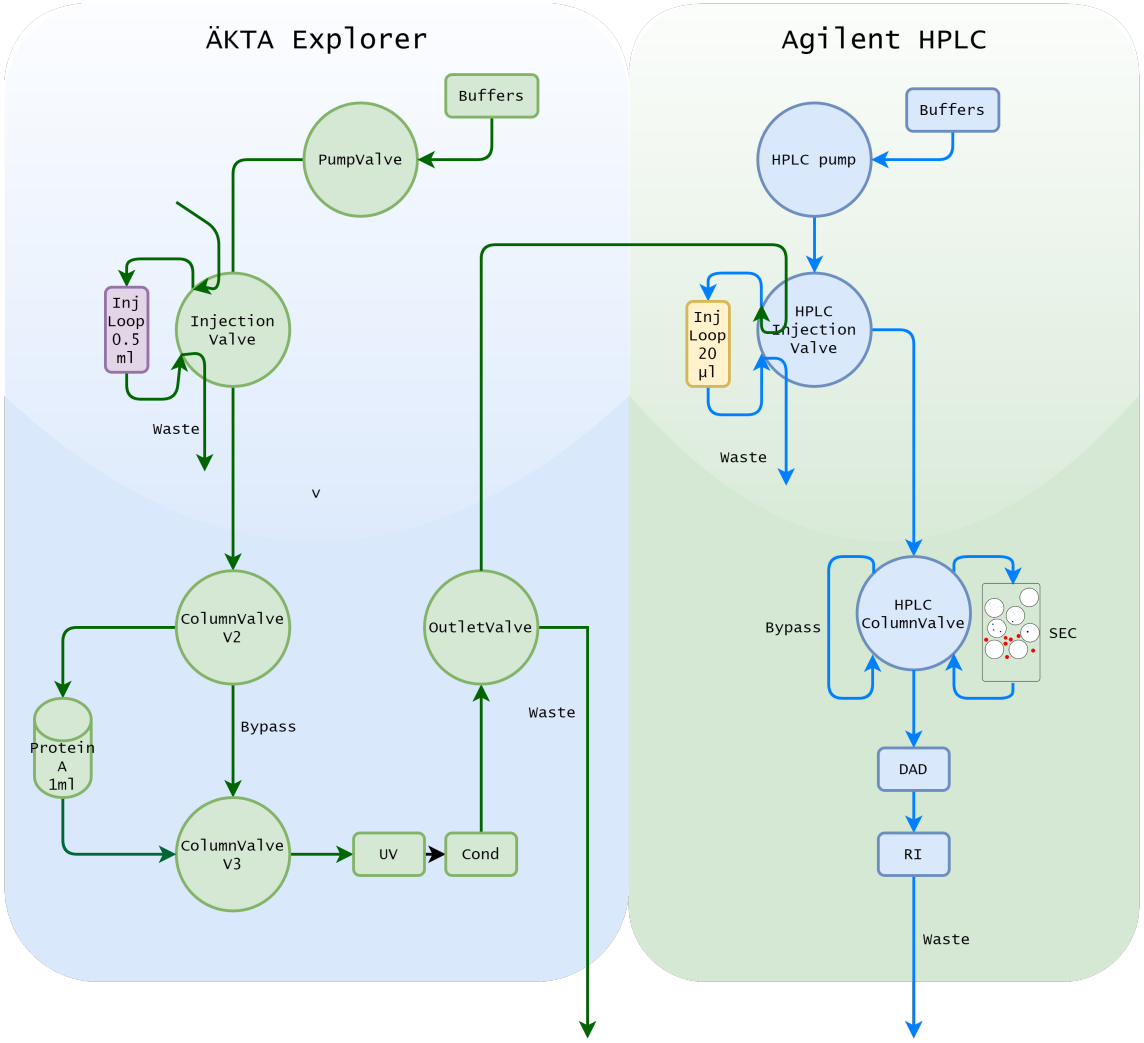
Advanced control of chromatography systems for automated analysis of monoclonal antibodies



Jerry Guan

Department of Chemical Engineering

Master Thesis 2021

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Master Thesis

Advanced control of chromatography systems for automated analysis of monoclonal antibodies

by

Jerry Guan

Department of Chemical Engineering
Lund University
Sweden
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Supervisor: **Doctor Niklas Andersson**
Co-supervisor: **PhD-student Simon Tallvod**
Examiner: **Professor Bernt Nilsson**

Front page picture: Process scheme of the experimental configuration used in this thesis.

Postal address
PO-Box 124
SE-221 00 Lund, Sweden
Web address
www.lth.se/chemeng

Visiting address
Naturvetarvägen 14

Telephone
+46 46-222 82 85
+46 46-222 00 00

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Finally, I would like to thank the rest of the Department of Chemical Engineering for creating such a pleasant workplace.

Thank You!

Abstract

Downstream processes for purification of biological pharmaceuticals such as monoclonal antibodies are moving from batchwise to not only fully continuous, but also fully automated production. A part of the vision for fully automated purification processes is to be able to automatically analyze from sample streams of the purification steps. Orbit is a software that allows for advanced control of ÄKTA chromatography systems from Cytiva which are used for purification of antibodies. In combination with another software called Satellite CDS, Orbit can also control analytical high performance liquid chromatography (HPLC) systems from Agilent Technologies.

In this master thesis, an ÄKTA Explorer system was connected to the injection loop of an Agilent HPLC system to form a dual system for sample preparation and analysis. The purpose of the ÄKTA was to collect sample and prepare the sample by a chromatographic step if needed. The HPLC was equipped with a size exclusion column for analysis of aggregates in antibody solution. Two instances of Orbit was used to control and integrate the two systems and experiments were made to test the functionality of the setup. The purpose of this master's thesis was to investigate the possibility of automatic analysis and for this system to be fully operational, a method that ensures that the sample that reaches the HPLC loop has the same concentration as the original sample is needed. This is important to get a representative analysis of the sample. The solutions to this problem could be either by having a larger volume of sample from the ÄKTA Explorer for creating a larger pool or an addition detector at the HPLC injection loop. For even further work, this analytical setup could be integrated into a downstream purification process.

Sammanfattning

Nedströmsprocesser för rening av biologiska läkemedel som monoklona antikroppar rör sig från batch-processer till kontinuerliga, och vidare till helt automatiska, kontinuerliga nedströmsprocsser. En del av visionen för automatiska reningsprocesser är möjligheten att automatiskt analysera från olika steg av reningen. Orbit är ett program som tillåter advancerad styrning av ÄKTA kromatografisystem för rening av proteiner från Cytiva. I kombination med ett ytterligare program som heter Satellite CDS kan Orbit även kontrollera HPLC-system från Agilent Technologies.

I detta examensarbete kopplades en ÄKTA Explorer till injektionsloopen på en Agilent HPLC för att bilda ett dubbelsystem med syftet att förbereda prov för analysis, samt att utföra analys. ÄKTA Explorern samlade prov och förberedde det om nödvändigt. HPLC-maskinen var utrustad med en kolonn som separerade beroende på molekylens storlek för att kunna analysera bildandet av aggregat i antikroppslösningen. Två körningar av Orbit användes för att styra och länka dessa två system och experiment utfördes för att testa funktionaliteten. Syftet med examensarbetet var att testa möjligheten för automatisk analys och för att detta system ska vara fullt funktionellt behövs en metod för att säkra att provet i HPLC-loopen har samma koncentration som originalprovet. Detta är viktigt för en reprensativ analys. Några lösningar till problemet kan vara att använda mer prov, vilket ger en större pool, eller en detektor vid HPLC-loopen. För ytterligare framtida arbete kan denna analytiska system integreras i en nedströmsprocess för rening av antikroppar.

Popular science summary

Antibodies are proteins which are naturally found in the immune system and their purposes are to identify and inhibit pathogens. Because of these useful characteristics, antibodies can also be created for use in pharmaceuticals. Monoclonal antibodies are laboratory made and as the name suggests, a clone of a single B-cell which are a type of white blood cell. Each variant of monoclonal antibodies are identical and highly selective as they solely bind at a specific site of a target antigen which makes them very attractive in the pharmaceutical market.

Monoclonal antibodies are produced from living organisms along with other impurities which creates the need to be purified before use. The future vision is to have a fully automatic purification process of antibodies. To ensure the quality of the purification steps, analysis is needed as the drug should not be harmful to the patient. Both analysis and parts of the purification process uses chromatography.

This master thesis investigated the possibility of an automatic analysis and sample ordering system which would be a part of the future vision of a fully automatic production of antibodies. For this purpose, two chromatography systems were used: one analytical high performance liquid chromatography system from Agilent and an ÄKTA Explorer from GE Healthcare, which is a chromatography system for protein purification. By connecting these two systems, a setup for automatic sample preparation and analysis was created. To control them automatically, a software called Orbit was used which allows for advanced programming of the two chromatography systems and also has the capability of linking them together. To test this setup, monoclonal antibodies were transferred over from the ÄKTA Explorer to the HPLC and were analyzed. The experiments done in this project showed promise for implementing a fully automatic analysis of antibodies.

Populärvetenskaplig sammanfattning

Antikroppar är proteiner som finns naturligt i immunsystemet för att identifiera och hämma patogener. Dessa egenskaper leder till att de även kan användas i läkemedel. Monoklonala antikroppar tillverkas i laboratorium och som namnet antyder, är de kloner av en enda B-cell som är en typ av en vit blodkropp. Varje variant av monoklonala antikroppar är identiska med varandra och binder på ett specifikt ställe på en antigen vilket gör dem attraktiva på den farmaceutiska marknaden.

Monoklonala antikroppar produceras från levande organismer tillsammans med andra föroreningar som behöver tas bort för att rena antikropparna. Framtidsvisionen är att kunna helt automatisera reningsprocessen av antikroppar. För att säkerställa kvaliteten på reningsstegen behövs olika analysmetoder så att läkemedlet inte blir skadligt för patienten. Både analys och delar av reningsprocessen använder separationsmetoden kromatografi.

Detta examensarbete undersökte möjligheten för ett automatiskt analyssystem som skulle kunna vara en del av framtidsvisionen för en helautomatisk produktion av antikroppar. För detta ändamål användes två kromatografisystem: ett analytiskt vätskekromatografisystem från Agilent(HPLC)och en ÄKTA Explorer från GE Healthcare, vilket är ett kromatografisystem för proteinrening. Genom att koppla dessa två maskiner skapades ett system för automatisk förbehandling av prov och analys. För att styra dessa maskiner automatiskt användes en programvara som heter Orbit som möjliggör avancerad styrning och programmering av de två kromatografisystemen och har också möjlighet att länka dem. För att testa detta system överfördes monoklonala antikroppar från ÄKTA Explorer till HPLC och analyserades. Experimenten som gjordes i detta projekt visade lovande resultat för att i framtiden kunna utföra helt automatiska analyser av antikroppar.

Contents

1 Introduction

The market of biopharmaceutical drugs for therapies is increasing and with that, the demands are increasing which means that more productive and efficient production processes are desired. With this, continuous downstream processes for the purification steps are becoming more and more common as moving from batch processes to continuous processes gives benefits such as lowering cost and increasing productivity (Gomis-Fons, Löfgren, et al., 2019). Monoclonal antibodies (mAbs) are used for therapeutic treatment of varying illnesses and the downstream production of these are also moving towards continuous processes. The steps of the purification process of mAbs are usually the following: cell culture harvest, capture chromatography using affinity, virus inactivation, polishing, virus filtration and then filtration. The polishing steps are also chromatographic steps to further remove impurities and usually cation-exchange (CEX) as well as anion-exchange chromatography (AEX) are utilized. (Shukla et al., 2007).

The Chromatography Group at the Department of Chemical Engineering at Lund University have been working on automating the downstream purification processes of monoclonal antibodies by developing a software in Python called Orbit which can automatically control the ÄKTA chromatography systems. To follow the quality of the different step in the purification process, there is a need to analyze the sample at these different points of purification. This is important especially for pharmaceutical products to ensure that the drug is safe for the patient. By analyzing impurities such as host cell proteins, aggregates, and modifications like glycosylation or charge variants at different points of the process, the effectiveness of the steps can be followed. Therefore it is desired to be able to sample from the mAb process e.g. the feed, and the different chromatography steps and send them from these points to order an analysis. For this purpose, a HPLC system have been integrated into Orbit.

The aim of this thesis was to investigate the possibility of connecting and controlling an ÄKTA Explorer system with a HPLC system via Orbit for automated sample preparation and analysis of monoclonal antibodies.

2 Background

Antibodies, also known as immunoglobulins (Ig), are a family of proteins produced by B cells which are a form of white blood cells. They are a part of the immune system and serves the purpose to locate and neutralize antigens. Monoclonal antibodies are made by cloning a single B cell and the method for producing mAb was discovered in 1975 by Köhler and Milsten where they managed to create identical antibodies that target a specific site on an antigen. This discovery earned them and Jerne the Nobel Prize in 1984 (Diefenbach-Streiber et al., 2010; Köhler and Milsten, 1975).Since then, monoclonal antibodies have become a large part of the fast growing market for biopharmaceuticals. However, it can be very expensive as manufacturing costs are high and the traditional batch processes for chromatography are inefficient compared to continuous processes due to less buffer use, smaller equipment and faster processes with continuous chromatography (Gomis-Fons, Schwarz, et al., 2020; Jozala et al., 2016). For analysis of the product of these processes, chromatography is a very common method.

2.1 Chromatography

Chromatography is a separation method based on a mobile phase carrying a mixture containing the molecule of interest, and a stationary phase which is often placed inside a column (Poole, 2013). The different interaction between the molecules in the mobile phase and stationary phase results in separation and giving different elution times. As the name suggests, in liquid chromatography, the mobile phase is liquid containing the sample mixture and a buffer solution. The liquid then moves through the column containing the stationary phase which is often an adsorbent material bonded to either the walls of a column or onto solid materials packed in the column (C.Harris, 2016). High performance liquid chromatography (HPLC) is a method where the mobile phase is pumped through the system at a higher pressure to achieve faster separations (Poole, 2013).

Chromatography can be preparative or analytical which are the two main uses.

Preparative chromatography refers to processes using chromatography to recover pure products e.g. the capture chromatography step in mAb purifcation. Analytical chromatography uses the separation of solutes for identification of an unknown attribute. This type of chromatography is often used to gain more information about the product and to validate the quality of the processes. When analyzing, the sample is not kept and is either destroyed by the detector or sent to waste after being analyzed (Poole, 2013).

There are different variants of liquid chromatography based on the interaction with the stationary phase. Examples are ion-exchange chromatography (IEX), size exclusion chromatography (SEC) and affinity chromatography. IEX is based on charge and can be separated into cation-exchange and anion-exchange. Cations or Anions are attached to the stationary phase and these attracts and interacts with molecules of the opposite charge in the mobile phase (C.Harris, 2016). Elution is done with high concentration of salt to replace the molecule in the sites on the stationary phase (Jandera and Lembke, 2011). This method is commonly used for separations of charged molecules e.g. ions, amino acids, nucleic acids and other proteins who bind to the stationary phase via their charged groups on the surface.(Cytiva, 2021; Jandera and Lembke, 2011). This project will mostly focus on size exclusion chromatography and affinity chromatography which will be further explained in chapters 2.1.1 and 2.1.2.

2.1.1 Affinity Chromatography

Affinity chromatography is a highly selective chromatography method based on binding interactions between the stationary phase consisting of a biological ligand with a specific affinity for a biomolecule in the mobile phase. When the mobile phase passes through, the specific molecule is captured by the ligand. The target molecules are then eluted at a later stage (C.Harris, 2016). Figure 2.1 shows an illustration of the method.

In regards to chromatography in regards to purification of monoclonal antibodies, affinity chromatography using protein A is an important step in the purification process as it is relatively fast and gives a high yield of product(Liu et al., 2010). Protein A is a ligand from bacterial cell walls that have an affinity for immunoglobulin and bind to a specific region on them called the Fc region. Impurities such as host

cell proteins, DNA, and other components in the raw extract will not bind are washed out. Further impurities can be removed by IEX or other chromatographic methods(Cytiva, 2021; Diefenbach-Streiber et al., 2010). Protein A has the strongest binding affinity at near neutral pH and disassociates at lower pH which means that the antibodies are eluted by switching to a low pH buffer (Hage, 2014).

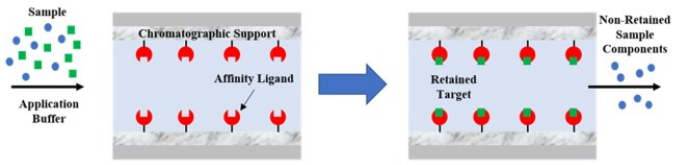
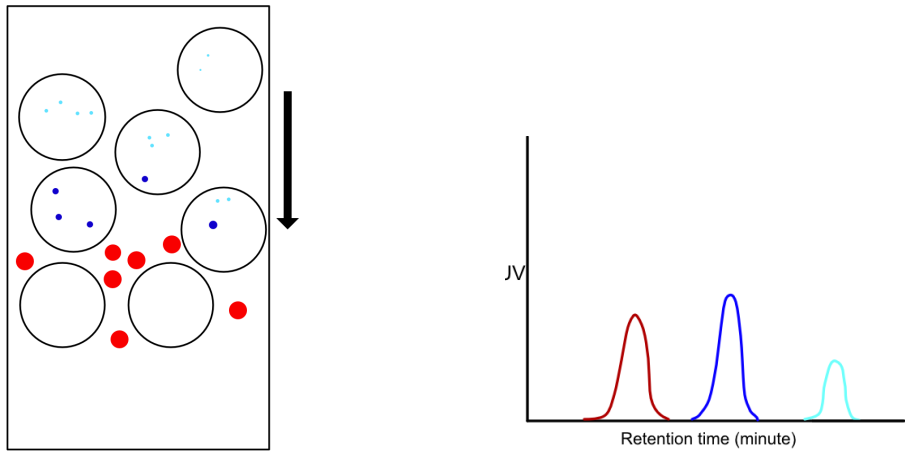


Figure 2.1 – An illustration of the principle of affinity chromatography where the target molecule is bound to the ligand while the other components are washed out (Rodriguez et al., 2020).

2.1.2 Size-exclusion Chromatography

SEC is a method where the solutes in a mixture are separated by size and unlike affinity chromatography, attractive interaction is not wanted for separation. Instead, the mobile phase passes through a porous material where the small molecules travels the long way through the pores while larger molecules are excluded and does not enter the pores. This means that smaller molecules take longer to elute as they have to travel a larger volume through the pores before leaving (C.Harris, 2016). This principle is illustrated in Figure 2.2. Instead of elution time or retention time, retention volume is used instead when talking about when the solute leaves the column (Jandera and Lembke, 2011). This makes SEC a very useful tool during mAb analysis and purification as protein monomers, aggregates and smaller fragments are separated. This allows for quantification of aggregates. SEC can also be used for desalting or buffer exchange as the smaller salts will take longer to elute than the larger biomolecules. As earlier mentioned, ideally there are no other interactions between the solutes and the stationary phase because then the selectivity depends only on the accessibility of the pores of the matrix and control of for example the pH of the buffer solution is used to deny interaction with the solute. One small limitation of SEC is that the solutes must differ by 10 % in molecular mass.(Jandera and Lembke, 2011).



(a) The principle of SEC, larger molecules(red) are too big to enter the pores streams through. Smaller molecules (blue and light blue) enters the pores and are retained for a longer time.

(b) The same phenomena as Figure 2.2a seen in a chromatogram, the larger molecule elutes first, followed by the smaller ones .

Figure 2.2 – The separation method of SEC illustrated by the author.

A calibration curve for the SEC column with known standards is useful for estimating apparent molecular weight (MW). It is done by measuring retention volume and plotting Log_{10}MW against elution time or retention volume, see Figure 2.3. An antibody generally has an MW around 150 000 Da (Diefenbach-Streiber et al., 2010). Normally the calibration curve isn't fully linear and can sometimes be approximated by a polynomial function. However, as the linear area is often in the range of interest, a linear approximation can be made (Jandera and Lembke, 2011; Podzimek, 2011).

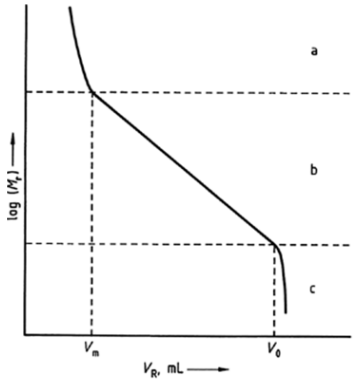


Figure 2.3 – An illustration of a calibration curve for SEC where the retention volume V_R corresponds to the logarithm of the relative molecular mass M_R. The top area is where molecules are too large to enter the pores, the middle area is linear and the main separation range, the bottom area is where molecules are so small that they travel through all the packing (Jandera and Lembke, 2011).

2.2 The Orbit software

Orbit is a program developed at the Department of Chemical Engineering at the Faculty of Engineering to be able to control ÄKTA systems. The software is written in Python and contains three main parts which are the system configurations, the interfaces and the unit libraries. The interfaces is the main part of Orbit and contains classes for the systems and the units such as tubes, valves or sensors. Besides the files that build up Orbit, a process file and a script file are needed to allow the user to execute commands. Over the recent years, more functionality have been added and one of those is to be able to connect Orbit to control an Agilent HPLC system. Also, any system that provides a communication interface with support for Python can be implemented(Andersson, Löfgren, et al., 2016; Andersson, Tallvod, and Nilsson, 2018).

Cytiva, previously GE Healthcare (Uppsala) are the supplier of ÄKTA systems and they provide the Unicorn software which is how the instruments are normally controlled. Unicorn is however quite limited and Orbit allows for more complex tasks like running machines in parallel or more advanced process controls. To run two ÄKTA systems or a HPLC and ÄKTA simultaneously one would have to manually create each method and start the machines at the same time, and even then the timings would need to match which causes a lot of difficulties. This is much more convenient in Orbit as two or several instances can communicate with each other. Orbit allows the user to specify the different phases of a process such as loading, elution or equilibriation and the user can control settings like valve positions and flow rate. These phases are then executed by the ÄKTA machine while the software gathers information and can handle calculations if wanted. The phases and the calculations are written in the process and the script files that were mentioned earlier. Orbit is connected to Unicorn via an Object linking and embedding for Process Control (OPC) - connection using the OpenOPC package in Python. When Orbit sends an action it is communicated to the machine and Unicorn via the OPC interface or via API (Andersson, Löfgren, et al., 2016).

With the Agilent HPLC system, Unicorn OPC is not available which means that another way to be able to control the HPLC-system with Orbit was needed and therefore Satellite CDS was developed. Satellite is built around Agilent Instrument

Control Framework, Agilent ICF, which allows control of their systems. With this a new interfaces ICF file was created with all the units for the HPLC. The Satellite software is written in C # and runs like a server that connects the HPLC with Orbit and allows Orbit to control the hardware in a similar way as with an ÄKTA system (Andersson, Tallvod, and Nilsson, 2018).

3 Materials and Methods

The section of materials and methods will cover the design process and experimental work. The two major parts of this work were programming in Orbit and the running of the systems in the laboratory and the two parts were closely connected.

For this work, two cases were looked at:

- Case 1: Sample preparation machine sends sample directly to HPLC loop for analysis
- Case 2: Sample preparation machine performs a chromatography step as pretreatment before the sample is sent to the HPLC loop.

With a SEC column and a protein A affinity chromatography column, concentration of mAb and amount of aggregates were the two forms of analysis that could be performed in this project.

3.1 Experimental work

This part will include the materials and experiments done in the lab. Instead of taking samples from a downstream process, injection of sample was done via a syringe into the injection loop of the ÄKTA as this thesis aims to build the sample preparation plus analysis setup.

3.1.1 ÄKTA Explorer

The ÄKTA Explorer by GE Healthcare seen in Figure 3.1 was used in this thesis for separation and direction of sample to the HPLC and it was controlled by an instance of Orbit. The system contains different modules e.g. buffer pumps, valves for different purposes and sensors for pH, UV and conductivity. A HiTrap MabSelect SuRe 1 ml column from Cytiva was connected at the column valve for protein A chromatography. The injection loops used for this project were 100 µl, 300 µl and 500 µl.

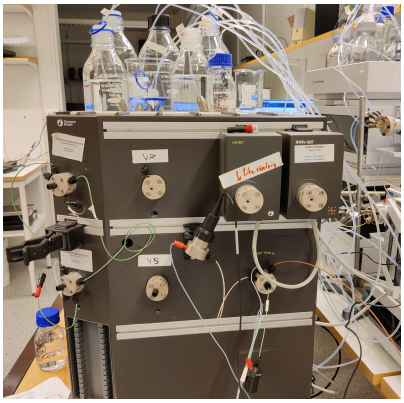


Figure 3.1 – A picture of the ÄKTA Explorer used in this thesis.

3.1.2 Agilent HPLC

The HPLC used in this project was an Agilent 1260 Infinity II LC System which is shown in Figure 3.2 and it was controlled by a second instance of Orbit. The system is equipped with a diode array detector (DAD) and the potential to add additional detectors. The benefits of a DAD is that it is capable of measuring every wavelength between 190 nm and 950 nm compared to the maximum of three wavelengths on an ÄKTA System (Agilent, 2018; Bio-Sciences, 2008). This could be of use in future work but was not taken advantage of in this project as it was mainly the absorption at UV 280 nm that was looked at.

The column used for this thesis was a BioSep-SEC-S3000 column which contains a silica resin and have the dimensions of 300 x 7.8 mm. The injection loop was 20 µl.

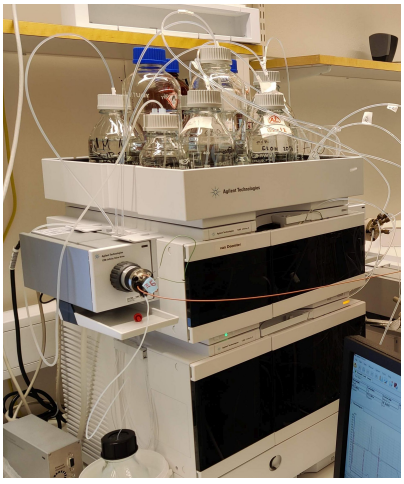


Figure 3.2 – A picture of the Agilent HPLC used in this thesis.

3.1.3 Calibration curve

An attempt to create a calibration curve was made according to the method mentioned in chapter 2.1 using dextran of known molecular weights at 100, 200 and 2000 kDa. Dextran was used since the apparent MW is known and can then be correlated to the retention volume. Since there were only three sizes of dextran available where one of them was very large, a calibration curve provided in a document by Phenomenex who are the maker of the columns was also used for comparison(BioSep, n.d.).

This provided calibration curve can be seen in Figure 3.3. The curve fit to the calibration was polynomial and not given so the results from the experiments were compared graphically and by a linear fit which was made by taking values from samples 2-10 which was assumed to be the linear range.

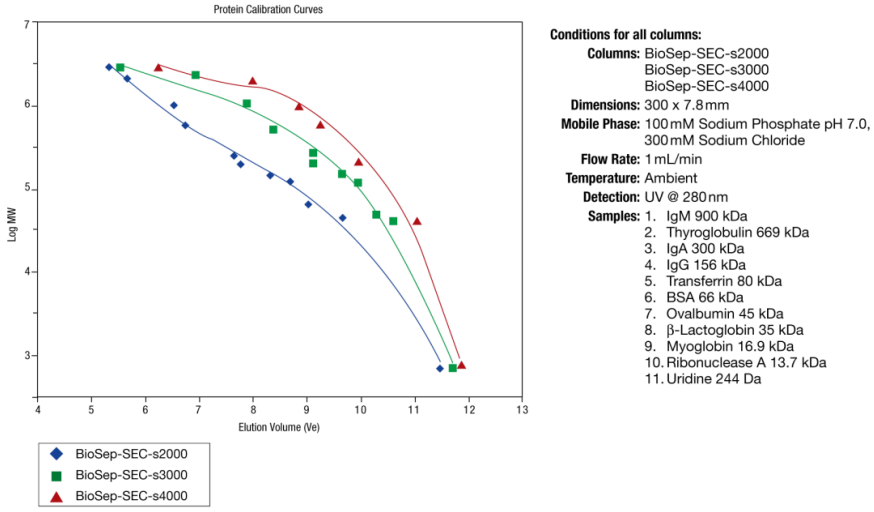


Figure 3.3 – Calibration curve for BioSep-SEC-S300 provided by the producer with conditions, parameters and sample molecular mass (BioSep, n.d.).

Relating the MW to the retention time might be interesting to identify possible species or determining MW of unknown species.

3.1.4 Buffer solutions

As the pH and the final concentration was known, then the Henderson-Hasselbalch Equation (3.1) was used in combination with Equation 3.2 to create an equation

system to calculate the concentrations for the components of the buffer solutions. The buffers used are presented in Table 3.1.

$$pH = pKa + log(\frac{[A^-]}{[HA]}) = pKa + log(\frac{[B]}{[A]}) \tag{3.1}$$

$$c_{tot} = c_A + c_B = [A] + [B] \tag{3.2}$$

Table 3.1 – Buffer solutions used in this project

Buffer use	**Buffer components**	**pH**
HPLC analysis	50 mM Na-phosphate, 150 mM NaCl	7.0
HPLC SEC cleaning	0.1 M NaH_2PO_4	3.0
Loading & Equilibrium	20 mM Na-phosphate, 150 mM NaCl	7.4
Wash 2 MabSelect	50 mM Na-acetate	6.0
Elution & Regeneration	50 mM Na-acetate	3.5
CIP MabSelect	0.1 M NaOH	-

3.1.5 Connection of hardware

The ÄKTA Explorer and the HPLC was connected with a capillary tube from outlet position 8 on the ÄKTA to the injection loop on the HPLC.

To ensure that the antibodies reached, and stayed in the loop of 20 µl, some experiments were needed to measure the volume between the injection site of the ÄKTA and the HPLC. This was done by measuring the dead volume from the injection site at the HPLC to the DAD detector as well as the dead volume from the injection site of the ÄKTA to the DAD detector of the HPLC. By measuring these volumes and subtracting the dead volume of the HPLC from the dead volume of the entire system, the volume between the systems is calculated. These experiments were made using acetone as the sample to minimize use of antibody solution. The experiments were ran with a flowrate of 0.5 ml/min and the dead volume was estimated from time of injection to sample peak on the HPLC detector.

The addition of a MabSelect column required new calculations as the system volume changed and the sample was binded into the column instead of flowing directly from the injection site. This was solved by calculating the distance from the UV sensor in the ÄKTA Explorer to the injection loop of the HPLC and another experiment running an antibody solution through the column was needed. The calculations were made by taking the volume between the systems minus the volume from injection to the UV which is seen by the contaminants that were instantly washed from the column. The elution time of antibodies was also needed which was also seen on the ÄKTA UV. By adding the volume of elution peak and the volume from the UV to the HPLC injection loop, an estimation could be made of when the signal should be sent to the HPLC.

3.1.6 Sample solutions

The antibody solutions used were supplied by GE Healthcare. They were stored in a freezer and all the samples needed to be thawed before use.

Solutions used were previously purified at 2.1 g/l mAb to test the protein A into HPLC stream, 0.17 g/l purified mAb to test the bypass path, and 0.5 g/l supernatant for purification and then analysis. A purified mAb was used first for protein A chromatography as it was faster to thaw than the supernatant solution.

3.1.7 Monoclonal Antibody Analysis

At first, the machines were run separately and the first sample used was the 0.17 g/l mAb. The sample was first injected in the HPLC for SEC analysis, and for the second run, 300 µl was injected into the ÄKTA and sent to the injection loop of the HPLC for analysis. This entire process only used the 50 mM phosphate buffer.

The 2.1 g/l mAb was used to test the MabSelect SuRe and elute the antibodies from it. The eluted sample was then sent to the HPLC. The loading buffer was used during injection, then eluted with elution buffer followed by CIP and equilibrium. The HPLC ran the 50 mM phosphate buffer as usual.

Finally the supernatant solution was purified, injecting 500 µl to capture the antibodies which were then eluted to the HPLC injection loop for analysis through SEC.

Similarly to the previous test, the same buffer solutions were used. Wash 2 buffer was not used as it was deemed excessive and the washing during loading was enough. The injection volume was increased to increase the amount of antibodies.

The data from the runs were saved by Orbit into a data file that could be opened in Excel and for the ÄKTA, data were also saved by Unicorn. The data from the HPLC for UV_{280} was plotted against time and by using the trapz function in MATLAB, the area of the peak were calculated according to equation 3.3. The area of the different peaks gives information about level of aggregation.

$$Abs = \frac{Area_{peak}}{Volume} \tag{3.3}$$

Unicorn provides an useful evaluation tool for calculating peak area and elution volume. It can also calculate concentration if the path length and extinction coefficient in Beer-Lamberts law is given, see equation 3.4. ϵ is the extinction coefficient which can be around 1.3 - 1.5 mg ml^{-1} cm^{-1} for antibodies (Maity et al., 2015). 1.4 was chosen for this project. l is the pathlength, which is 2 mm for the UV sensor in the ÄKTA Explorer and c is the concentration.

$$Abs = \epsilon cl \tag{3.4}$$

If the concentration of the raw extract is to be calculated, Equation 3.5 can be used. C_1 and V_1 are the starting concentration and the injection volume while C_2 and V_2 are the concentration and volume of the eluted mAb.

$$C_1V_1 = C_2V_2 \tag{3.5}$$

3.2 Process Design

As there are two cases for the sample preparation, the final configuration consists of two different paths for the sample to take from the ÄKTA system into the HPLC injection loop for analysis. This is controlled by the column valves as one path sends

sample directly into the HPLC loop and the other one passes a preparative method in case a sample needs to be treated before analysis. For this experiment a protein A capture column was used. The complete process scheme can be seen in Figure 3.4.

The flowrate of the ÄKTA Explorer was set to 1 ml/min except for loading which was 0.5 ml/min. When pump-washing, the flowrate was set to 5 ml/min for 2.5 minutes. The HPLC performed analysis at 0.8 ml/min.

The protein A chromatography step included 6 column volumes (CV) of loading, 7.7 CV of elution, 5 CV of CIP, 10 CV of equilibration, and 3 CV for regeneration. The HPLC ran analysis for 20 minutes.

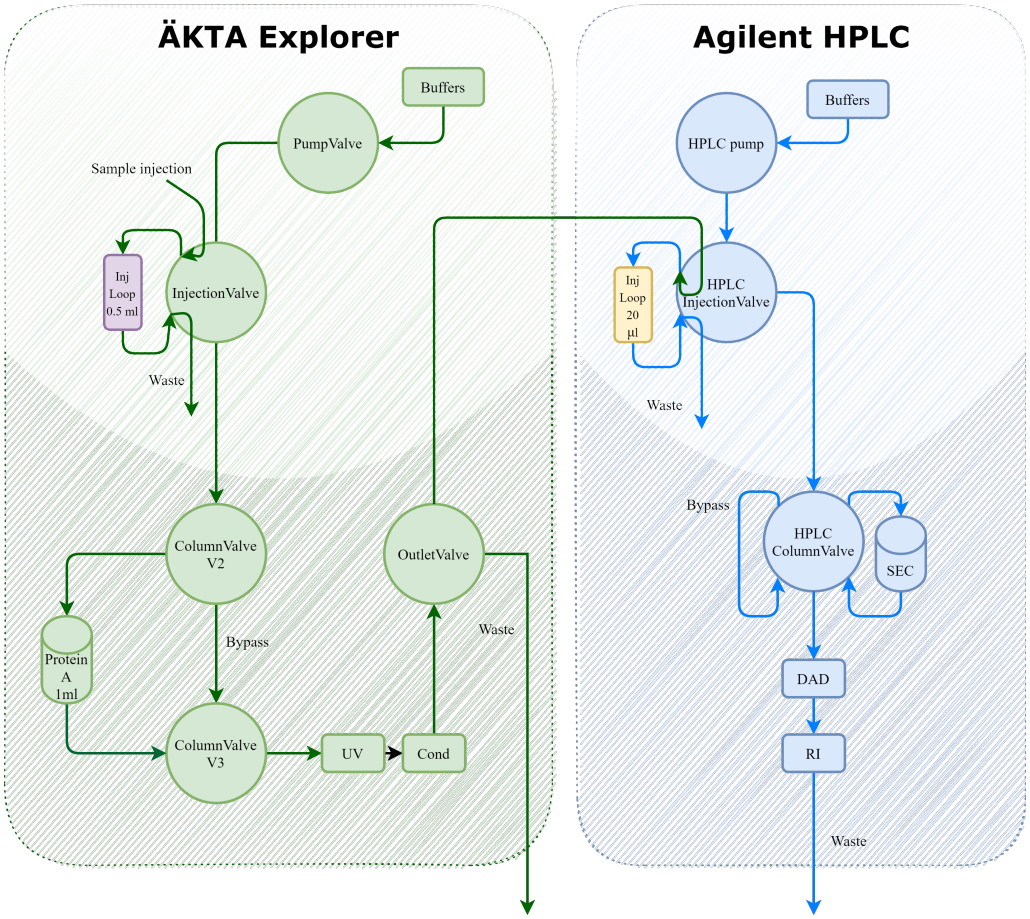


Figure 3.4 – A flowchart of the system. The HPLC is connected to the OutletValve of the ÄKTA which makes it possible to fill the HPLC injection loop.

3.3 Programming in Orbit

The goal of the programming work was to sync two instances of Orbit, one controlling the HPLC, and another one controlling the ÄKTA Explorer. The scripts contained information such as which buffer to use, when to inject, how long each phase should be and the position of the valves.

To start off, scripts were written to run the machines separately. When both machines successfully ran on Orbit, they were connected via an Orbit method called ExternalSync which allows different instances of Orbit to communicate. The computer connected to the ÄKTA Explorer was used as the server while the computer connected to the HPLC acted as a client and connected to the server using its IP address. By running these two scripts simultaneously, they can wait for each other by setting true and false variables with ExternalSync.

A script controlling only the HPLC was used to measure retention volume of dextran of different molecular weights and a script only controlling the ÄKTA was used to run protein A chromatography to ensure that everything worked and to be able to calculate the distance from the UV sensor during elution of mAb before connecting the ÄKTA with the HPLC.

The script file for HPLC contained phases for start up, injection, and two phases containing ExternalSync where one waits for the ÄKTA and the other phase sends a signal to confirm that the HPLC is ready.

In the final script file for the ÄKTA Explorer, the phases for the protein A path were: start up, loading, washing, elution, equilibration, regeneration, cleaning in place (CIP). The phases for the bypass path were: start up and injection. This script also included the ExternalSync method to be able to communicate with the other instance of Orbit. The script also included phases for pump wash, to make sure that the buffer solution is in the system. Both scripts can be seen in Appendix B and C

4 Result and Discussion

The experiments showed that it was possible to connect the HPLC and ÄKTA for automated analysis of antibodies controlled by Orbit. It was also possible to add a capture step and then send the purified monoclonal antibody for analysis. There are a few problems with the setup that need to be addressed before it can be fully integrated with a downstream process which will be discussed in the future sections in this chapter.

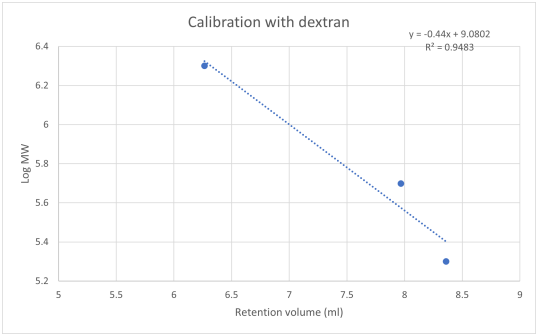
4.1 Experimental study

The experiments showed that it was possible to connect the HPLC and ÄKTA for automated analysis of antibodies controlled by Orbit. The few manual parts of this experimental version of the dual sample preparation - analysis system were the mixture of buffer solutions and the injection via a syringe to the ÄKTA. It was also possible to add a capture step for quantifying the antibodies and also to send the purified monoclonal antibody for analysis. The analytical experiments performed in this study were able to determine percentage of aggregate and the concentration of mAb which contributes to the information about the purity of the product as well as the effectiveness of a process step. For further analysis of the purification process, columns such as an analytical IEX column could be added to the other positions at the column valve of the HPLC. A desalting SEC column could also be added to the ÄKTA Explorer if a buffer exchange would be required before analysis.

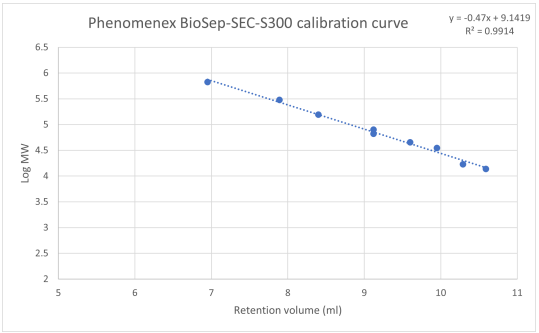
The benefit of the Agilent HPLC for analysis are the detectors and the possibility of additional detectors. By being able to detect multiple wavelengths, it is possible to gain information for example by comparing the difference of the spectrum for the target product and mixture as well as comparing different wavelengths to find where a species absorb the most. However, in this thesis, it was only the 280 nm wavelength that was used as proteins usually have absorbance maxima there and for simplicity. Other wavelengths could have been examined for possibility of higher signal.

4.1.1 Calibration of SEC column

Since the three dextran sizes available were all above the expected size for an antibody mentioned in chapter 2.1.2, the calibration curve will most likely not give a representative value for the molecular mass of the sample. It is also most probable that large molecules of 2000 kDa would ignore the pores and elute with the void volume. The two different calibration curves are presented in Figure 4.1. In Figure 4.1a it can be seen that the fit was decent as the R^2 value was 0.948, but with only 3 data points, it was not enough information and dextran of varying sizes within the separation range of the column would have been preferred for a calibration of the column. The Phenomenex curve in Figure 4.1b should give a more representative value of the MW and the buffer used includes the same components but at a higher concentration. The data points for this curve results in a better fit for the linear regression with a R^2 of 0.991. However, it also has some flaws as the retention times were estimated by drawing lines from the points down to the x-axis.



(a) Calibration curve 1 from the dextran experiment, linear fit: y = -0.44x + 9.0802.



(b) Calibration curve 2 of the linear region from the calibration curve shown in 3.3 in chapter 3.1.3 provided by the BioSep-SEC-S3000 manual. linear fit: y = -0.47x + 9.1419.

Figure 4.1 – Calibration curves of the SEC chromatography column from experiments and from the manual,

For this thesis, the calibration did not give much information as the monomer was the largest part of every analysis and there were only monomer, aggregate and fragments present. However, it could be used to locate the monomer and other species in a more fragmented or aggregated solution. If there are unknown molecules it could also be beneficial to gain information about their MW.

4.1.2 Sample preparation to HPLC analysis

The measurements of dead volume gave 0.42 ml for the HPLC and around 1.8 ml for the entire system. This means that the difference was around 1.38 ml which should be the volume between the two systems. This is a close estimation but might not be 100 % accurate as the acetone oversaturated the detector and the peak width was very wide so a value around the middle of the peak was used. The calculated volume between the systems was then used to set the time for when the ÄKTA should send a signal for the HPLC to start and resulted in sample detected at the HPLC which means the method was partly successful. More discussion about sample transferring will be discussed at a later point in this section.

The chromatogram from the HPLC for a mAb solution of 0.17 g/l can be seen in Figure 4.2. Orbit was running from the beginning and collecting data while waiting for the HPLC which means that the injection point was at 2 minutes 6 seconds. This gives an elution time of 10 minutes 48 seconds which equals to 8.64 ml with a flowrate of 0.8 ml/min. With calibration method 1, the MW was 190 kDa and with method 2 the MW was 121 kDa which confirms the suspicions that method 1 is not close to accurate while method 2 is close. Since 150 kDa is an average of mAb, the antibody used in these experiments could very well be smaller than that. The small peak before are aggregates, most probably dimers as it is a single peak very close to the monomer. There also seem to be some low molecular weight fragments which could be parts of antibodies but it could also be noise because of the overall low signal. The amount of aggregates was calculated to 8.2 %.

This analysis showed that it was possible to use Orbit to steer the process of taking a sample from the injection loop in the ÄKTA and then sending it to the HPLC for analysis. Overall the system showed success as the sample preparation system managed to send sample to the HPLC for analysis. One problem that was observed was that the concentration of antibodies was too low when injecting 100 µl of sample as it was not detected when sent to the HPLC. Such a problem can be avoided by either concentrating the sample or increasing the injection volume.

The system was able to run completely autonomously. However, some pre-work was needed such as finding out when the sample would reach the HPLC loop and this method may have flaws as not every analysis will look exactly the same and it is not

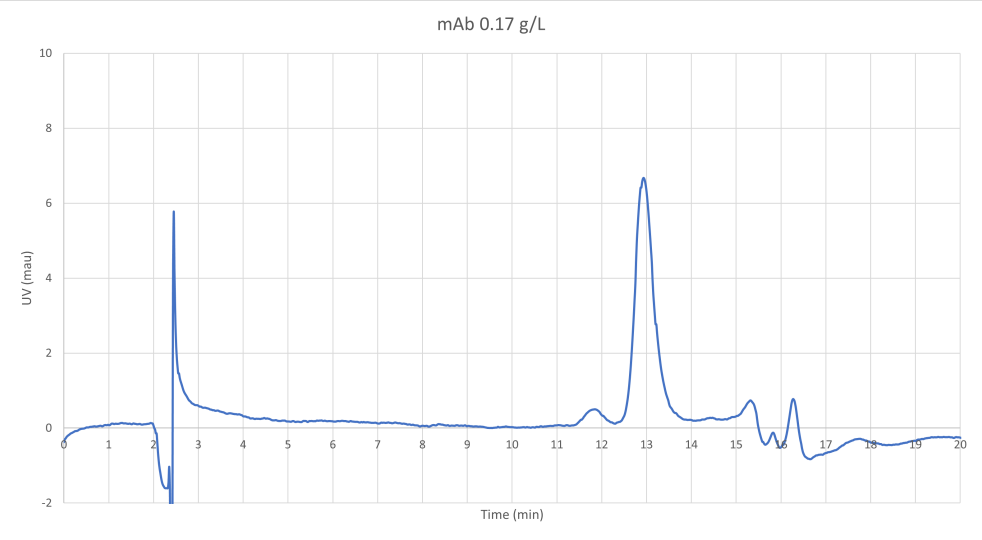


Figure 4.2 – Chromatogram from HPLC analysis of purified mAb received from the sample preparation system, injection started at 2 min 6 s. Retention volume was 8.64 ml

100 % percent certain that the loop will contain the entire amount of the sample. This would result in a lower concentration of mAb and aggregates as not all sample would be contained in the loop and could be one of the reason that the signal was rather low. If the mixture in the HPLC is broken up or uneven, for e.g. if not the highest concentration of sample has entered yet or have already passed the loop, then the analysis from the HPLC will not be fully representative. One solution could be an additional detector coupled to the HPLC injection loop to notify when sample starts to leave the loop. Another idea is to use a larger injection loop or even a super-loop in the ÄKTA which would create a larger pool which gives a more constant stream of sample and therefore makes it easier to transfer a high concentration of sample to the HPLC. By doing this, the margin for timing the sample is larger to ensure that a representative concentration of mAb reaches the HPLC loop.

4.1.3 Sample preparation with affinity chromatography step

For case 2, the addition of a chromatography step to prepare the sample for analysis was successfully implemented to the sample preparation system. In this case, an affinity chromatography column was used which purified the mAb for HPLC analysis and also gave information about the concentration of mAb. As previously mentioned,

the preparative step could also be a desalting column for buffer exchange to not disturb potential charge based analysis. From early experiments it turned out that washing with a solution at 6.0 pH did not contribute to the purification which meant that it was omitted from the experiments shown in this section.

The distance between the UV sensor and the HPLC loop was calculated to be 0.77 ml and the elution peak was 1.92 ml after washing which meant that the HPLC started at 2.7 ml after elution from the protein A column. These calculations resulted in the sample successfully transferred over to the HPLC, but as previously mentioned, it might not be a representative amount.

Test run

The first runs of this configuration were made with an already purified mAb. The purpose of this was to test the functionality of the system to see if the eluted mAb reached the HPLC and to find out the elution time. The setup and buffers worked as a tiny amount of impurities were washed out during loading and then a large peak of mAbs were bound and eluted as seen in Figure 4.3 . The monoclonal antibodies then made their way into the HPLC loop for injection bypassing the column as these experiment were only to confirm that the sample successfully entered the loop. The results in Figure 4.4 show that a large amount of the mAbs were successfully caught in the loop and then sent to the HPLC DAD-detector.

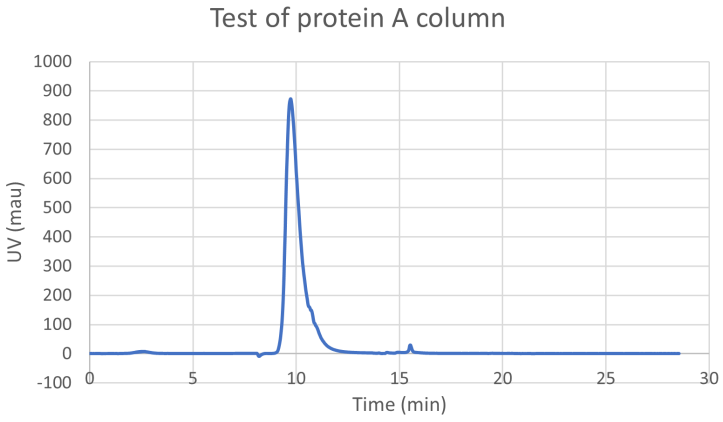


Figure 4.3 – Chromatogram from experiment testing the protein A column by loading it with purified mAb.

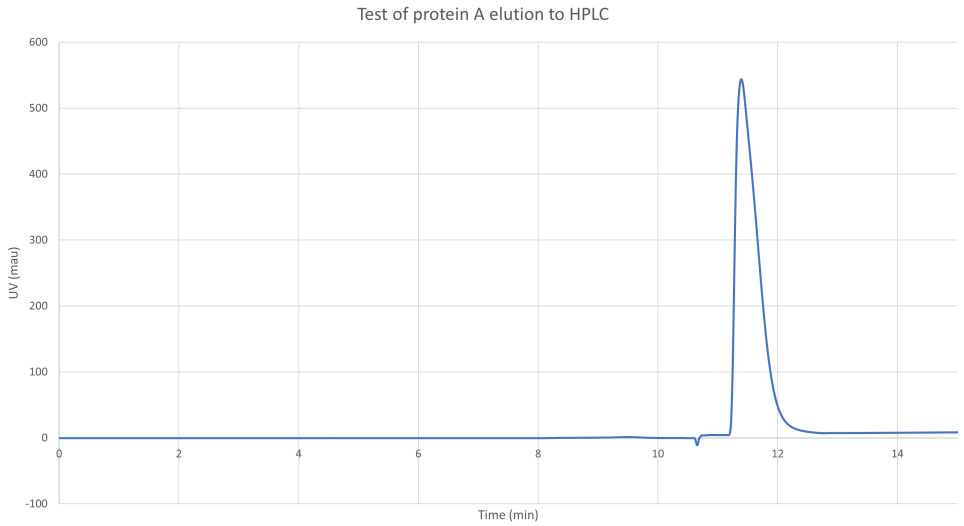


Figure 4.4 – Test of elution from protein A column to HPLC loop, injection started at 10 min 52 s.

Analysis of supernatant solution

In figure 4.5, the chromatogram of the protein A chromatography process can be seen. The first peak at around 4 minutes shows the impurities that were immediately washed out during loading, the second peak at around 15 minutes shows the elution of mAbs. At around 0.77 ml after this peak, the HPLC was started as the eluted sample had reached the injection loop. After elution, CIP was performed and washed out what was left in the column which gives the third peak at around 20 minutes.

The evaluation tool in UNICORN was used to calculate concentrations and a picture of the program can be seen in Figure 4.6. The concentration of the eluted mAb turned out to be 0.029 mg/ml and the starting concentration was calculated to be 0.16 mg/ml which is quite a bit less than the supposed 0.5 mg/ml. This difference in concentration could have several reasons; number one is that when freezing, the water will freeze before the antibody which makes them all concentrated in the middle of the container might thaw at a later time than the ice. This can also lead to a lower concentration of antibodies if the solution was not mixed enough to even the concentration. Another reason could be that some antibodies have denatured by reasons such as freezing and thawing.

In the chromatogram for HPLC analysis shown in Figure 4.7, the injection occured

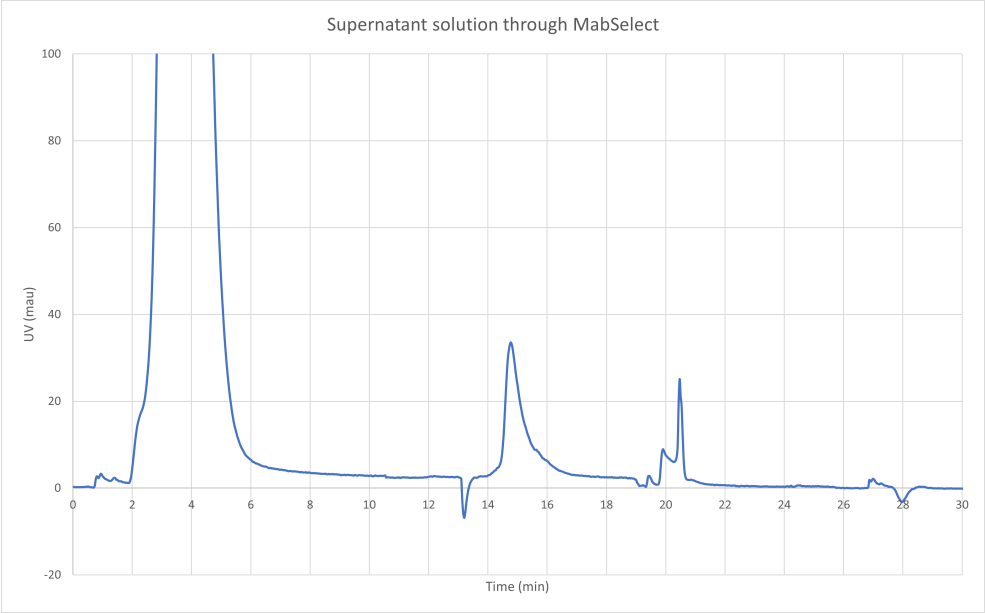


Figure 4.5 – Chromatogram from the purification of supernatant solution using HiTrap MabSelect SuRe on the ÄKTA Explorer.

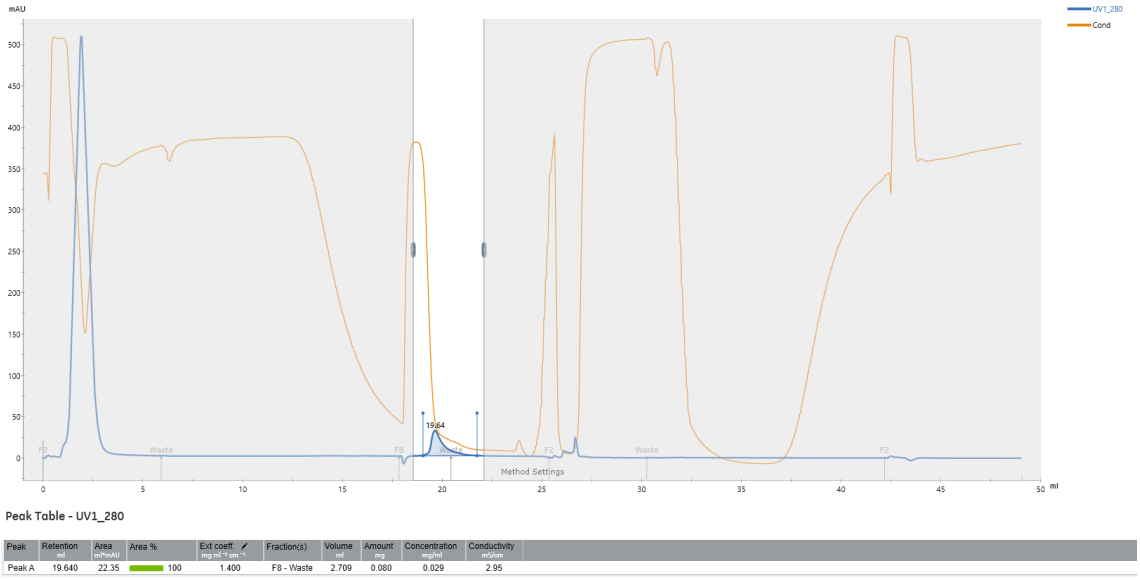


Figure 4.6 – Picture of the evaluation tool in Unicorn to analyze the elution peak. The evaluation tool can calculate elution volume and concentration

at 15 minutes and 52 seconds which means that the mAbs elute after 10 minutes and 42 seconds. This equals a retention volume of 8.56 ml and molecular weight of 206 kDa and 131 kDa in curve 1 and curve 2, respectively. Unlike the pure mAb solution, the supernatant showed no aggregates but low MW fragments were still present. Similarly to the previous case, the peaks could also be noise or caused by the buffer due to the lower signals. These experiments showed similar problems as case 1 with sample transferring and ensuring a high concentration of sample for a representative analysis which was discussed in the previous section.

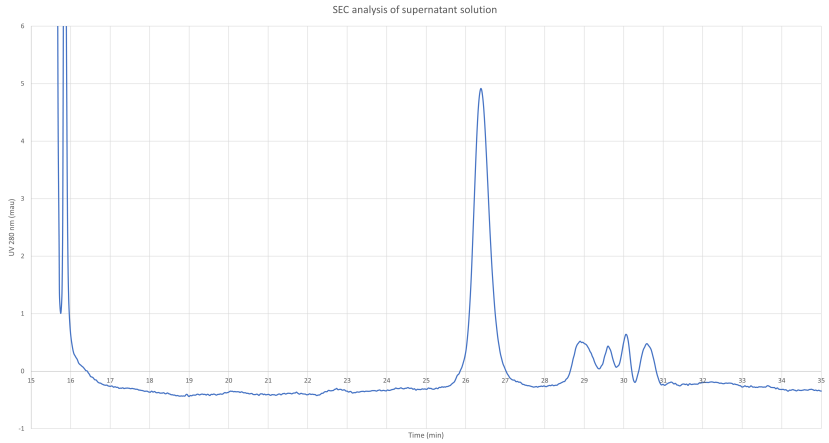


Figure 4.7 – Chromatogram from HPLC analysis of eluted mAb after affinity chromatography, injection started at 15 min 52 s. Retention volume was 8.56 ml

4.2 Orbit

The easiest way to control the chromatography processes was by setting a time limit on each phase. Communication between the two instances of Orbit was successfully implemented by using phases containing ExternalSync which set and waited for variables. These phases blocks processes while they wait for the flags and were placed at the start to make sure the connection works and that no machine starts without the other. Another one was placed between two elution phases during the elution of mAb from the affinity column: Elution to HPLC - Send variable to start HPLC - Elution to waste.

It was noted that Orbit was gathering data from the HPLC while it was waiting which made the x-axis of the graphs show the time for the entire run of the two systems instead of from when the HPLC runs started. This was a minor inconvenience and if

fixed, it would simplify the process of reading data from the experiments.

The programming work made the code easy to follow and understand but allowed for no dynamic control as one would have to wait until the phase ended until the next phase started no matter what. However, dynamic control would probably not be beneficial for this analytical system except for optimization of the phases of the affinity chromatography to possibly decrease the time for analysis. Dynamic control would be very useful if an UV detector was added to the HPLC loop as it could change the outlet valve of the ÄKTA as well as start the HPLC injection when the UV starts to form a peak as that means that sample is in the loop.

5 Future Work

Future work could be to integrate the system to an antibody purification downstream process for an automated analysis of different steps of the purification process. As earlier mentioned, a method of ensuring that the concentration inside the HPLC loop is the same as the sample in the ÄKTA is needed for this system to be fully operational and to perform representative analysis. The possibility to send samples to the autosampler of the HPLC could also be investigated. In this study a SEC column was used on the HPLC but other columns could be added and if needed, more detectors such as a mass spectrometer could also be added to the HPLC for a more comprehensive analysis

The scipts with Orbit could also be improved and less cluttered. If a detector is installed at the HPLC-loop, or if larger injection volumes are used, if-statements or additional functions for dynamic control could be added for communication between the two instances to signal when the loop is filled with sample.

6 Conclusion

This project evaluated the possibility of a sample preparation - HPLC analysis system and automating it. It was proven to be and promising for fully automatic analysis of biopharmaceuticals as sample was successfully transferred and analyzed with Orbit being a powerful tool for this purpose. However, as this thesis only investigated the possibility of the analytical setup, there are still some problems before it could be integrated and used for automatic analysis. The HPLC analysis might not be representative as a result of problems with timing the sample transferring between the two systems, suggestions to improve this would be injecting a larger volume into the ÄKTA for a larger and wider elution pool before which gives more room to coordinate the timing to the HPLC loop. In this thesis, analysis of aggregation and concentration of the mAb solutions were made but additional analytical tools such as an ion exchange chromatography column, or a mass spectrometer could be added in the future for more in depth analysis of its purity.

The HPLC shows benefits for analytical purposes such as a multi wavelength detector for observing, and comparing any wavelength between 190-950 nm. A mass spectrometer can also be introduced to the HPLC as mass spectrometry is a very common method to identify and quantify proteins.

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A Aggregate quantification

Level of aggregation in a sample was calculated using trapz() function in MATLAB. This was done by setting limits for the x-axis to approximate the width of the peaks. The script for this process can be seen below.

```
uv=mAB{:,2};
time=mAB{:,1};

plot(time,uv)
xlim([16 27])
ylim([-1 10])

limsagg = (time >= 19.7) & (time <=20.8);
plot(time(limsagg),uv(limsagg))
intagg = trapz(time(limsagg),uv(limsagg))

limsmono = (time >= 20.9) & (time <=22);
intmono =trapz(time(limsmono),uv(limsmono))
plot(time(limsmono),uv(limsmono))

aggper = intagg/(intagg+intmono)
```

B ÄKTA Explorer script

```
# -*- coding: utf-8 -*-
"""
Created on Tue Apr 13 14:34:53 2021

@author: lab
"""

from projects.explorerhplc.system_explorer import*
from projects.explorerhplc.process_explorer import Phase, Process, Sync
    , ExternalSync

S = SystemExplorer(mode='test')

runMode = 'real'

exSync = ExternalSync(n=1,port=51001)

LoadHPLCDone = Phase(name = 'LoadHPLCDone',  #sets to true, HPLC
    receives signal
                        SyncLoadHPLCDone = {'exSync' : exSync},
                        FlowRate = 0,
                        InjectionValve=1,
                        OutletValve=1
                        )
WaitForHPLC = Phase(name = 'WaitForHPLC', #TRUE wait for HPLC to set to
    FALSE
                        SyncHPLC = {'exSync' : exSync},
                        FlowRate = 0.0)

start1 = Phase(name='start1',
        Time=10,
        SetInlet = {'A':1, 'B':1, '%B':0},
                BufferA1=2,
        FlowRate=1,
                OutletValve=8)

pumpwash1 = Phase(name='pumpwash',
```

```
                    Time=2.2*60,
                    BufferA1=2,
                    FlowRate=5,
                    InjectionValve=1,
                    ColumnValve=1,
                    OutletValve=1)

inject = Phase(name='inject',
                       Time=60*1.6,
                       BufferA1=2,
                       FlowRate=1.,
                       InjectionValve=0,
                       ColumnValve=1,
                       OutletValve=8)

stop = Phase(name='stop',
                Time=10,
                BufferA1=1,
                FlowRate=0)

##### Phases for hi-trap protein A column #####
F_flow= 1.0
F_pumpwash=5.0
F_load=0.6

tload = 10*60
twash1= 7*60
twash2= 5*60
telute = 10*60
tregen=3*60
tcip= 5*60
tequi=7*60
tpumpwash=2.5*60

#20mM phosphate buffer load, wash1, equi A14
# 50 mM NaAc pH 6.0 wash2 A15
# 50 mM NaAc pH 3.5 elution, regeneration A13
#0.1 M NaOH cip B1

start2 = Phase(name='start2',
        Time=10,
               SetInlet = {'A':1, 'B':1, '%B':0},
```

```
77                BufferA1=4,
78                InjectionValve=1,
79          FlowRate=1,
80                OutletValve=2) #to pH meter
81
82  pumpwashload = Phase(name='pumpwashload',
83                     Time=tpumpwash,
84                     SetInlet = {'A':1, 'B':1, '%B':0},
85                     BufferA1=4,
86                     FlowRate=F_pumpwash,
87                     InjectionValve=1,
88                     ColumnValve=1,
89                     OutletValve=1)
90
91  load = Phase(name='load',
92                     Time=tload,
93                     SetInlet = {'A':1, 'B':1, '%B':0},
94                     BufferA1=4,
95                     FlowRate=F_load,
96                     InjectionValve=0,
97                     ColumnValve=4,
98                     OutletValve=2)
99
100 wash1 = Phase(name='wash1',
101                    Time=twash1,
102                    SetInlet = {'A':1, 'B':1, '%B':0},
103                    BufferA1=4,
104                    FlowRate=1,
105                    InjectionValve=1,
106                    ColumnValve=4,
107                    OutletValve=2)
108
109 pumpwashwash2 = Phase(name='pumpwashwash2',
110                    Time=tpumpwash,
111                    SetInlet = {'A':1, 'B':1, '%B':0},
112                    BufferA1=5,
113                    FlowRate=F_pumpwash,
114                    InjectionValve=1,
115                    ColumnValve=1,
116                    OutletValve=1)
117
118 wash2 = Phase(name='wash2',
```

```
119                     Time=twash2,
120                     SetInlet = {'A':1, 'B':1, '%B':0},
121                     BufferA1=5,
122                     FlowRate=1,
123                     InjectionValve=1,
124                     ColumnValve=4,
125                     OutletValve=2)
126
127 pumpwashelute = Phase(name='pumpwashelute',
128                     Time=tpumpwash,
129                     SetInlet = {'A':1, 'B':1, '%B':0},
130                     BufferA1=3, #50 mM Na acetate pH 3.5
131                     FlowRate=F_pumpwash,
132                     InjectionValve=1,
133                     ColumnValve=1,
134                     OutletValve=1)
135
136
137 elute = Phase(name='elute', #only used to test single machine
138                     Time=telute,
139                     SetInlet = {'A':1, 'B':1, '%B':0},
140                     BufferA1=3, #50 mM Na acetate pH 3.5
141                     FlowRate=1,
142                     InjectionValve=1,
143                     ColumnValve=4,
144                     OutletValve=2)
145
146 elutetoHPLC = Phase(name='elutetoHPLC',
147                     Time=2.7*60,
148                     SetInlet = {'A':1, 'B':1, '%B':0},
149                     BufferA1=3, #50 mM Na acetate pH 3.5
150                     FlowRate=1,
151                     InjectionValve=1,
152                     ColumnValve=4,
153                     OutletValve=8)
154
155 elutecont = Phase(name='elutecont',
156                     Time=5*60,
157                     SetInlet = {'A':1, 'B':1, '%B':0},
158                     BufferA1=3, #50 mM Na acetate pH 3.5
159                     FlowRate=1,
160                     InjectionValve=1,
```

```
                    ColumnValve=4,
                    OutletValve=2)

pumpwashcip = Phase(name='pumpwashcip',
                Time=30,
                SetInlet= {'A':1, 'B':1, '%B':100},
                FlowRate=F_pumpwash,
                InjectionValve=1,
                ColumnValve=1,
                OutletValve=1)

cip = Phase(name='cip',
                Time=tcip,
                SetInlet= {'A':1, 'B':1, '%B':100},
                FlowRate=1,
                InjectionValve=1,
                ColumnValve=4,
                OutletValve=2)

pumpwashequi = Phase(name='pumpwashequi',
                 Time=tpumpwash,
                 SetInlet = {'A':1, 'B':1, '%B':0},
                 BufferA1=4,
                 FlowRate=F_pumpwash,
                 InjectionValve=1,
                 ColumnValve=1,
                 OutletValve=1
                 )

equi = Phase(name='equi',
                 Time=tequi,
                 AutoZero = True,
                 SetInlet = {'A':1, 'B':1, '%B':0},
                 BufferA1=4,
                 FlowRate=1,
                 InjectionValve=1,
                 ColumnValve=4,
                 OutletValve=2
                 )

regen = Phase(name='regen',
                  Time=tregen,
```

```
                SetInlet = {'A':1, 'B':1, '%B':0},
                BufferA1=3,
                FlowRate=F_flow,
                InjectionValve=1,
                ColumnValve=4,
                OutletValve=1
                )

water = Phase(name='water',
                Time=2.2*60,
                SetInlet = {'A':1, 'B':1, '%B':0},
                BufferA1=1,
                FlowRate=5,
                InjectionValve=1,
                ColumnValve=1,
                OutletValve=1
                )



#cip1= [settings, pumpwashcip, cip, pumpwashequi,equi]
case1 = [WaitForHPLC,start1,inject,LoadHPLCDone, stop]
case2 = [WaitForHPLC,settings, equi, load,pumpwashelute, elutetoHPLC,
    LoadHPLCDone, elutecont, pumpwashcip,cip, pumpwashequi,equi,
    LoadHPLCDone, WaitForHPLC]


phases = case1

P = Process(name='mab_toSEC',
              phases=phases,
              S=S)

options = {'mode':runMode,
           'timeFactor':1.,
           'sampleSignals':['uv1'],
           'sampleTime':2.0,
           'loops':[(None, P.e_peakIntegration, None)],
           'logData':True,
           'logRun':True,
       'exSync':exSync
           }
```

```
243
244
245 P.run(options)
```

C HPLC script

```
# -*- coding: utf-8 -*-
"""
Created on Tue Apr 13 11:34:41 2021

@author: lab
"""
from projects.explorerhplc.system_hplc import *
from projects.explorerhplc.system_explorer import*
from projects.explorerhplc.process_hplc_analysis import Phase, Process,
    Sync, ExternalSync
from orbit.process import NoSystem

collength = 300 #mm
coldia = 7.8 #mm
Vcol=14.335 #ml
"""
Inject - position 1: Load, position 2: inject
ColumnValve - position 1: bypass, position 2: empty, position 3:
   SEC3000-column, position 4: empty
Buffers: A1: dH2O,
B1: Na2PO4 pH 3 for SEC CIP, B2-2:dH2O, B2-4: Phosphate buffer,

"""
def method(S,HPLC):

    runMode = 'real'

    if runMode=='test':
        extIP = 'tcp://localhost:51001'
    else:
        extIP = 'tcp://130.235.4.141:51001'

    exSync = ExternalSync(address=extIP, identity=1)


    start = Phase(name='start',
                  Time=10,
```

```
                    SetColumnValve=1,
                    SetBuffer={'A1','B2-4'},
                    PercentB=100.,
                    Flowrate=0.8)


waitForAKTA = Phase(name='waitForAKTA',
                    SyncAKTA={'exSync':exSync}) #TRUE, wait for False

HPLCReady = Phase(name='HPLCReady',
                  SyncHPLCReady={'exSync' : exSync},
                  Inject=1)                      #set to false

equil = Phase(name='equil',
                  Time=Vcol*2/0.8*60,
                  SetColumnValve=3,
                  Inject=1,
                  SetBuffer={'A1', 'B2-4'},
                  PercentB=100,
                  Flowrate=0.8)

inject = Phase(name='inject',
                    Time=60*20,
                    SetColumnValve=3,
                    Inject=2,
                    SetBuffer={'A1','B2-4'},
                    PercentB=100.,
                    Flowrate=0.8)

stop = Phase(name='stop',
                    Time=10,
                    SetColumnValve=1,
                    Inject=1,
                    SetBuffer={'A1','B2-2'},
                    PercentB=50,
                    Flowrate=0)


water = Phase(name='runwater',                #wash 2 CV with water
                  Time = Vcol*2/0.5*60,
                  SetColumnValve=1,
                  Inject=1,
```

```
78                      SetBuffer={'A1','B2-2'},
79                      PercentB=50,
80                      Flowrate=0.5)
81
82
83
84      sampleanalysis = [HPLCReady, waitForAKTA, start, inject, stop,
    HPLCReady, waitForAKTA]
85
86      phases = sampleanalysis
87
88      P = Process(name='HPLC_mab_SEC',
89                  phases=phases,
90                  S=S,
91                  HPLC = HPLC)
92
93 #hplc1:flowrate, hplc7:280nm, hplc8:254nm, hplc9:210nm, hplc10:230nm,
    hplc18:RI, hplc27:entire DAD spectra
94      options = {'mode':runMode,
95                 'timeFactor':1.,
96                 'sampleSignals':['hplc1','hplc7','hplc8','hplc9','hplc10
    ','hplc27','hplc18'],
97                 'sampleTime':1.0,
98                 'loops':[],
99                 'logData':True,
100                'logRun':True,
101           'exSync':exSync
102                }
103
104     # Set initial settings (optional)
105     P.I0 = []
106     P.run(options)
107
108
109 if __name__ == '__main__':
110
111     S = NoSystem(mode='test', connection='opc')
112     HPLC = SystemHPLC(mode='write',address='130.235.4.151')
113     instr = method(S, HPLC)
```