypsin produces tryptic peptides going into solution. Peptides normally need to be cleaned and desalted, via Solid Phase Extraction (SPE) prior drying in Eppendorf tubes and re-suspension in 0.1 % Formic Acid (FA) to be injected a Nano-LC-MS sys-
tem. Even if there are differences between oSED and in-solution, the optimized and detailed protocol regarding the reduction, alkylation and finally protein digestion using trypsin in solution digestion [77] was followed also in oSED.

Worth noticing is that even during sample preparation in e.g. bottom-up proteomics, unspecific bindings of peptides to surfaces occur. Experiments made by me (data not shown) indicated that peptides can show a significant increase in intensity when adding acetonitrile (ACN) to the re-suspension buffer after the SPE step. These strategies of altering the solute on composition during different stages of the sample preparation to minimize this effect [78] should be taken into consideration during method development.

Mass spectrometry

The principle behind one of the most powerful and versatile techniques available for chemists, relies on the detection of molecular ions in gas phase. Three main parts of a MS system are required, an ion source, an analyzer and a detector, see Figure 15. The function of the ion source is to transform the molecules in a liquid sample into gas phase ions. The task of the analyzer is to manipulate the ions in order to select some of them, based on the charge divided by mass of the individual ions (m/z) and transfer them to the detector. The detector then counts the actual ions that have been selected in the analyzer. Not only does the actual analyzer differ, but also are there a number of different ionization techniques available.

Figure 15. Schematic view of the major parts (under lined) in mass spectrometer with some examples (dot lists) system and additional systems required to inject analytes and retrieve data (top in figure)
As mentioned under Methods for protein identification, MALDI is an often used ionization method where a matrix solution containing molecules that form crystals when drying. These crystals will be able to transfer the energy from the laser photons to the proteins or peptides which will both desorb and ionize the sample. The detailed mechanism is still not known but it generates mainly single charged ions. When coupled to a Time-Of-Flight (TOF) mass analyzer, the charged molecule is accelerated in an electrical filed towards the detector. And the flight time is proportional to the \( m/z \). This is the predominant set-up for protein adsorption measurement using MS [79,80]. MALDI is fast method which not only can identify different proteins, but also give information about the lateral distribution over the surface of the adsorbed proteins. In fact, experiments using MALDI-TOF to determine binding constants between Low-Density PolyEthylene (LPDE) and angiotensin I, bradykinin, and porcine insulin have been reported [81]. Some drawbacks with MALDI are however are reduced number of proteins that can be identified at a single area [80], some proteins/peptides are difficult to ionize, and therefore hard to detect, and sometimes poor reproducibility. For surface adsorption studies it seems convenient with the inherent use of a surface for sample application; however MALDI needs a uniformly flat surface to provide accurate mass determination, which makes the use of any arbitrary surface impossible for quantitation.

![Oxidation Reduction Diagram](image)

Figure 16. Schematic picture of the ESI principle for the formation of (in this case) positively charged molecular in gas phase, originating from a solution.

The second dominant ionization method is ESI [61,82], which is the most used ionization method when analyzing peptides in solution [62]. The sample, dissolved in volatile buffer solution (generally low pH for positive ESI to enhance charging of analytes already in the liquid phase), flows out from a small tip in the end of a capillary creating a spray. The negative (or posi-
positive) potential between the tip and the orifice of the inlet to the mass analyzer both drain (or provide) the solution of electrons and accelerate the charged droplets toward the heated orifice as seen in Figure 16. In positive ESI the excess positive charge in combination with fast evaporation of the volatile solution forces the droplets to fast form smaller droplets and finally form positive ions in gas phase [83]. ESI has also gained increased popularity due to the development of nano-LC system, which use low flow rates (< 200 nl/min). The lower flow rates improve the droplet formation, and it also enables the use of smaller aperture size of the sprayer tip. This smaller size reduces the initial droplet size in the spray, and thus reduces the number fission steps required before releasing ions into gas phase. This results in faster ion emission [84]. The combination of Nano-LC and ESI can handle small sample volumes with low concentrations on small micro RP columns, making it possible quantitatively analyze peptides [85] and proteins even for clinical applications [86].

Analyzers in MS utilize different physics in order to select, transfer the induced \( m/z \) entering the MS. All analyzers have different pros and cons and are suitable for different applications, as for the ion source. The previous mentions TOF is common as well as quadrupoles and ion traps, and combinations of different analyzers. There is also additional capabilities to some analyzers e.g. the linear ion trap used in Orbitrap Velos comprise of two parts, one high pressure cell which can be utilized to fragment ions by Collision Induced Dissociation (CID) and a low pressure cell which is adjacent to dynode detectors for \( m/z \) detection. Detectors, such as dynode and faradays cup enhance the signal of the charged ion hitting the detector by induce secondary emission of even more electrons. Another type of detectors, which also acts as analyzers, are Fourier Transform Ion Cyclotron Resonance (FTICR) [87] and Orbitraps [88] which detects an image current of the circulating ions which can be transformed via Fourier Transform (FT) to \( m/z \). Data will be produced.

Data acquisition

As a detector an MS instrument is really versatile, but one needs to know what to look for and with what approach on a given MS-system, since the setting of each instrument will determine the data that will be produced. In “bottom-up” proteomics there are several fundamental principles to detect proteins somewhat more or less optimal depending on instrument [57]. In this work mainly one MS instrument been used, the Orbitrap Velos Pro. A LTQ Velos Pro has an linear ion trap, where a part of it fragment ions and one part analyzes, before a C-trap that can store and injects selected ions into the more mass accurate Orbitrap analyzer. The resolution of the linear ion trap is lower compared to the Orbitrap, but it is faster and has greater storage.
capacity and preforms collision induced dissociation CID for e.g. MS^n experiments. Mainly two methods have been used in my work for detection and quantification of proteins on the instrument.

When studying complex biological matrixes investigated in a qualitatively (identification) mode, Data Dependent Acquisition (DDA) is often used, as in **Paper IV** and **Paper V**. Here the top ten m/z from the full MS^n scan (whole mass range) are selected for further fragmentation into MS/MS (MS^2) spectra (**Figure 17**).

**Figure 17.** The main three data gathering steps in the mass spectrometer and what purpose those fulfill. Quality control (QC) of data and sample and also Quantification parameters related to the peptide/protein of interest.

These MS^2 spectra will be used for further peptide identification done by specific software se: Data analysis. In order to detect new peptides instead of the same abundant m/z fragment, the recent fragmented (including isotopes) are excluded for further fragmentation for a defined timeframe, optimally the width of the chromatographic peak.

The second approach when *a priori* information about what proteins to detect Selected Ion Monitoring (SIM) is preferred due to higher reproducibility of, less data, and increased sensitivity. This will utilize the selectivity of the MS to only do fragmentation of selected m/z (peptides representing the protein of interest) represented by the yellow bar in the middle figure in **Figure 17**, which is stored in an inclusion list. In order to improve the signal to noise ration the full scan can also be divided into smaller equally increment-ed m/z windows covering the whole mass range, as used in **Paper VI**.

### Data analysis

MS instruments will produce enormous amounts of data that need to be processed in order to be able to detect and quantify what molecule that went in to the system. In the field of “bottom-up” proteomics, the data quantity is even further increased, since each protein is cleaved in to multiple peptide
fragments, and each peptide fragment detected in MS$^1$ is further fragmented to create an MS$^2$ pattern, specific to the amino acid sequence of that peptide. This fragmentation is often performed many times each second, which during a 90 minute LC gradient will give thousands of MS$^2$ spectra to interpret, which was the case in Paper IV. This amount of data is of course impossible to manually analyze within reasonable time frames, instead computers are used to match, score and identify the detected MS$^1$ and MS$^2$ spectra against “in silico” generated tryptic peptides [89-91]. Computer generated tryptic peptides are derived from information about the amino acid sequence from databases comprising of proteins likely to find in the sample. The overall path of peptide identification looks like:

The MS raw-file $\rightarrow$ Select spectrum $\rightarrow$ Select properties of computer generated peptides $\rightarrow$ Match and score probabilities of a correct match. $\rightarrow$ Link result of detected peptides to raw-file data and present it graphically.

In this step in DDA the “right” cut of values of the probability score need to be set, so the “true” matching are presented not to stringent (that will make the system reject a lot of true identifications), and not to relaxed (that will falsely identify a MS$^2$ spectra belonging to a peptide). A lot of effort is put in to this primarily mathematically step in order to be able to present “how true” the peptide matching really are to the proteomics scientists (that are not experienced and interested in detailed statistics). In DDA sets containing many proteins with high rate of peptides, an machine learning algorithm Percolator [92] uses high scoring peptide spectrum matches (PSM) for positive examples and decoy databases (of nonexistent amino acid sequences) for negative examples for optimizing the algorithm, which greatly improve the numbers of identified proteins. This evaluation method was used in Paper IV and Paper V. After this “final” stage, a list of identified proteins are given, along with which peptides and what modifications they had (first row in Figure 18.). Added to the list is also data about the scoring of the peptide match and how many times the peptides where identified in the raw file.
When a priori information is available regarding proteins of interest the MS can be guided to only search for selected masses in SIM mode. To speed up the selection of what peptides to add to the inclusion list in the MS, a function in the program Skyline [93] evaluates DDA data sets for some specified criteria’s, and you will be given quite good candidates. The suggested peptides however need to be further evaluated in some aspects to minimize the risk of: co-eluding peptides with isobaric m/z, gives noisy MS2, peptides having many modifications decreases intensity, many charge states decreases intensity, co-eluting with many other selected m/z reduces the possibilities of MS2 (row two in Figure 18.). When the first ten peptides are narrowed down to five repeat the process to end up with three god peptides for each protein of interest. This was the approach used in Paper VI. Depending on the used MS system there might a good idea to correlate the triggering of a MS2 scan specific to the m/z on the inclusion list, with the actual retention time of the peptide to focus the system even better on the task. After a run where at least some MS2 scans for each peptide is triggered, the calculation of the area under curve (AUC) for the selected MS1 m/z (precursor ion) can be performed. This area is also measured for calibration runs with different constrictions of the same peptide, or with the same peptides incorporated heavy isotopes on one amino acid. The absolute amount of that peptide can then be measured. The final list (before R or Excel) include the names and masses of the identified peptides and their AUC-value for each run along with many optional data depending on grouping of data, added standards, spiking etc. when using the PinPoint and Skyline software. Even if the basic idea with both PinPoint and Skyline is to speed up the analysis one must be aware that among all settings in that “black box” there might be a ticked box messing with the data.
Contribution to the field

The overall aim was to try to evaluate if there was a screening method that could identify adsorbed proteins on an arbitrary surface and link that protein adsorption pattern to material parameters as well as adsorption behaviors seen in less complex solutions. In Paper IV, the time dependent behavior of the adsorption was visualized with the number of different detected proteins that adsorbed during the course of time (Figure 13 and Figure 19). This curve has a great resemblance to many adsorption curves describing how a single protein adsorbs on to a surface (time vs. mass). As the intention also was to give an “easy way to interpretation” of mass spectrometry data form adsorbed proteins the selection of a sequence coverage to relate to the amount of detected proteins where done. This rather imprecise parameter was found to correlate well with the PSM, which is somewhat better, but sufficient for the screening purpose of complex adsorption. The approach is a rather holistic way of gain information in the adsorption characterization process, as in Paper III, but I hope the method in its simple form might inspire to do some less detailed measurement in order to eventually grasp a systematic knowledge faster. In Paper V when comparison of polycarbonate surfaces and polycarbonate surfaces coated with Pluronic F-127 was done, the fast insight was that a quite different adsorption behavior.

![Figure 19. A schematic heat map of identified proteins identified adsorbed onto native and modified membranes. Black squares indicates detection, partly gradient filled squares (B) symbolizes a random like adsorption behavior. From Paper V](image_url)

Not only different between proteins, but also by the general adsorption pattern for each surface. When the similar, but more in detail evaluated experiment was performed in Paper V, it was obvious that coating of the surface heavily reduced the overall adsorption as well as the individual proteins. The proteins that did not follow the trend were too few to do reliable statis-
tics on and many of the proteins did not have a clear pattern of adsorption. Any Vroman effects were not detected in any of Paper V and Paper IV. This is can be due to the somewhat imprecise measuring parameter, but follow-up experiment seems to be unavoidable to address this issue. Both experiments did however arise the question if the proteins that where adsorbed on the surface where affected and desorbed when addition of chemicals were made. DTT which actually cleaves disulfide bonds was thought to have the largest impact, on increased desorption. Using tubing actually made it impossible to add the DTT and IAA in the same solution, so they were added in sequence.

![Figure 20.](image)

*Figure 20.* Figure 2: a) Bradykinin; b) [Arg8] Vasopressin; c) Transthyretin; d) Alpha-1-acids-glycoprotein, Where left to right: Protein rinse, is the flow thru of protein standard. Tube Reaction, reacts in the tube, Tube Rinse reacts in a vial and Peptide Elution, is the final step after adding trypsin. From Paper VI

From Paper VI the use of a targeted LC-MS/MS method concluded that most of the peptide from the sampling method originating from the tryptic activity on the adsorbed protein (Figure 14). Here we could also conclude that protein desorption actually took place, but at much less levels than anticipated, while the added peptides desorbed at higher degree fractions, which also was anticipated (Figure 20). Paper VI also confirms that the method can be used both for identification and screening purposes. Even more detailed experiments can probably give insights to if oSED can be used as a “shaver” for adsorbed proteins, acting on layers in the solution interface first.
The preformed studies have adsorption in common, as big pharmaceutical companies and Geckos, but are the similarities over there? No they are not. It is quite obvious that every targeted characterization of an interaction in a more complex adsorption system will contribute to deeper knowledge of the total system. But there are benefits with holistic approaches to adsorption characterizations. The sheer thought of measuring the adsorption of a single protein on a membrane at a time can give a scientists head ache. Even if the exact interaction energies are not determined and the surface saturation capacity of each interaction site is unknown, the overall performances between different surfaces are easily shown. The question that needs to be answered is how to combine findings with the detailed interaction adsorption characterization with those found by identifying molecules. The answer is most likely; Do it. Use many approaches and gain deeper understanding about the adsorption system. In Paper III this was found to be true.

In Paper I the improvement of the ECP method both reduced errors in adsorption isotherm parameters and made the method now capable of determine isotherms having inflection points. Strategies and experimental considerations to perform determinations of adsorption isotherms of type II, type III and type V was presented in Paper II.

Process chromatography aims at increased production at lower costs; here objective function loaded with a correct adsorption isotherm will give benefits. The market for protein based pharmaceutical is now over $100 billion each year. The previous not so optimized steps of separating an polishing of proteins has now gained interest in a more fundamental thermodynamic knowledge of the separation system in order to improve[8]. Here a holistic view might narrow down what steps that are needed to be in depth evaluated. Process chromatography has during the later years regained its interest for Supercritical Fluid Chromatography (SFC) separations, due to the cheap mobile phase (CO₂) and environmental friendly chemicals. Here the adsorption isotherm determination can be a challenge as other parameters than measured in LC need to be verified [94], more detailed knowledge will maybe give more insights of the phase system with in a SFC column. Two different techniques, two different approaches in the first step (Identification and interaction).

Paper IV showed that identification of proteins and peptides can characterize the time dependent pattern for vCSF protein adsorption.
In **Paper V and VI** the general statement of; smaller proteins adsorbs first, and the bigger adsorbs later according to the Vroman principle, was not noticed. This is most likely due to the somewhat imprecise measuring parameter, and a more specific study of the Vroman effect in highly complex solutions, using oSED in combination with targeted MS and long time series, would be perfect for validation. The use of targeted methods could be utilized after initial screening experiments using DDA only.

Most probably is the protein adsorption from complex mixtures not “layer” in ordinary sense, but rather a gradient of adsorbed proteins, ranging from high (maybe irreversible) proteins at the surface, to lower protein concentration (highly reversible) further out from the surface [95]. These gradients are of course dependent of the unique properties of the surfaces, solutions and proteins involved in the adsorption system. The oSED method might be useful to determine the composition and relative strength of such gradients. That might however first require time studies on the trypsination mechanism on adsorbed proteins, to be really useful. In this future enzymatic study, the orientation of proteins on surfaces might be validated at the same time.

Only focusing on detailed and well defined parts may sometimes hamper insights of the bigger picture. However I also hope that this wider form characterization will reveal areas that other techniques and more detailed measurements can take the fundamental knowledge further. As the instruments of today is much better, faster and often more complicated, the eager to solve difficult specific questions might be a driving force away from a path where details and overview cooperates. I have during my work with this thesis realized that too much focus on details can tend to be misleading, but also that to big chunks of complexity, slows down the overall gain of understanding.
Summary in Swedish

Adsorption av molekyler på ytor sker ständig runt om kring oss. Att vi inte är medvetande om det betyder inte att vi inte påverkas av det. Det mest vanliga adsorptionsfenomenet är att vattenmolekyler fastnar på en yta och blir sittande. Den nu stationära vattenmolekylen stannar kvar tills någon typ av yttre påverkan får den att släppa, och en ny vattenmolekyl troligen tar dess plats. Systemet är i jämvikt. Detta adsorptionsförlopp betyder att adsorption är reversibel (omvändbar), och ytan kan bli helt fri från vatten. Inom många kemiska analyser blandas kemikalier i olika vätskor och inom analytisk kemi (som kanske hörs på namnet), analyseras många gånger dessa vätskor i olika instrument. Om vi då tänker på att molekyler nu kan adsorbera på ytor, kan det ju vara en risk att de molekyler som vi vill mäta i mycket utspädda lösningar helt enkelt fastnar på ytan i våra flaskor pipetter, istället för att mätas i instrumentet.

som finns i vätskan försvinner vid olika tidpunkter då den åker igenom kolonnen vid en konstant temperatur. Olika sätt att mäta och räkna fram den här adsorptionsisotermen ger olika ”rätt” bild av vilka krafter som verkar mellan molekylen och ytan, vilket gör att man måste ha system för hur man ska testa och även hur man ska räkna för att kunna avgöra riktigheten. Det är även så att vissa tekniker för att mäta och räkna adsorptionsisoternen inte funkar om molekylerna ytan och vätskan beter sig tillräckligt ”konstigt”. Den tidigare modifikationen av ECP till ECP-slope tekniken, gjorde det nu möjligt att mäta dessa ”konstiga” isotermer. I Artikel II förklarar vi hur man ska göra och vad man ska tänka på för olika modeller som beskriver adsorptionsisotemer. I Artikel II användes inte ”riktiga” experiment utan datorer räknade fram hur det skulle se ut när en molekylnr tog sig igenom kolonner för att vi sen räknar på dessa kurvor för att kunna bestämma adsorptionsisotemer. De försök som gjordes i Artikel III syftade till att mäta hur olika molekyler dras till olika ytor olika mycket beroende på laddning, storlek, och hydrofobicitet hos molekylen, samt hur ytbehandlingar som gör att kolonnen skall tåla höga pH (basiskt), påverkar. Först gjordes försök genom att bestämma adsorptionsisotemer för några molekyler med olika egenskaper. De molekyler som var konstant positivt laddade kunde man se till exempel se att de tog längre tid på sig genom kolonnen vid ett högt pH, vilket vi förstod berodde på att vissa av ytbehandlingen av kolonnerna blev väldigt negativa, så molekylerna drögs till ytan istället för att följa med igenom. Hydrofoba molekyler betedde sig ganska lika mellan de olika kolonnerna. Vi använde även en metod (Hydrofob Subtraktions Metoden(HSM)) som består av ett antal testmolekyler som ger ett mätvärde på vad kolonner presterar. Denna metod pekade åt samma riktning som de bestämda adsorptionsisoterner, men klarade inte att bestämma ”hur hårt” eller ”hur många” av dessa interaktioner som fanns. Dock förenklade HSM arbetet med att välja en liknande kolonn av ett annat märke då väldigt många koloner testats och lagts upp i en databas.

Inom biokemi och läkemedelsforskning är man många gånger intresserade av att mäta på proteiner, som är en typ av molekyl som tillsammans med fett och kolhydrater utgör huvuddelen av kroppens byggstenar. Proteiner kan fungera som allt ifrån reglerätta byggstenar i muskler, som budbärare till celler att göra något, eller som små molekylära fabriker som klipper och klistrar ihop nya proteiner, fett eller kolhydrater i syfte att bygga något eller ge oss energi. Dessa proteiner är på något sätt alltid påverkade av sjuksommar och kan därför bli fler av något slag, eller nästan helt försvinna. På senare tid har man insett att det inte bara är våra gener (som är bärare av arvsanlag) som är betydelsefulla för om vi kommer bli sjuka eller inte, utan även proteiner. Ett problem är många gånger att de proteiner man är intresseade av att mäta finns i en väldigt liten mängd och ibland väldigt svåråtkomliga i kroppen. Detta gör att man har väldigt liten mängd att mäta på. För att identifiera och mäta dessa små mängder av proteiner krävs ofta avan-
cerad utrustning och många olika tekniker för provtagnings och metoder för att preparera proverna.

Vad har då detta med adsorption att göra? Jo, nu är det så att proteiner har en naturlig fallenhet för att vilja fastna på ytor, vilket gör att de värden som man faktiskt mäter, kanske inte helt stämmer med hur mycket man hade i kroppen. Vad kan man då göra åt detta? Jo, man kan försöka identifiera de egenskaper hos proteiner och ytor som gör att de dras till varandra, för att sen försöka ändra på ytan eller den vätskan som proteinerna är i, och på så sätt minska adsorptionen på ytorna. Detta skulle då kunna innebära säkrare mätningar för att kunna undersöka om förändringar av mängden eller typen av proteiner är resultatet av en sjukdom eller skada och inte ett mätfel orsakat av adsorption.

Den andra inriktningen är här att med en modifierad metod kunna identifiera adsorberade proteiner på en godtycklig yta (filter, flaska, pipettspets, etc.) med hjälp av mass spektrometri. Detta görs genom att tillsätta kemikalier som bryter upp de hopvecklade proteinerna som sitter fast på ytan och möjliggöra för att kunna klippa dessa proteiner i bitar (med hjälp av ett protein!!) för att analysera dessa bitar i en masspektrometer. Detta kan lite liknas vid att ha en stor tavla (som man inte kan se på av någon anledning), klippa den i små bitar och sen noga analysera bitarna (som man kan se) och sedan med hjälp av datorer pussla ihop dessa bitar till den ursprungliga tavlan. Arbetet har i huvudsak gjorts på modellytor där jag mätt hur tiden påverkar vilka proteiner som adsorberar (Artikel IV). Utöver det har jag tittat på om metodens kan detektera vad som händer om man modifierar modellytan med andra molekyler som kan fungera som små molekylära ”hårstrån”. Detta förändrar då vilka proteiner som fastnar på ytan samt mängden av dessa (Artikel V). En ytterligare undersökning gjordes för att se om tillsatsen av de ”uppbrytande” kemikalierna gjorde så att proteinerna släpptes från ytan, vilket i så fall skulle kunna försvåra mätningar (Artikel VI) på vissa typer av material (ex. vis slangar).

Som sammanfattning kan sägas att, kombinera metoder som är väldigt precis med lite mer generella mätningar, kan ofta ge en bättre total förståelse för adsorptions system. Även ska tilläggas att adsorption finns överallt, men kan ibland vara väldigt svår att mäta, speciellt om det är fler än en typ av molekyler som adsorberar till ytan eller till varandra. Svårigheter är dock till för att övervinna.
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