Chromatography

from Greek χρῶμα chroma "color" and γράφειν graphein "to write".


General chromatography:
- textbook experiment 2, beginning on page 25

Affinity chromatography:
- experiment #10, pages 157-162.

spinach leaves pigments
- Mobile phase = nail polish remover (acetone, ethyl acetate, butyl acetate), stationary phase = coffee filter paper.
- In 9:1 pet ether: acetone:
  - carotene $R_f = 0.98$
  - xanthophyll $R_f = 0.90$
  - chlorophyll a $R_f = 0.83$
  - chlorophyll b $R_f = 0.71$


Background

- Chromatography is a technique that allows for the separation of molecules based on their differential migration through a porous medium.
- Molecules partition between the stationary phase and the mobile phase, and this 'relative mobility' determines the relative rate of migration.
  - $R_f = (\text{dist. traveled by sample})/(\text{dist. to solv. front})$
  - The closer $R_f$ is to 1, the faster the migration
  - The closer $R_f$ is to 0, the slower the migration
  - $R_f = 1 - \alpha$. $\alpha$ is the partition coefficient

$$\alpha = \frac{[\text{ in stationary phase}]}{[\text{ in stationary and mobile phase}]}$$

- In column chromatography, the stationary phase is a resin.
- The mobile phase is a buffer solution that flows over the resin carrying analyte with it. (More generally, the mobile phase can be mixtures of solvents other than water. Even biomolecules may be separated in water:acetone and water:acetonitrile mixtures.
- The analyte is characterized by the relative extents to which it interacts with the mobile vs. the stationary phases.
many types of chromatography

- exploit different interactions.
- For purposes of separation, exploit a feature that distinguishes different compounds present in a mixture, such as size, charge, polarity ...
- For the example of plant pigments the solid phase is cellulose (HCOH)$_n$ and the mobile phase is 10% H$_3$COCH$_3$ and 90% light hydrocarbons (pentane-heptane).

\[ R_f = 0.98 \]
\[ R_f = 0.90 \]
\[ R_f = 0.83 \]
\[ R_f = 0.71 \]

Chromatography video.

http://www.youtube.com/watch?v=9GiLjH9Oym8
Chromatography of many kinds

- See tables 2-1, 2-2 in text book
- Gel filtration: retardation of large molecules
- Ion-exchange: electrostatic retention of molecules with a charge opposite to that of the resin.
- Hydrophobic interaction: non-polar surface groups cause molecule to be retained on column (also called reverse phase).
- Affinity: exploits a specific affinity of the molecule for a ligand that can be built into the column or attached to it. (Also requires a release mechanism).

Different resins: Size-Exclusion

- Relies on the ability of a given sized molecule to enter the uniformly sized pores of a solid matrix. Molecules that are too big are excluded from the mobile phase within the pores, and exit the matrix faster. Smaller molecules have to go through a larger volume of mobile phase, and so exit later.
- The volume accessible to the molecule dictates its rate of migration.
Ion-Exchange

- Relies on the differential electrostatic affinities of molecules carrying a surface charge for an inert, charged stationary phase.
  - Cation exchange has negative stationary phase
    - Molecules with positive charge will interact with resin.
  - Anion exchange has a positive stationary phase
    - Molecules with negative charge will interact with resin.

- Molecules are eluted by either changing the pH or the ionic strength
  - pH: the molecule is adsorbed to the column at a pH that gives it a charge opposite that of the resin, and is eluted by a change in pH that causes it to lose its charge or affinity for the resin.
  - Ionic strength: as the concentration of counter-ions in the mobile phase increases, they electrostatically compete with adsorbed molecules bound to the charged resin. Molecules with lower net charge elute with lower salt concentrations.

- Carboxymethyl (CM) cellulose is a cation exchanger
Ion-exchange chromatography

- Carboxymethyl (CM) cellulose is a cation exchanger
- Diethylaminoethyl (DEAE) cellulose is an anion exchanger
- Elute by increasing ionic strength or by changing pH (chromatofocussing).

Blue dextran MW = 2,000,000  neutral
Cytochrome C MW = 12,000  pI = 10.7
DNP-glycine MW = 241     pI = 3

In ion-exchange mode which should elute first?
In gel filtration mode which should elute first?

Resolution: separation vs. spreading

- \[ R = \frac{2d}{W_1 + W_2} \]

Resolution on CM sephadex

- High-\(\alpha\) analytes tend to have more zone spreading (continuously released from the stationary phase into mobile phase passing by).
- A long column is not the solution.
- Employ gradient elution instead of (unchanging) isocratic resolution.
Affinity Chromatography

Use a resin that mimics a ligand of the protein. Eg. blue columns in which the resin is decorated with molecules of cibacron blue.

This is mis-recognized as a substrate analog by enzymes that bind NADH and purine nucleotides.

Such enzymes bind to the resin more strongly than random proteins do. The latter can be washed away and then the proteins binding the resin can be eluted with authentic substrates, cofactors or analogs (eg. SAM, ATP, GTP, NAD⁺, NADH, FMN ...)

Ni²⁺-affinity chromatography

- General protocol involves three steps
  - Bind interesting protein to chromatography resin.
  - Wash away all uninteresting proteins.
  - Elute interesting protein from chromatography resin (also called matrix).
- We will use a ‘batch’ method rather than a chromatographic application.
- Lower resolution but much faster.
- Depends on ability to find conditions where the target protein binds completely and everything else does not. Modern molecular biology makes this easy ...

Figure 2-7 Structure of Cibacron Blue F3GA.
Ni²⁺ column IMAC

Only proteins that can tightly bind to Ni²⁺ will be preferentially retained on the column. Such proteins are rare in nature, so a protein engineered to have this capability is generally unique, and the only protein to be retained on such a column.
Electrophoresis, 12% acrylamide, stained with Coomassie brilliant blue

The experiment

1. Use Ni column to purify flavoenzyme.
2. Use SDS-PAGE to visualize the proteins present in different fractions of the column purification.

Fractions to save and characterize by SDS PAGE:
- Cells prior to induction with IPTG*
- Crude extract (lysed cells minus debris)
- Column flow-through
- Column wash (second one)
- Eluate

* provided by T.A.
1. Running the Ni column (from the QIagen manual)

1. **Position the required number of Ni-NTA Superflow Columns (1.5 ml) on the QIArrack.**
   Note: First break the seals at the outlet of the columns before opening the screw cap!
   Before use, Ni-NTA Superflow Columns should have been stored in an upright position.
   Check that the resin is contained in the narrow part of the column body before opening the columns.
   If the resin is attached to the sides or to the cap of the column, resuspend the resin
   by inverting the column and allow resin to settle before proceeding with step 2.

2. **Remove the storage buffer from above the resin either by using a pipet or by allowing it to drain through by gravity flow.**
   The columns will not run dry by gravity flow.

3. **Equilibrate the columns by pipetting 10 ml Buffer NPI-10 into each column, and allow buffer to drain through completely by gravity flow.**

4. **Transfer the cleared lysates into the equilibrated columns and allow the columns to drain by gravity flow.**

5. **Perform the first wash step by pipetting 10 ml Buffer NPI-20 into each column. Allow the buffer to drain through completely by gravity flow.**

6. **Perform a second wash step by repeating step 5.**
   Very rarely, imidazole concentrations of 20 mM can interfere with binding of 6His-tagged proteins to the resin. If binding is inefficient, reduce the imidazole concentration in the wash buffer (e.g., to 10 mM).

7. **Place an elution vessel under each column outlet.**

8. **To elute the 6His-tagged proteins, add 3 ml Buffer NPI-250 to each column, allow buffer to flow through completely, and collect flow-through in the elution vessels.**
   Approximately 80% of the bound 6His-tagged protein is eluted within the first fraction. If desired, a second elution step can be performed to increase recovery, by repeating step 8.
   The second eluate can be collected into the same or into a second elution vessel.

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### Producing the protein and getting it out of cells (this will be done for you)

- Grow E. coli bearing expression plasmid to $\text{OD}_{600} \approx 0.5$
- Add IPTG to initiate expression
- Grow 3 hr for protein production
- Harvest by centrifugation, discard supernatant.
- Resuspend cell pellet in lysis buffer, centrifuge down cells again.
- Resuspend again in lysis buffer, add lysozyme (1mg/ml), incubate 30 min.
- Sonicate in bursts to minimize heating.
- Centrifuge out debris at 10,000 x g for 20-30 min.

**Lysis buffer (1 liter):** prevents non-specific binding

- 50 mM NaH$_2$PO$_4$
- 6.90 g NaH$_2$PO$_4$$\cdot$H$_2$O (MW 137.99 g/mol)
- 300 mM NaCl
- 17.54 g NaCl (MW 58.44 g/mol)
- 10 mM imidazole
- 0.68 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

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B: Protein purification under native conditions using gravity flow

Reagents and equipment to be supplied by user

- QIArrack (cat. no. 19015)
- Elution vessels (e.g., 4-14 ml polypropylene tubes)
- Buffers NPI-10, NPI-20, and NPI-250

Buffer compositions can be found in the Ni-NTA Superflow BioRobot Handbook, which can be downloaded in convenient PDF format at [www.qiagen.com](http://www.qiagen.com).

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Manual purification of 6xHis-tagged proteins (QB60_10_05 Oct-05) page 3 of 7

uploaded as:

6xHisTaggedProteins–NiNTASuperflowColumns.pdf
2. Samples to collect for SDS PAGE

Draw arrows into your flow chart showing when these fractions are collected and set aside
- Cells prior to induction with IPTG*
- Crude extract (lysed cells minus debris)
- Column flow-through
- Column wash (second one)
- Eluate

* provided by T.A.

Fractions will be treated with SDS-PAGE sample buffer, denatured by incubation at 90 °C, and run on a polyacrylamide† gel along with molecular weight standards.

† polyacrylamide is a neurotoxin.

SDS PAGE (see experiment 4 in text)

- The text describes the principles and apparatus.
- We will use purchased (precast) gels.
- You will simply need to add samples into the wells, add buffers, hook up to power and supervise.
- Gels will be removed from glass plates and stained with our old friend Coomassie brilliant blue.
- All materials will be made for you. Your execution plan simply needs to provide a sketch of your planned loading arrangement (what samples in which lanes).
- We WILL use the mobilities of molecular weight standards to determine the molecular weight of our test protein.
- (see pages 74-77)