"Purification of Proteins using Ion Exchange Chromatography"

(Developed by Professor Cramer, Steven Evans and Alex Freed)

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Goal: The objectives of this lab are: 1) experimentally determine the adsorption isotherm parameters for the three proteins used in this lab; 2) perform linear gradient column simulations outside of the lab to establish conditions that are amenable to separating the proteins of interest using the determined isotherm parameters and satisfying specified constraints; and 3) validate these simulation results using a linear gradient experiment.

Introduction: In this senior laboratory you will be performing experiments and simulations of the chromatographic separations of proteins, similar to those performed in the biotechnology industry. Chromatography is a separations technique based on differences in the adsorptive or partitioning properties of mobile phase feed components with a porous stationary phase chromatographic material. The migration rate of the solutes is determined by the equilibrium distribution of components between the flowing fluid and the stationary porous medium.

In this lab, you will be using a mode of chromatography called ion exchange chromatography which separates proteins based upon their electrostatic interactions with a charged chromatographic material (Cramer and Natarajan, 1999). In the first part of the lab, isocratic (constant salt concentration) experiments will be performed on a BioRad chromatographic workstation to determine the adsorption isotherm parameters for the proteins. After experimentally determining these parameters, you will employ the parameters (outside of the lab) in a chromatographic simulation software to determine an optimal linear gradient separation method. In the final part of the lab, the students will return to the lab to experimentally validate these simulated separation conditions, again using the BioRad chromatographic workstation.

The protein system for these separations will consist of three commercially available proteins (α-chymotrypsinogen A, ribonuclease A, and lysozyme). The column to be used for the separation contains sulfopropyl sepharaosefast flow (SP Sepharose FF, GE Healthcare).

For the simulations, the Steric Mass Action (SMA) isotherm for protein ion exchange chromatography will be employed. This isotherm was developed by Brooks and Cramer (1992) and enables the prediction of multicomponent protein equilibrium at various salt concentrations. This isotherm has been shown to accurately describe the behavior of proteins in ion exchange systems under isocratic elution (Gallant et al 1995), linear gradient (Gallant et al 1996), step gradient (Gallant et al 1995) and displacement (Natarajan et al 2000) chromatographic modes of operation. The single component SMA isotherm is given by:

$$C_i = \left[\frac{Q_i}{K_{SMA}}\right] * \left[\frac{C_{salt}}{\Lambda - (\sigma_i + \nu_i) Q_i}\right]^{\nu_i}$$

Where $C_i$is the concentration of species in the mobile phase, Q_i is the concentration on the stationary phase, K_{SMA} is the SMA equilibrium constant of the i^{th} component, C_{salt} is the concentration of

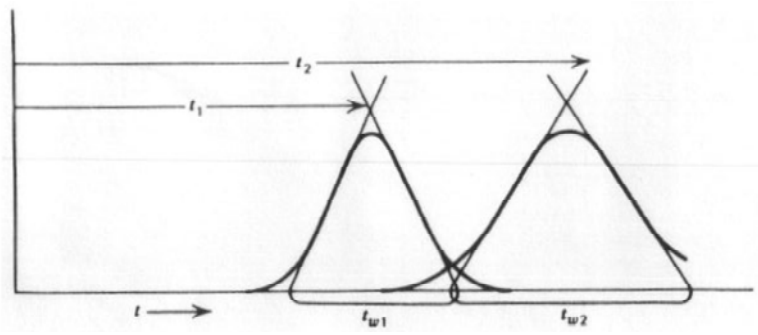
the counter ion, Λ is the total bed ionic capacity of the stationary phase, σ_i is the steric factor, and υ_i is the characteristic charge.

There are three isotherm parameters for each protein: K_{SMA}, υ_i, and σ_i. The K_{SMA} and υ_i parameters are considered "linear" isotherm parameters since they can be determined directly from experiments carried out in the linear region of the isotherm (i.e. at low mobile phase concentrations). These are the two parameters that will be experimentally determined for each protein in this laboratory. The steric factor σ_i is the nonlinear isotherm parameter and describes the behavior at high solute concentrations. The following nonlinear SMA parameters (which due to time constraints and material costs will not be measured experimentally) can be assumed for this laboratory: σ(α-chymotrypsinogen A) = 31.7, σ(ribonuclease A) = 17.2, and σ(lysozyme) = 17.0.

The liquid chromatography column packed with the SP Sepharose FF stationary phase has a length of10 cm and a column inner diameter of 0.46 cm. Further, the resins can be assumed to have the following properties: interstitial porosity (ε_e) of 0.40, intraparticular porosity (ε_p) of 0.70, bed capacity (Λ) of 525mM.

A useful concept that you will need to use in this lab is the resolution of two peaks. The equation for the resolution between two chromatographic peaks (R_s) with retention times of t_1 and t_2, respectively is given below where t_{w1} and t_{w2} represent the widths at the bases of the two peaks (note: these widths can be determined either directly from the baseline or by drawing lines through the inflection points as indicated in the figure). The figure at the right below gives a graphical representation of what the terms in the equation relate to.

$$R_s = \frac{(t_2 - t_1)}{(\tfrac{1}{2})(t_{w1} + t_{w2})}$$



The total porosity (ε_T) of a chromatographic column can be calculated using the equation below where the term (ε_p) represents the intraparticular porosity and (ε_e) represents the interstitial porosity of the column.

$$\varepsilon_T = \varepsilon_e + (\varepsilon_p)(1 - \varepsilon_e)$$

The chromatographic efficiency of the column can be calculated using any of the peaks from the chromatogram. The efficiency of the column is related to the number of theoretical plates in the column, N, which is equivalent to $16(t_r / t_w)^2$ where t_r equals the retention time of the peak and t_w is equivalent to the width of the base of this peak, in units of time. Alternatively, N can be obtained as $5.54\ (t_r / t_H)^2$ where t_H is the width of the peak at half of the maximum peak height.

Part I: Experimental Isotherm Determination

The TA's will have already prepared two ionic buffer solutions and primed the system with these solutions. As these are ion exchange separations being performed, one solution should contain a low amount of salt (buffer "A") and one should contain a larger amount of salt (buffer "B"). Buffer A should be a 50 mM sodium acetate solution at pH 5connected to inlet line "Inlet A1". Buffer B should be the 50 mM sodium acetate solution containing 1 M NaCl connected to inlet line "Inlet B1". Inlet lines A2 and B2 are connected to a DI water reservoir. The column should already be appropriately connected to the BioRad chromatographic workstation. A dilute solution containing the three proteins of interest (α-chymotrypsinogen A, ribonuclease A, and lysozyme) should be available as well. This solution should have been appropriately filtered to protect the column from fouling. The elution order for the three proteins in this system should be the following, from least retained to most retained: α-chymotrypsinogen A, ribonuclease A, and lysozyme. You will need to perform three isocratic (constant salt) retention measurements to obtain some information about the isotherm parameters of each of these three proteins. One injection of a solution containing all three of these proteins yields retention time information about all three of these proteins since we will assume that the protein-protein interactions are negligible under such dilute conditions.

Also, it is advisable to begin these isocratic measurements at higher salt concentrations in the running buffer and sequentially decrease the salt concentration in subsequent experiments. This is due to the fact that if a salt concentration is chosen that is too low, the retention of the three proteins will be too long, resulting in wasted time. On the other hand, if the initial salt concentration is too large, the proteins will move too rapidly through the column and may elute in the "flow through" of the column with negligible retention. If this occurs one can readily decrease the salt concentration in the next isocratic chromatographic run. Once a satisfactory run is carried out at relatively high salt concentrations, reduce the salt concentration and repeat the run. (note: a decrease of roughly 20% salt concentration relative to the first run may be a good starting point for this second run). Finally, once the second run is completed, carry out the third run at a lower salt concentration. (If there is time, you can do additional runs, but once the elution time for the lysozyme exceeds 60 minutes do not do any additional experiments). A suggested range of salt concentrations is roughly from 40-20% B.

As described above, the chromatographic efficiency of the column can be calculated using any of the peaks from the chromatogram. Determine the efficiency of the column based on the lysozyme peaks for chromatographic run. (note: Should this efficiency drop significantly over the course of the experiment notify the TA).Also calculate the minimum resolution between the two most similarly retained peaks for each run.

After performing three isocratic runs with the feed mixture containing all three of the proteins one can calculate the linear SMA parameters by constructing a log (k') versus log (salt) plot. The k' value is the dimensionless retention factor defined as:

$$k' = (t_r - t_o)/t_o$$

wheret_r is the retention time of the solute, $t_o$is the retention time for an unretained solute (corresponding to the dead time of the column). The retention time of the unretained solute can be determined by carrying out one experiment at high salt concentrations where all three proteins co-elute at this time (a value of 0.5 M salt can be used for this).The salt value is the concentration of the counter ion (Na) in the running buffer. In addition to generating this plot, also calculate what the retention time for an unretained solute should be based on the porosities, column volume and volumetric flow rates. Compare this to the value obtained experimentally and discuss.

This log-log plot should have a negative slope, and the absolute value of this slope is equivalent to the characteristic charge of the protein (ν), one of the linear SMA isotherm parameters. The y-intercept from the log (k') versus log (salt) plot is equivalent to $\log(B*K_{SMA}*\Lambda^{\upsilon})$,where the characteristic charge is calculated from the slope, and B is equivalent to the phase ratio of the stationary phase. The phase ratio can be calculated from the total porosity of the resin and is equivalent to

$$B = (1-\varepsilon_T)/\varepsilon_T$$

The total porosity can be calculated from the intraparticular and interstitial porosities using the equation given previously.

Once the linear SMA isotherm parameters are determined for the three proteins, use these values in concert with the steric value given above to construct the isotherms for these three proteins at several different salt conditions. Discuss the resulting isotherms.

Part II: Chromatographic Simulations(Performed between the 1st and 2nd Laboratory Sessions)

The chromatographic simulator (developed by Alex Freed) will be used to generate model separation results using the isotherm parameters calculated from the experimental separations performed in the laboratory and the column properties given in this manual. This simulator numerically solves the coupled partial differential equations which described the lumped mass transport chromatographic model (Natarajan and Cramer, 2000).

This simulator will be in the form of a folder which contains three files: one file which is an executable file entitled "Chrom_student.exe", one text file entitled "input.txt" and one Microsoft Excel files entitled "Result.xls". First input your parameters into the input file placing them exactly where the numbers appear in the original. Clicking on the "Chrom_student" file will run the simulator which will then automatically dump the results into the result.xls file (note: the result file cannot be written to properly if it is opened while the calculations are being performed).

Opening the Input file allows you to alter the input parameters for the simulator. This file must be saved before the Chrom_student.exe file is run if any new changes are to be implemented by the simulator. The following is a list of the first parameters at the top of this file and indicates the purpose of the parameters: Components (the number of components in the system to be separated), G_start (the starting concentration of salt for the gradient in units of mM), G_end (the final concentration of salt for the gradient in units of mM), and t_grad (the amount of time in minutes over which the gradient is to be delivered, this parameter is related to the steepness of the gradient slope that is being run). Lamda is the total ion capacity of the column which is given above and porosity is the total porosity of the column.

Below these parameters are the list of components and their three SMA isotherm parameters. The equilibrium constant and characteristic charge were measured experimentally in the lab and the steric factor was given above, enter these values into the file (make sure that there is a tab between the entered values, it does not need to line up with the headings). K is the K_{SMA} and nu is the characteristic charge obtained from your analysis, and sig is the sigma isotherm parameter given above.

Opening the Result file after a simulation has been performed will yield a matrix of output elution data, you can then simply plot the data using excel and the chromatogram will be obtained for the given parameters. If you want to save the data use another name since each subsequent simulation will dump the results into the results.exe file. Using this simulation software, find the fastest separation that will give the best resolution for the most similarly retained proteins. Adjustable parameters for your

simulations include the initial salt concentration (G start) for a linear gradient separation, the final salt concentration (G end) for a linear gradient separation, and the slope of this gradient. Once you have established the desired conditions for the linear gradient separation, print out the resulting chromatogram as well as the input parameters used to generate this simulated chromatogram. Feel free to print out other results as well and to use the code in any "creative way" that you choose.

Part III: 2nd Laboratory Session for Validation of the Model Predictions

Perform the linear gradient separation, initially using the condition established in your simulation study and determine if these simulated conditions were indeed sufficient to experimentally resolve these three proteins of interest. Then carry out one additional condition, if necessary, to further optimize the separation (note: Data can be exported from the BioRad system by using the "File" and "Export Data" menu functions. At this point the user is requested to indicate which data is to be exported. The data is exported in a "*.txt" format which any number of data processors, such as Microsoft Excel, should be able to handle.)

Description of Equipment Procedures:

Turn on the PC and monitor.

Turn on the BioRadBioLogicMaximizer using the power switch in the front bottom left of the instrument.

Turn on the BioRadBioLogicDuoFlow using the power switch in the front of bottom left of the instrument directly above the Maximizer power switch, it is essential for the computer communication that both of these power buttons be switched to the on position.

Turn on the BioRadBioLogicQuadTec UV-Vis detector using the power switch on the back of the instrument being careful to not knock over any bottles or get tangled on the tubing.

Click on the green BioLogicDuoFlow icon on the desktop.

At this point you should see the manual control screen with three windows at the top for manually controlling the Pump, the Fraction Collector, and the QuadTec Detector. At the bottom of this screen are the real time values for the flow rate, gradient percentage, pressure, UV, pH, conductivity, etc in both numerical and graphical forms.

The flow rate can be started by typing in a flow rate value and hitting the start button. Any subsequent changes one needs to hit the set button for the changes to take effect. While the instrument is running, there is a stick figure icon of a person "running."

Make sure that the waste reciprocal is emptied and that the fluid flow is appropriately going to the waste stream (note: verify that the actual volumetric flow rate is the same as indicated on the instrument by collecting the fluid in a graduated cylinder for a specified time).

From the manual screen there are a number of icons at the top of this page.

Click on the "Browser" icon and then under "Users, Steve, Test", double click on "0%B-Isocrat".This has now opened up the Method entitled "0%B-Isocrat". At the top of the screen the user can still click on Manual to get back to manual control of the instrument or click on the browser to open another method.

A range of isocratic methods have been pre-programmed. Each method (for different salt concentrations) consists of a "Zero Baseline" block for the detector, an "Isocratic Flow" block to deliver 1 mL/min, a "Load/Inject Sample" block using the "Static Loop" option to deliver a volume of 0.1

mL,another “Isocratic Flow” block for 100 mL at a flow rate of 1 mL/min, and an “End of Protocol block.”

Once the method is selected, click on the “Run” icon at the top of this method development window which will open the “Run” window. A window will pop up asking you to input the “Run Name” under which the data collected will be stored. The method has not, however, begun and this is important as we need to do two things before we run the method: 1) turn on the detector lamp using the software (the detector itself was turned on earlier but not the lamp), and 2) fill the sample loop with our sample of interest.

Click on the “Manual” button at the top of the screen and turn on the QuadTec detector by clicking on the “On” button, the little light bulb should light up indicating that the detector lamp is on. Real time values for the absorbances at various detector wavelengths should also be seen at the bottom of this manual screen. For this experiment we will only use a wavelength of 280 nm.

Next we fill the sample loop with our sample. Wash the syringe and the sample loop with DI water. It is important when loading the 50 µL sample loop that a sufficient volume of fluid is flushed through the sample loop to assure complete filling of the loop. There should be no bubbles in the syringe or in the sample loop as bubbles in either of these locations can potentially end up in the column where the resin will form channels or become fouled. It is also crucial that the great care is taken when loading the sample into the sample loop while using the needle. The needle should not be bent or forced into the injection chamber. The injection port should be located in the “L” position at this point as well, indicated beneath the images of the injection port and just above the needle entry location.

After all of the peaks have eluted from the column the user can hit abort or simply let the method run to completion. Once the run is complete you can click on the “Browser” icon at the top of the screen, scroll down through “Users”, “Steve”, “Test”, and find the injection you performed under an existing method or a method that you created. Under this method should be the file name for the injection that you just performed. If you happen to do this while another injection is running a separate “BioLogicDuoFlow Offline” analysis window will appear which performs the same functionalities and has the same screen lay out. The same file cannot be opened in both the “Online” and “Offline” screens simultaneously.

Right click on this file and click on “Add Run to Compare Trace”. Within the “Test” folder there will appear a “Compare1” file. Clicking on the “Compare1” file then allows you to open the data from the chromatography run for inspection. At the bottom of the screen, while you will still have “Compare1” highlighted, you can then click on “Open Run”.

From here you can export your data by clicking on “File” and then “Export Data”, your data will be exported to the location of your choosing in the form of a text document which can be manipulated in a data processing program such as Microsoft Excel. The labels of the y-axis are changed by using the drop down menus next to each axis and the scale of the axis is adjustable by using the scroll bar.

You will then have to choose one of the pre-programmed methods to run for the three isocratic experiments measuring the isotherm parameters. Before this method can be run you must manually start the flow rate and choose the desired %B that is given in this method. The column must equilibrate at this salt concentration, indicated by a steady conductivity value, prior to hitting the start button. Also, remember to re-load the sample loop with the protein mixture this time prior to hitting the start button.

For the linear gradient experiment, Click on the “Browser” icon and then under “Users, Steve, Test”, double click on “LinearGradientTemplate”.This has now opened up the Method entitled “LinearGradientTemplate”. Double clicking on any of the items in this method allows the user to view the parameters associated with that particular block of the method. In order to alter the method, you

must click on the “Edit Method” icon at the top of the screen which will prompt you to enter a name for the new method to which the existing method will be copied to. This ensures that the original method for which data that was previously collected remains retained in its original form while allowing the user to alter any key parameters for the collection of new data.

In this new method edit any of the parameters as needed such as the initial %B, final %B and gradient time. The initial %B should be changed in blocks 2 through 4 (“Isocratic Flow, “Load/Inject Sample”, and “Linear Gradient”). The final %B should be changed in blocks 4 and 5 (“Linear Gradient” and “Isocratic Flow”). The gradient time should be changed in block 4 in the volume of mobile phase over which the gradient is delivered.At this point, when you are satisfied with the new gradient method that you obtained from your column simulations, you can click on the “Run” and continue as described above for the isocratic separation.

BioRad Instrument Maintenance

Turn off the UV lamp when the experiment is finished

The small plastic syringe can be used to flush the back side of the pump heads with water to clear out any waste build up

Literature Cited

Brooks, C. and Cramer, S. M., Steric mass action ion exchange displacement profiles and Induced salt gradients, AIChE Journal 38 (12), 1969-1978, 1992.

Gallant, S.; Kundu, A. and Cramer, S. M., Modeling nonlinear elution of proteins in ion-exchange chromatography, Journal of Chromatography, 702, 125-142, 1995.

Gallant, S.; Kundu, A. and Cramer, S. M., Optimization of step gradient separations - consideration of nonlinear adsorption, Biotechnology and Bioengineering, 47, 355-372, 1995.

Gallant, S.; Vunnum, S. and Cramer, S. M., Optimization of preparative ion-exchange chromatography of proteins-linear gradient separations, Journal of Chromatography, 725, 295-314, 1996.

Cramer, S. M. and Natarajan, V., Chromatography, ion exchange, in the Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation, 612-627, 1999.

Natarajan, V. and Cramer, S. M., A methodology for the characterization of ion-exchange resins, Separ. Sci. Technol., 35 (11) 1719-1742, 2000.

Natarajan, V.; Bequette, B.W. and Cramer, S. M., Optimization of ion exchange displacement separations.I. Validation of an iterative scheme and its use as a methods development tool”, J Chromatogr. A, 876 (1-2) 51-62 April 21, 2000

Expectations

Interim Discussion. Please come prepared to discuss your isocratic chromatographic data as well as the linear gradient simulations results using the chromatographic software. Please bring your experimental chromatograms, efficiency calculations, resolution calculations, retention results for an unretained solute, log (k') plot, isotherms at each salt concentration, and simulated chromatogram. Be prepared to discuss the key concepts related to this laboratory. At our Interim Discussion session, we will discuss report details further.

Chromatography Lab Report Requirements

Determine the following

Column Efficiency, Retention for an retained solute, SMA parameters for the three proteins, Minimal Resolutions for each isocratic chromatogram, Isocratic chromatograms at three different salt concentrations (at least three is required), Adsorption isotherms at each salt concentration, and simulated linear gradient chromatogram at your optimal condition (note: also show several simulations to illustrate the trends in the linear gradient simulations as the initial salt, final salt or gradient slopes are changed).

II. Report Format
A. Abstract: what was studied and what results were obtained
B. Table of Contents
C. Introduction: present a brief overview of ion exchange chromatography, include a theoretical section which presents the basic equations which will be employed in this report.
D. Apparatus and Procedure: include an apparatus section which presents a schematic diagram for the experimental apparatus using standard piping and equipment symbols, also include a detailed procedures section which describes the various procedures employed in the experiment.
E. Results and Discussion: present all experimental results as well as a detailed discussion of the results.
F. Conclusions: summarize the salient results, include recommendations for improving the experiment.
G. References
H. Figures

III. Covering Memo: who did what (Group Leader's responsibility)