Lecture 20 Introduction to chromatography

Aim:

To understand the principles of chromatographic separation

Introduction:

Chromatography is a set of techniques that separate the molecules based on their partitioning between a stationary and a mobile phase. Let us see, using a discontinuous equivalent of the chromatographic process, how differential distribution of molecules between the two phases leads to their separation. Consider a separation experiment being carried out using *n+1* separation funnels, labeled *0, 1, 2, …., n*. All the separating funnels have a constant volume, *V* of a solvent, say chloroform that makes the stationary phase (Figure 20.1). The given sample is dissolved in the stationary phase (chloroform) present in the 0^{th} separating funnel. Let us now add to this funnel the same volume, *V* of another solvent that is immiscible with chloroform (say water); water makes the mobile phase of the system. We shake the mixture well and then allow the separation of aqueous and organic phases. At equilibrium, the chemical potential (μ), activity (α), and concentration (c) of a substance X is given by Nernst's distribution:

$$\mu_X^s = \mu_X^m \qquad \text{---------- (20.1)}$$

$$\Longrightarrow \alpha_X^s \propto \alpha_X^m \qquad \Longrightarrow c_X^s \propto c_X^m \qquad \text{---------- (20.2)}$$

where, subscript X represents the substance while the superscripts *s* and *m* represent the stationary and mobile phases respectively.

The proportionality constant, also known as the partitioning coefficient, is the ratio of the probability of a molecule to be in stationary phase to that in the mobile phase:

$$K_p = \frac{P(s)}{P(m)} \approx \frac{\alpha_X^s}{\alpha_X^m} \approx \frac{c_X^s}{c_X^m} \qquad \text{---------- (20.3)}$$

where, *P(s)* and *P(m)* represent the probabilities of the substance to be in the stationary and mobile phases, respectively. If *p* and *q* represent the fractions of the substance present in the mobile and stationary phases, respectively; K_p can be written as:

$$K_p = \frac{q}{p} \qquad \text{---------- (20.4)}$$

After equilibration, the mobile phase of *separating funnel 0* is transferred to *funnel 1* and fresh solvent is added to *funnel 0*, and the phases are equilibrated again. The mobile phases of *funnels 0 and 1* are simultaneously transferred to *funnels 1 and 2*, respectively. This cycle is repeated to continue with the process with rest of the funnels. This experiment is diagrammatically represented in figure 1 for five separating funnels (0 – 4).

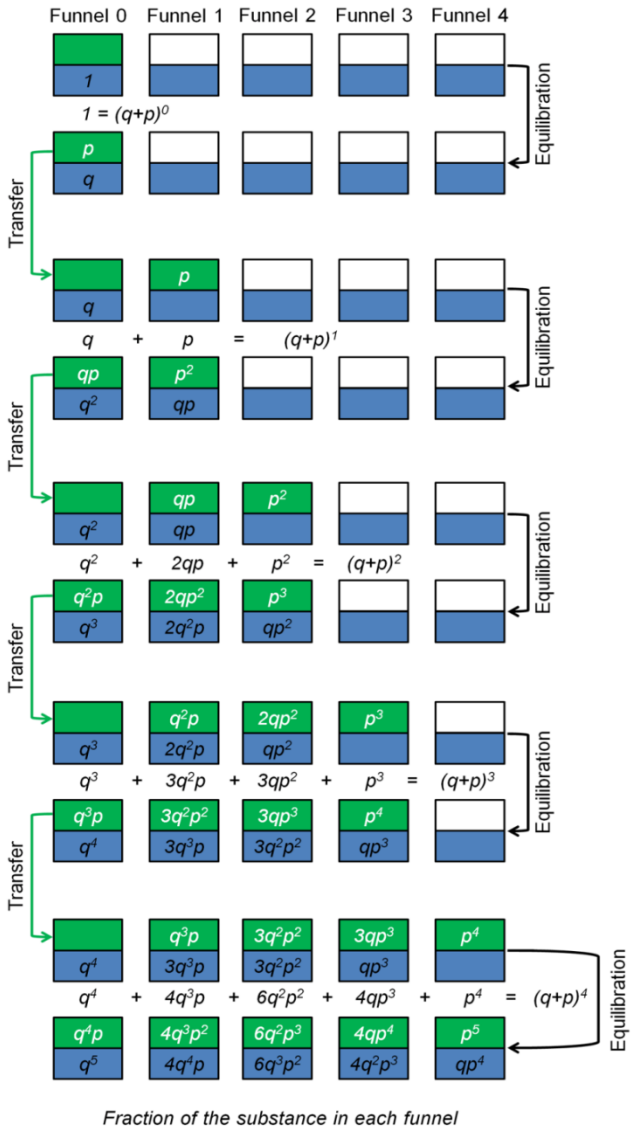

Figure 20.1 Distribution of a substance between stationary and mobile phases after transfers and equilibrations (please refer to text for details)

It is clear from figure 20.1 that the fraction of the substance in the funnels after *n* transfer/equilibration cycles corresponds to the binomial expansion of $(p+q)^n$. Therefore, the fraction of the substance present in funnel *r* after *n* transfers ($r \leq n$) is given by:

$$f_{r,n} = \frac{n!}{r!(n-r)!} p^r q^{n-r} \qquad \text{---------- (20.5)}$$

If c_0 is the original concentration of the substance in the funnel 1 (after addition of mobile phase), the concentration in funnel *r* is simply given by following expression:

$$c_{r,n} = f_{r,n} \times c_0 \qquad \text{---------- (20.6)}$$

$$= \frac{n!}{r!(n-r)!} p^r q^{n-r} c_0 \qquad \text{---------- (20.7)}$$

If we need to know the funnel having the highest concentration of the substance, we simply have to take the average of binomial distribution. The funnel number *x* having the highest concentration is, therefore, given by:

$$x = \sum_{r=0}^{n} r f_{r,n} \qquad \text{---------- (20.8)}$$

$$= \sum_{r=0}^{n} r \times \frac{n!}{r!(n-r)!} p^r q^{n-r} \qquad \text{---------- (20.9)}$$

$$= \sum_{r=0}^{n} r \times \frac{n!}{r!(n-r)!} p^r (1-p)^{n-r} \qquad \text{---------- (20.10)}$$

$$= n \times p \qquad \text{---------- (20.11)}$$

Consider a mixture having three chemical components having partition coefficients, 10, 1, and 0.1:

$$K_p(A) = 10 \qquad K_p(B) = 1 \qquad K_p(C) = 0.1$$

Therefore, $pA = 10$, $pB = 1$, and $pC = 0.1$. Figure 20.2 shows the distribution of these three components after 5, 10, 20, and 40 equilibration/transfer cycles.

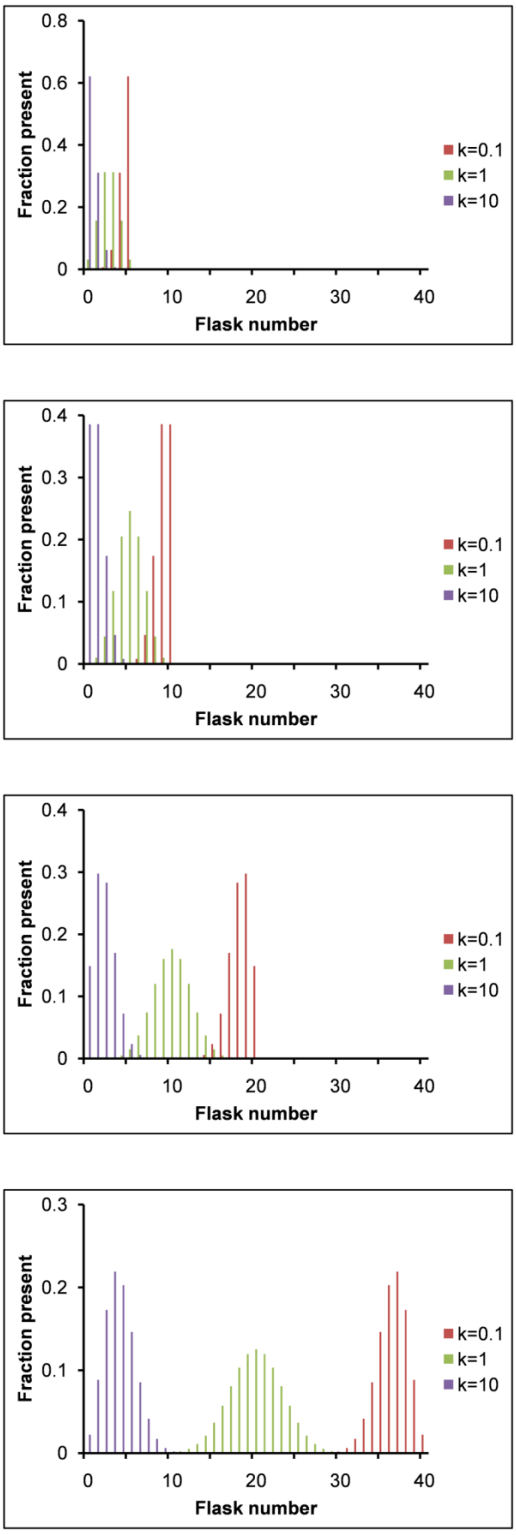

Figure 20.2 Separation of three components having different partition coefficients for the two phases

In chromatography, the stationary and mobile phases are not discrete but continuous. Paper chromatography, where filter paper was used as the stationary phase, was the very first chromatographic method. The paper chromatography technique developed

with time and other stationary phases also emerged. Introduction of silica gel as a stationary phase led to the development of thin layer chromatography and subsequent functionalization of silica resulted in the development of various other chromatographic methods.

Today, most chromatographic methods use a column where stationary phase is a hydrated gel while the solvent/buffer flowing through the column makes the mobile phase. The gel is made up of small, homogeneous beads. Let us have a look at some important terminology we would be using in subsequent lectures:

Column: Column of the gel, not the actual vessel holding the gel

Column volume (V_t): Total volume of the column; for a cylindrical column of radius, *r* and height, *h*, the volume is given by: $V_t = \pi r^2 h$

Void volume (V_0): The volume of the space occupied by the solvent outside the gel beads.

Included volume (V_i): Volume of the solvent present inside gel beads.

The unhydrated gel matrix usually occupies ~1% volume of that of the hydrated one. V_t can therefore be approximated as:

$$V_t = V_0 + V_i \quad \text{(Figure 20.3)}$$

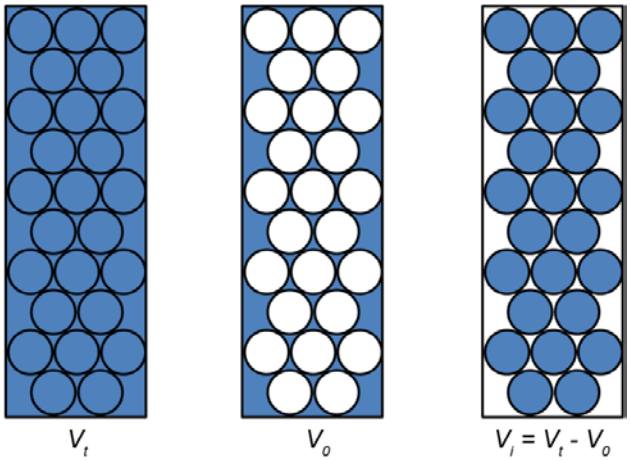

Figure 20.3: Terminology of the column volumes

Lecture 21 Thin Layer Chromatography

Aim:

To separate the mixture of amino acids (serine, phenylalanine, and lysine) by thin layer chromatography

Introduction:

Thin layer chromatography, abbreviated as TLC, is an analytical tool that is frequently used in chemistry laboratories to study the purity of organic compounds or to separate and analyze the components of complex mixtures. TLC is a solid-liquid form of chromatography *i.e.* the stationary phase is a solid while the mobile phase is a liquid. The stationary phase is prepared by coating a very thin layer of a polar adsorbent on a rectangular solid support. The solid support can be a glass, plastic, or a metal plate such as Aluminum. Silica ($SiO_2 \cdot xH_2O$) or alumina ($Al_2O_3 \cdot xH_2O$) are the most commonly used adsorbents (Figure 21.1) and are coated as uniformly thin layers on the solid supports. Analytical TLC plates usually have ~ 250 μm thick adsorbent layers. The plates used for preparatory separation can be up to 5 mm thick. A binding agent such as calcium sulfate or gypsum is usually incorporated to ensure the binding of the adsorbent to the solid substrate.

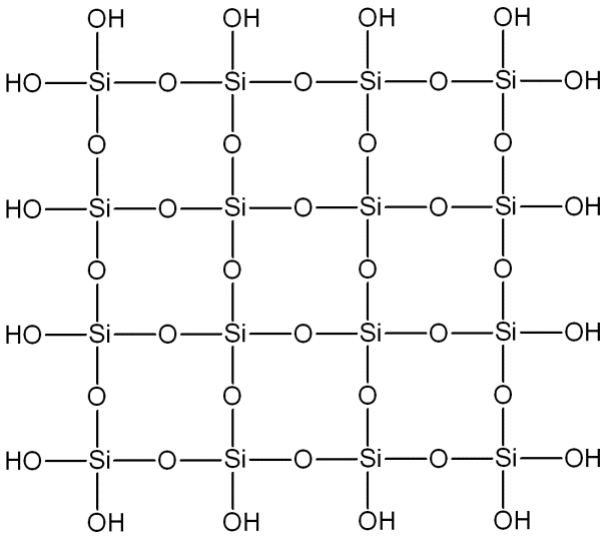

Figure 21.1: Structure of silica

A typical TLC experiment can be divided into following stages:

Preparation of plates

TLC plates are commercially available and are not very expensive. One can, however, prepare the TLC plates by oneself. The commercially available TLC plates usually have plastic or glass as the stationary phase support. The plates are sometimes purchased as large square sheets and one has to cut the desired size (usually a rectangular plate of 1–2 cm × 5 cm) from these large sheets. The plate of desired size can easily be cut using a scissors if the plates have plastic support. For glass backed TLC plates, one has to carefully cut the small plates using a diamond glass cutter. Cutting the glass plates is a straightforward procedure but requires some practice otherwise you can end up breaking the glass:

a. Take the large TLC plate and place it on a clean, smooth, and dry surface with the adsorbent layer facing down.
b. Take a ruler and draw parallel lines with a pencil to mark the places where you want the cut.
c. Place the ruler on the first line and firmly score the plate with one sweep of the diamond glass cutter (***Note 1***).
d. Place the plate with the score along the edge of your work-bench for the support and try to break the plate in a clean straight line.
e. Generate smaller plates repeating the steps a – d.

Once the plate of required dimensions is available, a line, parallel to the smaller side and at ~1 *cm* distance from one of the two small edges, is drawn using a pencil. The compound or the mixture of compounds to be analyzed is dissolved in an appropriate volatile solvent and spotted on the line. The spot on the plot is allowed to air-dry.

Development

A solvent or a mixture of solvents, called the eluant is taken in a wide-mouth container called the ‘development chamber’. A filter paper wetted with eluant is placed inside the development chamber to create a solvent saturated atmosphere. The dried TLC plate is placed in the development chamber such that the solvent front is slightly below the sample spot (ensure that the plate does not touch the filter paper placed in the development chamber). The solvent is taken up by the TLC adsorbent

due to capillary action. The stationary phase is polar (silica or alumina gel) while the eluant (mobile phase) is relatively non-polar. The molecules are therefore partitioned between the stationary and the mobile phase based on their polarities. As the solvent front reaches within 1 *cm* of the top edge of the plate, the plate is taken out using tweezers and the solvent front is marked with a pencil. The plate is then air dried and spots are visualized.

Visualization

If the compounds under analysis are colored, they can easily be visualized without any assistance. Most organic compounds, however, are not colored. In most cases, the spots can be detected using ultraviolet light. TLC plates wherein silica gel is impregnated with fluorescent compounds are commercially available. The plates have compounds that fluoresce when excited with the UV light of wavelength 254 nm. The plate, after the developing step, is illuminated with the UV light. The analytes that absorb UV quench the fluorescence and appear as dark spots in the glowing green background. The spots are outlined using a pencil and the plate is taken out of the UV light. In case this method does not work, the bands may be observed by placing the plates with iodine vapors; a large number of organic compounds form a dark-colored complex with iodine. A large number of TLC stains are available for detecting specific classes of molecules some of which are listed in Table 21.1

Table 21.1 List of some of the TLC stains used for biomolecular analysis

Stain	Analytes detected	Remarks
Ninhydrin	Amines such as amino acids, amino sugars	o Reagent: Dissolve 0.3 g ninhydrin in 100 ml 1-butanol and add 3 ml glacial acetic acid. o Spray and heat to 110 °C
Ninhydrin / pyridine / glacial acetic acid	Peptides	o Reagent: 1 *g* ninhydrin in 95 *ml* pyridine and 5 *ml* glacial acetic acid o Spray and heat at 100 °C for 5 minutes
Thymol / sulfuric acid	Sugars	o Reagent: Add 0.5 *g* thymol in 95 *ml* ethanol, and add 5 *ml* conc. sulfuric acid with caution. o Heat at 100 °C for 15 *min*.
Aniline phthalate	Reducing sugars	o Reagent: 0.93 *g* aniline and 1.66 *g* o-phthalic acid dissolved in 100 *ml* of water saturated *n*-butanol. o Dry the plate with hot air → heat at 110°C for 10 *min*. → spots show different colors, some spots give fluorescence on excitation at 365nm.
Bromthymol blue		o 0.1% bromthymol blue in 10% aqueous ethanol made just alkaline with NH_4OH o Spray the plates and dry.
Sulfuric acid-Phosphomolybdic acid	Sterols, steroids, fats, fatty	o Reagents: (*i*) 50% (v/v) sulfuric acid, (*ii*) 10% phosphomolybdic acid in 100% ethanol o Spray the dried TLC plate with 50% (v/v) sulfuric acid solution → Heat at 120° C for 3–5 *min*. → Prepare 10% phosphomolybdic acid in 100% ethanol → filter through the Whatman#1 filter paper → spray the plate → Heat at 120° C for 3–5 *min*.
Aluminium chloride	Flavonoids	o Reagent: 1 *g* Aluminum chloride in 100 *ml* of 95% ethanol o Yellow fluorescence when excited ~360 *nm*.
Aklaline permanganate	Sugars	o Reagent: 1% $KMnO_4$ in 2% (w/v) Na_2CO_3 o Spray on the plate → heat at 100 °C for a few *min*.
Potassium dichromate / sulfuric acid	Universal reagent for organic compounds	o Reagent: 5 *g* potassium dichromate into 100 *ml* conc. sulfuric acid o Spray the plate; if required, heat to 150 °C o Should not be used with the polymer bound TLC plates as this will char the binder, Use only on gypsum bound plates
Potassium permanganate / sulfuric acid	Universal reagent for organic compounds	o Reagent: 1.6% potassium permanganate in conc. sulfuric acid o Spray the plate → heat at 180 °C for 15–20 *min*. o Should not be used with the polymer bound TLC plates as this will char the binder, Use only on gypsum bound plates

Analysis

The TLC plates can be used to calculate what is called the retardation factor or the R_f value (***Note 2***). R_f value of an analyte is the ratio of the distance traveled by the analyte to that travelled by the eluant front (Figure 21.2).

$$R_f = \frac{Distance\ travelled\ by\ the\ analyte}{Distance\ travelled\ by\ the\ eluant}$$

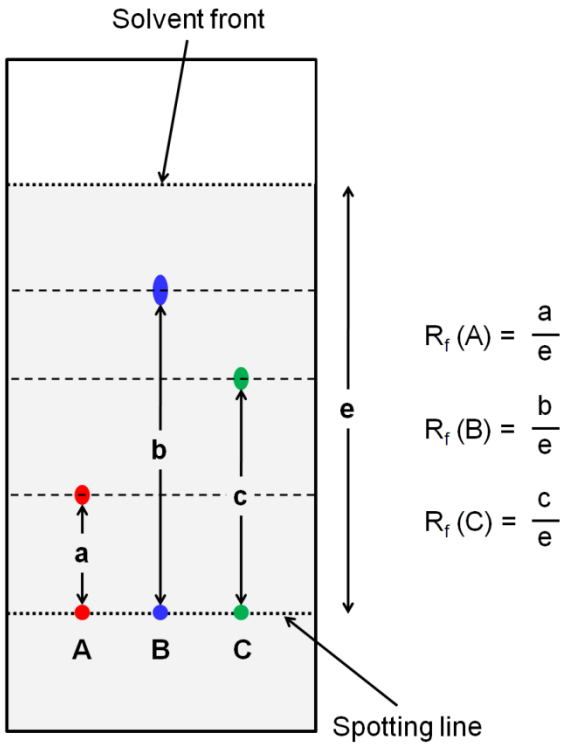

Figure 21.2 Definition of the R_f value

Materials

Equipments:

1. Weighing balance
2. Hot air oven
3. Chemical fume hood

Reagents:

1. Silica
2. Sodium acetate

3. 1-Butanol
4. Glacial acetic acid
5. Ninhydrin
6. Serine
7. Phenylalanine
8. Lysine
9. Serine–Phenylalanine–Lysine mixture

Glassware, plasticware, and other items:

1. Wide mouth glass bottle with lid (Development chamber)
2. 25 *ml*, and 50 *ml* measuring cylinders
3. 10 *ml* glass pipette
4. 100 *ml* glass flask
5. Glass capillaries
6. Glass rods
7. Filer paper
8. Tweezers
9. Glass microscopic slide
10. Ruler
11. Pencil
12. A pair of gloves

Preparation of reagents and TLC plate:

TLC plate:

1. Prepare 20 *ml* of 20 *mM* sodium acetate solution.
2. Weigh 10 *g* of silica and add it to the 20 *ml* sodium acetate solution.
3. Mix thoroughly to obtain the slurry.
4. Wear the gloves and take 4 – 5 clean glass microscopic slides.
5. Place thin pieces of paper along the long sides of the slide as shown in figure 21.3, the thickness of paper determines the thickness of the adsorbent layer.

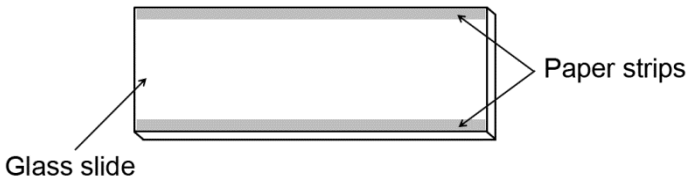

Figure 21.3: A diagram showing paper strips of equal dimensions placed along the long side of the glass slide

6. Pour the silica slurry on one edge of the slide and roll the glass spreader (glass rod) towards the other edge in a single stroke.
7. Air-dry the slides until they become white and smooth.
8. Bake the slides in a hot air oven at 120 °C for 30 minutes.
9. Take out the coated slide and allow them to come to the room temperature.
10. The TLC plate is ready to use

Eluant: Butanol : Glacial acetic acid : Water (4 : 1 : 1)

1. Pour 40 *ml* of 1-butanol, 10 *ml* of glacial acetic acid, and 10 *ml* of water in a 100 ml flask.

0.2% Ninhydrin solution:

1. Weigh 0.2 *g* of ninhydrin and dissolve it in 97 ml of 1-butanol.
2. Add 3 ml of glacial acetic acid.

Procedure:

Spotting of samples on the TLC plate

1. Take a pencil and draw a light line, parallel to and 1 *cm* away from, one of the small edges. Be careful so that the silica layer does not get scraped off (***Note 3***).
2. Prepare 1% (w/v) standard solutions of serine, phenylalanine, and lysine in water.
3. Using glass capillaries, load the serine standard solution, phenylalanine standard solution, lysine standard solution, and the given mixture of amino acids as four small spots on the spotting reference line (Figure 21.4).

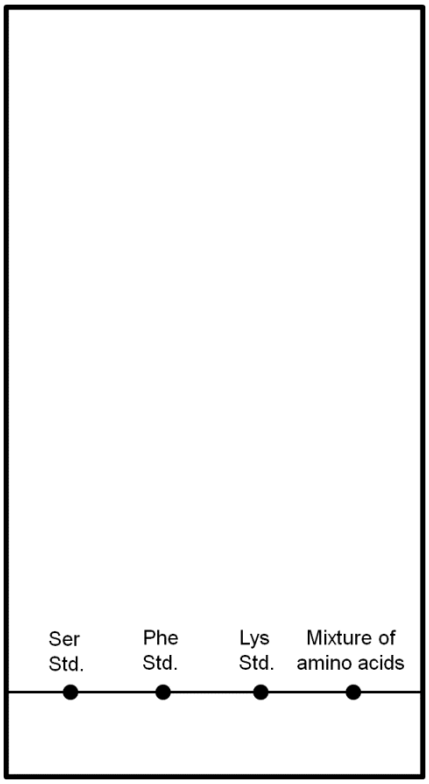

Figure 21.4: A thin layer chromatographic plate with spotted samples

4. Load each of these solutions 5 times with intermittent drying using a hot-air blower or simply in the air.
5. Air-dry the TLC plates for 10 minutes when the sample loading is complete; the plate can also be kept in the hot air oven at 70 ºC for 2-3 minutes for complete drying of the plate.

Development

6. Pour the eluant in the development chamber to get an eluant column of ~0.5 – 0.8 cm.
7. Take a piece of filter paper, dip it in the eluant and stick it inside the wall of the development chamber (Figure 21.5).
8. Close the development chamber and leave it aside for two hours to saturate the chamber with solvent vapors.
9. Place the dried TLC plate in the development chamber; ensure that the plate does not touch the filter paper kept inside the development chamber (Figure 21.5).

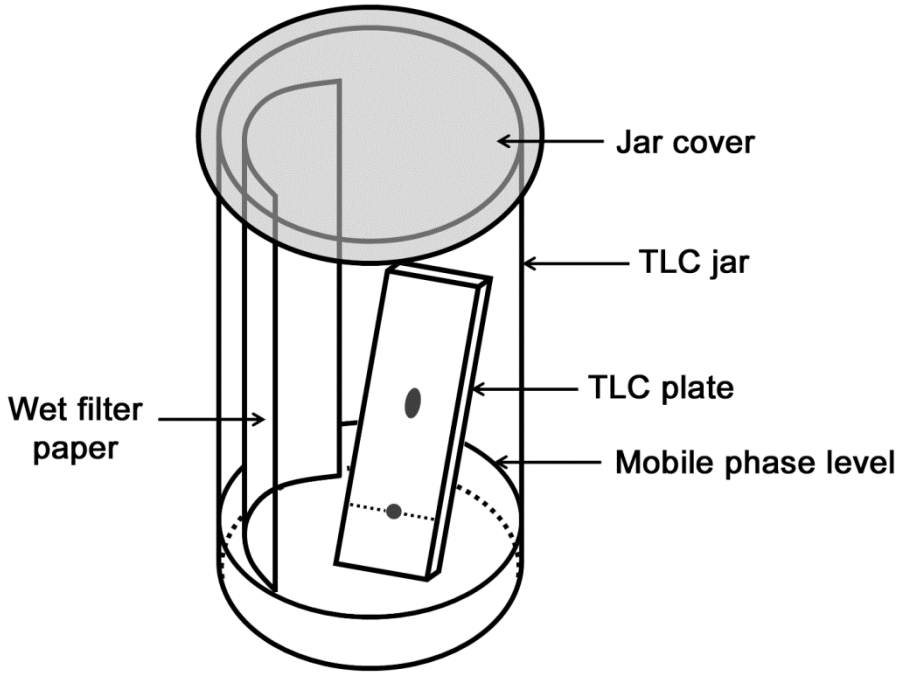

Figure 21.5: A thin layer chromatographic experiment set-up

10. Allow the eluant to rise till the eluant front reaches the desired height (~1 *cm* from the top edge of the plate).
11. Take out the plate and mark the solvent front using a pencil.
12. Air-dry the plate for 20 minutes followed by 2 minutes drying in a hot air oven at 70 ºC.

Visualization and analysis

13. Evenly spray the developed and dried TLC plate with the 0.2% ninhydrin solution (***Notes 4 and 5***).
14. Heat the plate at ~120 ºC for 3-5 minutes for drying the plate; purple-blue spots should appear under white light (***Note 6***).
15. Outline the spots using a pencil and measure the distance travelled by the spots relative to the spotting reference line.
16. Calculate the R_f values of the spots as shown in figure 21.2 and compare them with the standard spots.

Notes:

1. It is tempting to score the glass multiple times to get a deeper cut but this usually makes it difficult for the glass to break along the scored line.

2. If the relative mobility of the components is very low, a run-over chromatography may be required to obtain separation of the components. In such cases the eluant reaches the top edge of the plate and starts dripping as an overflow. In such cases, it is not possible to determine the R_f values. However, R_s, the ratio of the distance travelled by the analyte to that travelled by a standard, can be determined.

$$R_s = \frac{Distance\ travelled\ by\ the\ analyte}{Distance\ travelled\ by\ the\ standard}$$

3. Never use pen to mark the plate; the dyes present in the ink may appear in the chromatogram.
4. The staining with ninhydrin should be done in a chemical fume hood and inhalation or contact with the skin should be avoided.
5. Ninhydrin reacts with α-amino acids as shown in figure 21.6 to give purple-blue color except for proline that gives yellow-orange color.

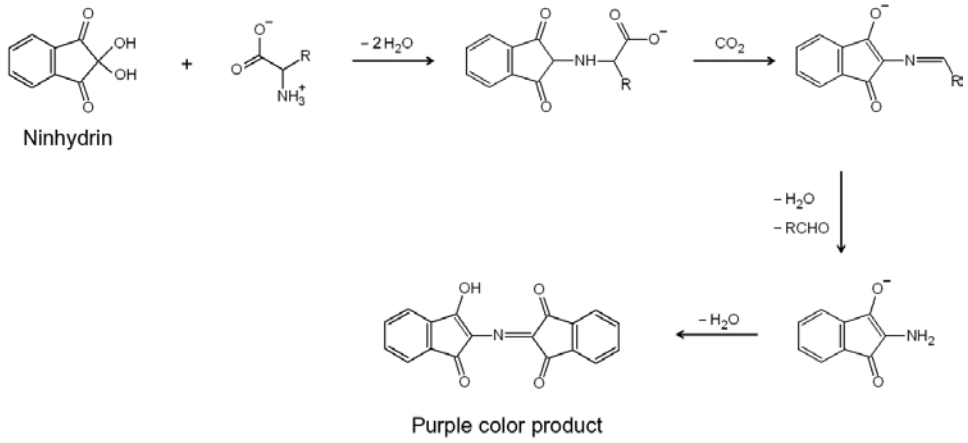

Figure 21.6: Reaction of ninhydrin with an α-amino acid

6. Proline is a secondary amine and gives yellow-orange color.

Lecture 22 Packing a gel filtration column and determination of void volume

Aim:

To pack a gel filtration column and determining the void volume of the packed column

Introduction:

Gel filtration chromatography is also known as gel permeation, molecular sieve, and size exclusion chromatography. The molecules are separated based on their size. The column matrix is made up of small spherical porous beads. The smaller molecules can enter the pores present in the beads while the molecules larger than the maximum pore size of the bead are completely excluded. The access to the pores is determined by both the shape and the molecular weight of the molecules; the separation, therefore, is based on the ability of the molecules to enter the porous beads. Properties of some of the routinely used gel filtration matrices are shown in table 22.1.

Table 22.1: Gel filtration matrices commercially available for separation in the range from 100 Da to subcellular particles

Gel	Material	Useful working range (Mol wt. in kDa)
Sephadex G-10	Dextran	Up to 0.7 kDa
G-15		Up to 1.5 kDa
G-25		0.1 – 5
G-50		1.5 – 30
G-75		3 – 70
G-100		4 – 150
G-150		5 – 300
G-200		10 – 600
Biogel P2	Polyacrylamide	0.1 – 1.8
P-4		0.8 – 4
P-6		1 – 6
P-10		1.5 – 20
P-30		2.5 – 40
P-60		3 – 60
P-100		5 – 100
P-150		15 – 150
Superdex 75	Cross-linked agarose	3 – 70
Superdex 200		10 – 600
Sepharose CL-4B		60 – 20,000
Sepharose CL-6B		10 – 4,000
Sephacryl S-100 HR	Cross-linked dextran/bisacrylamide	1 – 100
Sephacryl S-200 HR		5 – 250
Sephacryl S-300 HR		10 – 150

Sephacryl S-400 HR		20 – 8,000
Sephacryl S-500 HR		40 – 20,000
Sephacryl S-1000 SF		500 – 1,00,000

Materials:

Equipments:

1. Peristaltic pump
2. Vacuum pump
3. A UV/Visible spectrophotometer (if elution is not monitored by an HPLC instrument)

Reagents and chemicals:

1. Sephadex G-100
2. Blue dextran 2000
3. Buffer (Phosphate-buffered saline: 50 *mM* phosphate buffer, pH ~7.0 – 7.4 + 150 *mM*NaCl)

Glassware, plasticware, and other materials:

1. Gel filtration chromatography column tube along with the adaptors and tubings
2. A column extension (packing reservoir)
3. A filtration unit
4. Glass rod
5. A laboratory stand
6. 0.22 μm filter
7. Buffer reservoir
8. 1.5 *ml* microfuge tubes for collecting fractions.
9. 1 *ml* capacity glass cuvettes

Procedure:

Preparation of the gel

1. Determine the bed volume of the column as follows:
    a. Fix the column tube in a vertical position on a laboratory stand.
    b. Insert the bottom adapter to the desired level in the column tube, tighten the adapter, and attach the stop plug.
    c. Fill the column tube with distilled water up to the point the final gel is to be packed.
    d. Remove the column cap and collect all the water in a measuring cylinder.
    e. The volume of the collected water gives the column bed volume, V_t.
2. Prepare at least 20 bed volumes ($10V_t$) ofbuffer and filter it through 0.22 μm filter in a filtration unit (Figure 22.1A).

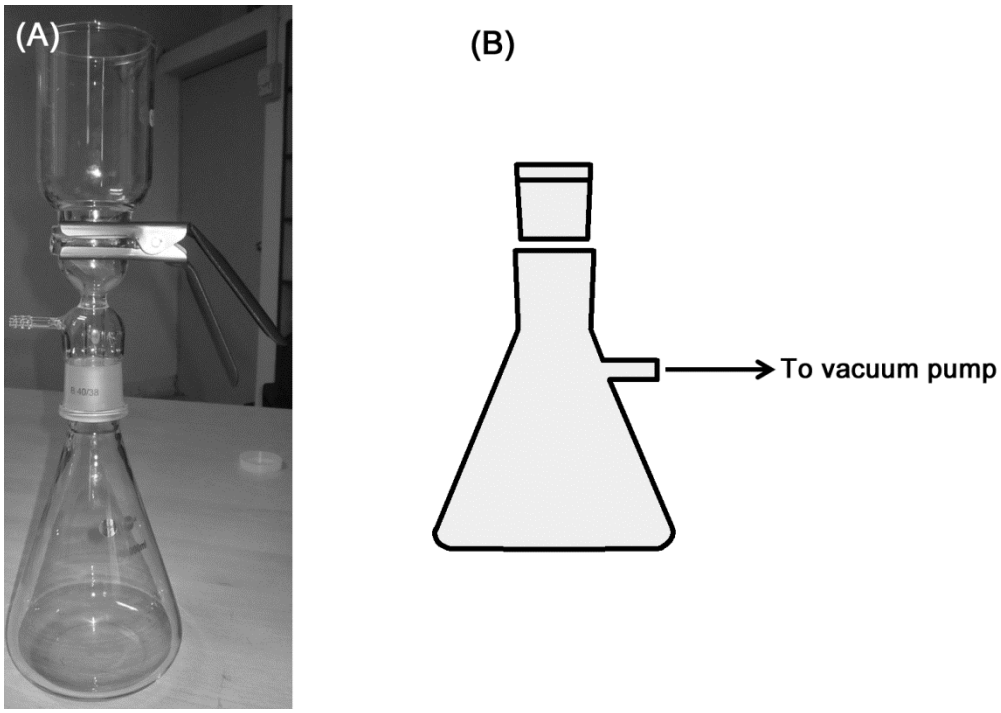

Figure 22.1: A buffer filtration unit (panel A) and an Erlenmeyer flask for degassing (panel B)

3. Calculate the amount of dry matrix required for packingthe column:
    a. The swelling factor of the gel matrix is provided by the manufacturer.
    b. The amount of dry gel required in grams $= \frac{Bed\ volume\ (ml)}{Swelling\ factor\left(\frac{ml}{g}\right)}$
4. Weigh slightly more than the calculated amount of the gel matrix.
5. Take 2 bed volumes ($2V_t$) of buffer in an Erlenmeyer flask with a thick side-arm for applying vacuum(Figure 22.1B).
6. Transfer the dry gel matrix into the buffer present in the Erlenmeyer flask.
7. Stir the suspension gently using a glass rod (*Important*: Do not use a magnetic stirrer as it can disrupt the soft gel particles).
8. Cover the flask with a rubber stopper that fits well in the flask mouth; seal the side arm with parafilm.
9. Allow the gel to swell overnight at room temperature.
10. During swelling, the gel settles down in the beaker. The upper buffer region may contain broken beads and looks hazy.Remove the hazy buffer portion by decanting.
11. Suspend the settled gel in 2 – 4 foldexcess of buffer and allow ~95% gel to settle down. Decant the buffer that contains non-settled gel particles. Repeat this process until the gel matrix settles as a sharp zone (usually 4 – 5 times is sufficient).

Packing of the column

12. Dilute the gel slurry two-fold by adding approximately the same volume of buffer as that of the settled gel.
13. Degas the gel by applying vacuum on the side-arm of the Erlenmeyer flask for 15 *min* (Figure 22.1B). Swirl the flask occasionally to release the air bubbles formed.
14. If more than 50% of the column tube needs to be packed, attach an extension on top of the column tube that can, together with the column, hold the entire volume of the gel slurry.
15. Mount the column tube on a stable laboratory stand. The column along with its attachments is shown in figure 22.3.

16. Remove air from the bottom adapter tubing of the column by attaching a buffer-filled syringe and forcing the sufficient buffer volume up though the bed support-net.
17. Insert the bottom adapter to the desired level in the column tube, remove the syringe, tighten the adapter, and attach the stop plug.
18. Ensure that the column is vertical using a carpenter's level or a plumb-line.
19. Swirl the gel slurry and pour the entire slurry into the column along a glass rod that is in contact with the inner wall of the column or column extension.
20. Fill the remaining space completely with the buffer by pouring it carefully along the glass rod so that the gel layer is not disturbed.
21. Using a syringe, inject the buffer through the upper adapter tubing while holding the outlet upwards so that the net becomes wet and air bubbles trapped in the net escape though the outlet.
22. Gently insert the top adapter in the column at 45° angle to avoid trapping any air, remove the syringe, and tighten the adapter.

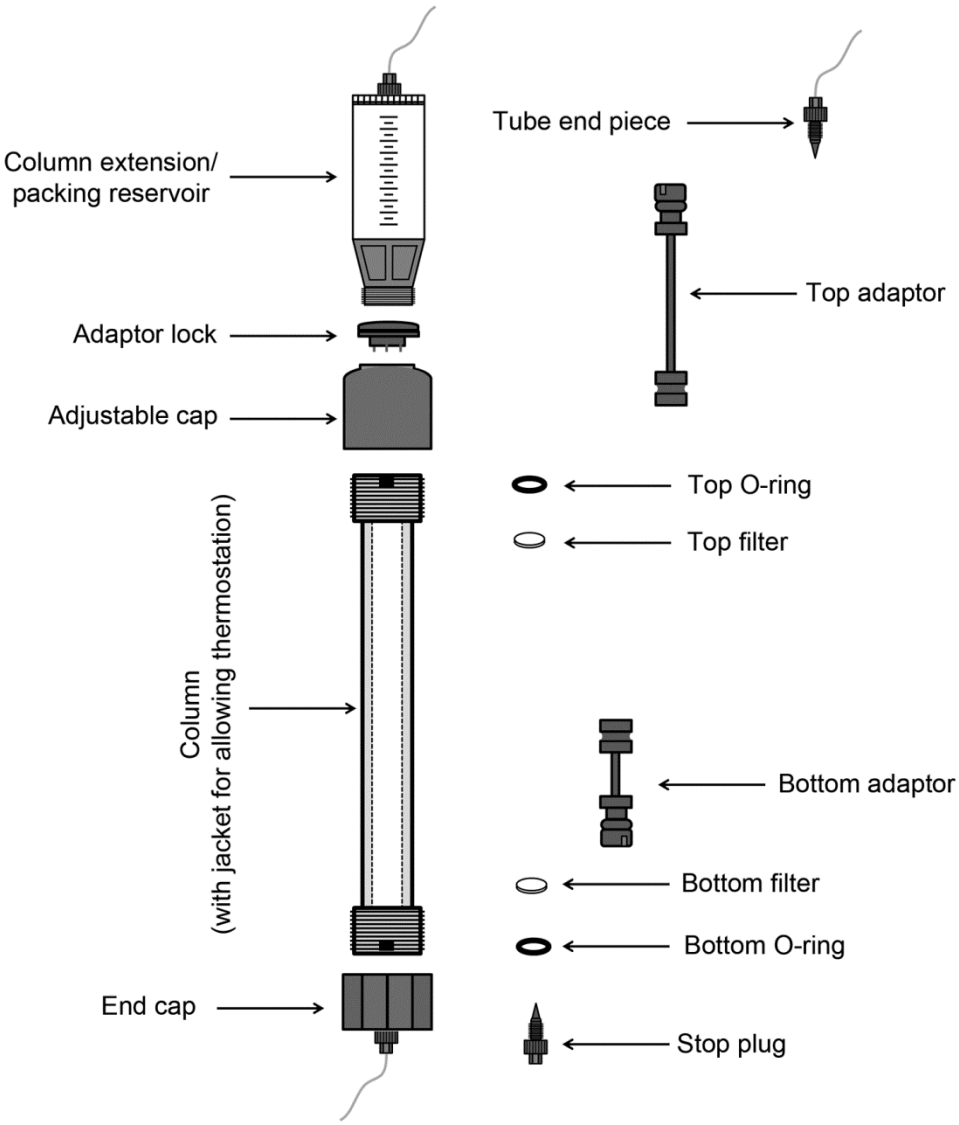

Figure 22.3 Column and its attachments

23. Push the adapter little down, without touching the gel bed. This causes removal of some buffer from top of the column assembly ensuring no air inside the column.
24. Fill the buffer reservoir with the buffer and place it at a height higher than the pump.
25. Connect the buffer reservoir to the pump and purge the pump with the buffer.
26. Keep a low flow rate and allow the buffer to ooze out of the pump outlet tube.
27. Stop the flow and make a drop-to-drop connection of the pump outlet to the inlet of the column tube or the column extension tube.

28. Remove the bottom stop plug.
29. Start the pump at a flow rate lower than the maximum flow raterecommended by the manufacturer for the gel material.
30. Let the flow continue until the height of the gel becomes constant (typically 3-4 bed volumes of the buffer).
31. Stop the pump and remove the column extension along with the adapter. This creates a vacant space in the column tube.
32. Fill the vacant space in the column tube by carefully adding the buffer.
33. Adjust the bed height, if required:
    a. If the bed height is more than that is desired, gently swirl the upper part of the gel bed with a glass rod and aspirate out the excess slurry.
    b. Allow the gel to settle down.
34. Fix the inlet adapter on column tube as discussed in steps 21 – 23.
35. Resume the flow conditions for 1 hour.
36. Stop the pump and lower the adapter down so that it is flush with the bed surface.
37. *The column is packed*. Inspect it visually for any cracks, trapped air bubbles, or any particulate material.

Determination of void volume

38. Connect the outlet of the pump to the column via an injection valvehaving a 500 μlcapacity loop (Figure 22.4).
39. Allow one bed volume to run through the column at the flow rate used for column packing.
40. Meanwhile, prepare a 1 *mg/ml* ‘blue dextran 2000’ solution in the buffer and filter it through 0.22 μm filter.
41. Bring the injector valve into the load mode.

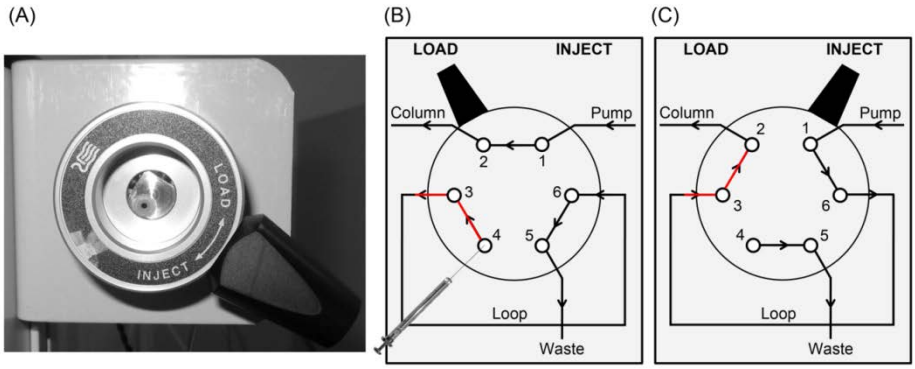

Figure 22.4: Injector valve (A); the diagram showing tube connectivities in load (panel B) and inject (panel C) mode

42. Inject blue dextran solution (≤2% of the bed volume) in the injection loop and change the injection valve into the injection mode.Take out the injection syringe.
43. Start collecting 1 *ml* fractions until the blue dextran is completely eluted. The migration of blue dextran can easily be tracked within the column. Stop collecting fractions once the blue coloured dye is completely eluted.

Results and analysis:

44. Switch ON the UV/Visible spectrophotometer and allow it 30 minutes warm up.
45. Set the instrument to absorbance mode and wavelength to 620 nm.
46. Zero the reading taking first fraction as a blank.
47. Measure absorbance of all the fractions (washing of cuvette in-between is not required for any of the fractions).
48. Record the absorbance in an observation table (Table 22.2)

Table 22.2: Table for recording absorbance of collected fractions

Fraction number	A_{620}
1	0.000
2	
3	

⋮	
⋮	
n	

49. Plot A_{620} against elution volume to obtain the chromatogram of blue dextran 2000. A typical chromatogram is shown in figure 22.2.

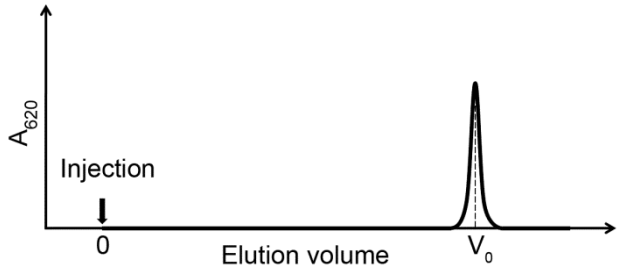

Figure 22.5: A chromatogram illustrating the void volume of a gel filtration column

50. From the chromatogram, it can be deduced that the void volume of the column is V_0.

Lecture 23 Protein molecular weight determination using gel filtration chromatography

Aim:

To determine the molecular weight of a given protein using gel filtration chromatography

Introduction:

Gel filtration chromatography separates the molecules based on their size; it is therefore also known as size exclusion chromatography. The gel bed is made up of the beads; the beads themselves are made up of cross-linked three-dimensional network of molecules having pores (Figure 23.1). The porosity of the beads is determined by the extent of cross-linking of molecules. Molecules, that are smaller than the maximum pore size of the bead, can penetrate the beads. Molecules, that are smaller than the smallest pore, can penetrate all the pores and are said to be completely included. Similarly, the molecules that are larger than the largest pore, cannot enter any of the pores and are said to be completely excluded. It is therefore not possible to resolve the components that are completely included or completely excluded using size-exclusion chromatography. This defines the separation limits of a size exclusion column.

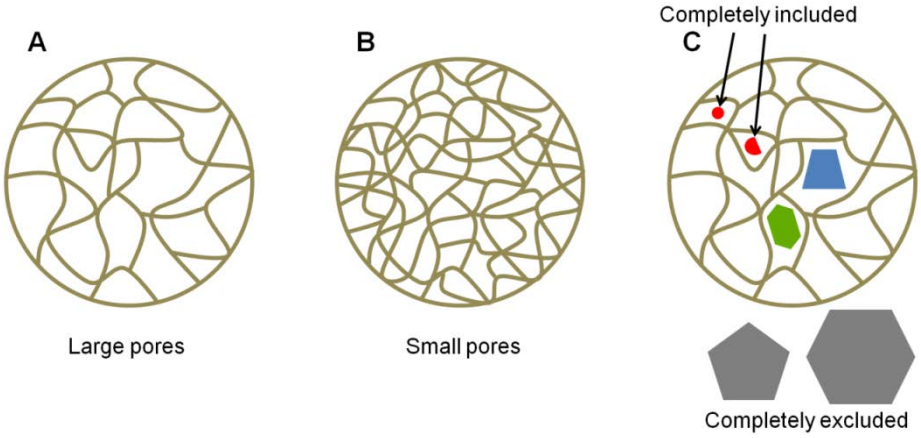

Figure 23.1 The diagrammatic representation of the beads with different porosities. Molecules completely included (shown in red) and completely excluded (shown in grey) are also shown in panel C.

The molecules that are completely included elute at V_t while those that are completely excluded elute in the void volume, V_0.

The extent to which the molecules enter the pores depends on both their shape and molecular weight. The molecules that lie in the separation regime *i.e.* those that are neither completely included nor completely excluded elute based on their molecular weight; the largest molecule elutes first while the smallest elutes last as shown in figure 23.2.

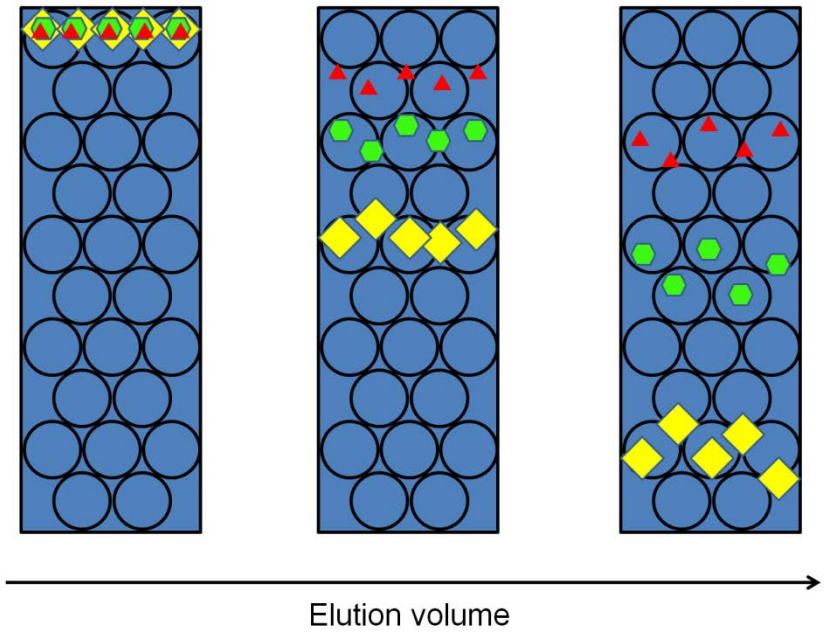

Figure 23.2: A diagram showing separation of molecules based on size in a gel filtration column

Materials:

Equipments:

4. A UV/Visible spectrophotometer (if elution is not monitored by an HPLC instrument)
5. Peristaltic pump

Reagents and chemicals:

1. Buffer (Phosphate-buffered saline: 50 *mM* phosphate buffer, pH ~7.0 – 7.4 + 150 *mM* NaCl) filtered through 0.22 μm filter (prepared in previous experiment)
2. Protein standards (***Note 1***)
    a. Aprotinin, bovine lung (6.5 kDa)
    b. Cytochrome c, horse heart (12.4 kDa)
    c. Carbonic anhydrase, bovine erythrocytes (29 kDa)
    d. Albumin, bovine serum (66 kDa)
    e. Alcohol dehydrogenase, yeast (150 kDa)
    f. β-amylase, sweet potato (200 kDa)
3. Blue dextran 2000 (1 *mg/ml*)

Glassware, pasticware, and other materials:

1. A packed Sephadex G-100 column (packed in previous experiment) (***Note 2***)
2. 0.22 μm filter
3. Buffer reservoir
4. 1.5 *ml* microfuge tubes for collecting fractions.
5. 1 *ml* capacity quartz cuvettes

Procedure:

51. Mount the column tube on a stable laboratory stand.
52. Fill the buffer reservoir with the buffer and place it at a height higher than the pump.
53. Connect the buffer reservoir to the pump and purge the pump with the buffer.
54. Keep a low flow rate and allow the buffer to ooze out of the pump outlet tube.
55. Stop the flow and connect the outlet of the pump to the column tube via an injection valve (injection loop of 500 μl volume).
56. Remove the bottom stop plug of the column.
57. Start the pump at a flow rate used for packing the column.
58. Equilibrate the column with the buffer by allowing two bed volumes to flow though the column.

59. Determine the void volume using blue dextran 2000 as mention in previous lecture.
60. Equilibrate the column again with two bed volumes of buffer.
61. Weigh 1 *mg* of each of the standard proteins and dissolve them together in 1 *ml* gel filtration buffer. This gives a 6 *mg/ml* protein solution having equal amount of all the six standard proteins.
62. Filter the standard protein mixture through 0.22 μm filter.
63. Bring the injector valve into the load mode.
64. Inject the protein mixture (≤2% of the bed volume) in the injection loop and change the injection valve into the injection mode. Take out the injection syringe.
65. Start ollecting 1 *ml* fractions until the V_t volume is collected.

Results and analysis:

66. Switch ON the UV/Visible spectrophotometer and allow it 30 minutes warm up.
67. Set the instrument to absorbance mode and wavelength to 280 nm (***Note 3***).
68. Zero the reading taking first fraction as a blank.
69. Measure the absorbance of all the fractions (washing of cuvette in-between is not required for any of the fractions).
70. Record the absorbance in an observation table (Table 23.1).

Table 23.1: Table for recording absorbance of collected fractions

Fraction number	A_{280}
1	0.000
2	
3	
⋮	
⋮	
n	

71. Plot A_{280} against elution volume to obtain the chromatogram of standard proteins. The chromatogram should give six distinct peaks; label the peaks from 1 – 6 with increasing elution time.
72. Equilibrate the column with two bed volumes of buffer and repeat the procedure for the given protein of unknown molecular weight.
73. Plot A_{280} of the unknown protein fractions against elution volume to obtain its chromatogram (***Note 4***).
74. Calculate the elution volumes of the reference proteins as well as the unknown protein.
75. Using elution volumes, V_0, and V_t, calculate the K_{av} of the proteins (Table 23.2):

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \qquad \text{---------- (23.1)}$$

76. Calculate the log_{10}(Mol. wt.) of the standard proteins (Table 23.2).

Table 23.2: Analysis of the chromatograms

Peak number	**Protein**	**Mol. wt (kDa)**	**Log (Mol. wt.)**	**Elution volume**	K_{av}
Standard 1	β-amylase	200	2.30	V_1	$K_{av_1} = \frac{V_1 - V_0}{V_t - V_0}$
Standard 2	Alcohol dehydrogenase	150	2.18	V_2	$K_{av_2} = \frac{V_2 - V_0}{V_t - V_0}$
Standard 3	Albumin	66	1.82	V_3	$K_{av_3} = \frac{V_3 - V_0}{V_t - V_0}$
Standard 4	Carbonic anhydrase	29	1.46	V_4	$K_{av_4} = \frac{V_4 - V_0}{V_t - V_0}$
Standard 5	Cytochrome c	12.4	1.09	V_5	$K_{av_5} = \frac{V_5 - V_0}{V_t - V_0}$
Standard 6	Aprotinin	6.5	0.81	V_6	$K_{av_6} = \frac{V_6 - V_0}{V_t - V_0}$
Unknown	Unknown	To be		V_u	

		determined			

77. Plot the K_{av} values against the log_{10}(Mol. wt.) for the reference proteins to obtain the standard curve.
78. Fit the data using linear regression (***Note 5***).
79. Determine the log_{10}(Mol. wt.) for the unknown protein using the regression line equation.
80. Take antilog of the log_{10}(Mol. wt.) to determine the molecular weight.

Notes:

7. The standards for molecular weight determination should be of the same shape as the molecule whose molecular weight is to be determined *i.e.* globular proteins for a globular protein under question, fibrous proteins for a fibrous protein under question, peptides for a peptide under question. In case such standards are not available, other suitable reference molecules can be used. The standard proteins used in this experiment are well established markers for gel filtration and are commercially available.
8. The column matrix and the proteins for the standard plot are chosen based on some idea about the molecular weight of the unknown protein. If there is no such information available, Sephacryl HR, the matrix with fractionation range of 40 – 20,000 kDa, can be used.
9. Each of the standard proteins used in this experiment has either tyrosine or tryptophan or both and can therefore be monitored through absorbance at 280 nm.
10. The absorbance of a protein at 280 nm is the outcome of the light absorbed largely by tryptophan and tyrosine residues and by disulfide linkages to a small extent. If the unknown protein lacks these chromophores, the protein will not absorb the 280 nm light. It is therefore important to record an absorption spectrum of the unknown protein from 210 – 300 nm. If the spectrum lacks an absorption band ~ 280 nm, the absorbance of the protein fractions should be recorded at 214 nm for plotting the chromatogram.

11. Molecules of similar shapes and densities display sigmoidal relationship between their K_{av} values and the log_{10}(Mol. wt.). Over a considerable K_{av} range (~0.1 – 0.7), however, the relationship is linear.

Lecture 24 Protein purification using ion exchange chromatography

Aim:

To purify hen egg white lysozyme from a given protein mixture using ion exchange chromatography.

Introduction:

Ion exchange chromatography is perhaps the most commonly employed chromatographic method for separating the proteins, polypeptides, nucleic acids, and other charged molecules. The principle underlying ion exchange chromatography is the reversible exchange of the charged molecules (ions) present in the solution with those electrostatically bound to the insoluble support medium. The molecules in ion exchange chromatography, therefore, get separated based on their charge. The stationary phase is constituted by the inert polymeric beads that are functionalized with ionizable groups. Based on the type of ionizable group present on the stationary phase, an ion exchange chromatographic column is termed as an anion exchanger (positively charged stationary phase) or a cation exchanger (negatively charged stationary phase). Quaternary amines are the most common functional groups for anion exchange while carboxyl and sulphopropyl are the most common cation exchanging groups (Table 24.1)Sulphonicand quaternary amino groups are completely ionized over a wide range of pH and are therefore termed as strong exchangers. Weak ion exchangers start losing their chargebelow/above certain pH(below pH 6 for cation exchangers and above pH 9 for anion exchangers).

Table 24.1 Ion exchangers and their functional groups

Anion exchanger	pH range	Functional group
Diethylaminoethyl (DEAE), weak	2 – 10	$-OCH_2CH_2N^+H(CH_2CH_3)_2$
Aminoethyl (AE), intermediate	2 – 9	$-OCH_2CH_2NH_3^+$
Quaternary aminoethyl (QAE), strong	2 – 10	$-OCH_2CH_2N^+H(C_2H_5)_2 - CH_2CH(OH)CH_3$

Cation exchanger	pH range	Functional group
Carboxymethyl (CM), weak	3 – 10	$-OCH_2COO^-$
Phospho, intermediate	2 – 10	$-PO_4H_2^-$
Sulphopropyl (SP), strong	2 – 12	$-CH_2CH_2CH_2SO_3^-$

DEAE and CM continue to be the most widely used ion exchange materials for protein purification in the physiological pH range. Proteins have several ionizable groups; at neutral pH, amino terminus of the protein and the side chains of lysine and arginine carry +1 charge each while C-terminus of the protein and the side chains of aspartate and glutamate impart -1 charge each. As the charges at the protein termini cancel each other out, the net charge on the protein at physiological pH is determined by the number of arginine, lysine, aspartate, and glutamate residues.

Proteins bind to the ion exchange matrices through reversible electrostatic interactions. Depending on their net charge at the working pH, the protein of interest may bind to a cation exchanger (if protein net charge is positive) or to an anion exchanger (if protein net charge is negative). Separation is achieved due to different affinities of the protein molecules to the ion exchange matrix. The bound molecules are usually eluted by increasing the ionic strength of the elution buffer (***Note 1***). The molecules that are weakly bound to the column elute first followed by the ones that are strongly bound. Ion exchange experiments are usually performed in thefollowing stages:

1. *Preparation of ion exchanger*: This includes treating the anion exchange gel material with1 *N*HCl and cation exchange gel material with 1 *N*NaOH. This results in the ionization of all the ionizable groups present in the gel material. Repulsion between the identical charges also facilitates swelling.
2. *Packing the column and equilibration of matrix*: The column is packed with the binding buffer *i.e.* the buffer that allows binding of the sample molecules.

The packed column is equilibrated with 2–3 bed volumes of the binding buffer.

3. *Sample loading and binding of the molecules to column*: The sample is prepared in the binding buffer and loaded on the column. This results in the binding of molecules to the counter-ionic matrix. If the sample has NaCl concentration > 50 *mM*, the samples should be dialyzed against the binding buffer.
4. *Elution of the bound molecules*:The bound molecules are desorbed and eluted either by changing the pH of the buffer or by increasing the salt concentration. A change in pH causes the molecules to reach their isoelectic points at a particular pH wherein they no longer interact with the column matrix. High salt concentration provides ions that compete with the analyte molecules for the matrix. Fractions of defined volumes are collected as soon as elution step starts.
5. *Analysis of the collected fractions*: The collected fractions are analyzed using the suitable methods. Protein fractions can be analyzed by recording absorbance at 280 nm or by any other protein estimation method.
6. *Removal of the compounds that do not elute by the elution buffer*: Some compounds may bind very tightly to the column; they can be removed by washing with very high concentration of the salt
7. Regeneration of the column: The column is regenerated by washing with 1 *N*HCl or 1 *N*NaOH.

In this experiment we are going to perform the ion exchange chromatographic experiment with hen egg white lysozyme. Hen egg white lysozyme has an isoelectric point of ~11.3 *i.e.* it is a cationic protein at neutral pH. We shall therefore be performing cation exchange chromatography.

Materials:

Equipments:

6. A UV/Visible spectrophotometer (if elution is not monitored by an HPLC instrument)
7. Peristaltic pump
8. A gradient mixer

Reagents and chemicals:

1. Sepharose ion exchanger (Carboxymethyl-cellulose)
2. 20*mM*phosphate buffer, pH 7.0
3. 0.1 *N*NaOH
4. 3*M*NaCl solution

Glassware, pasticware, and other materials:

1. Glass column (30 *cm* × 2.5 *cm*)
2. Laboratory stand

Procedure:

81. Follow the manufacturer's instructions for swelling the ion exchanger matrix. Alternatively follow the following steps:
    a. Weigh 10 *g* of CM-cellulose and suspend it in 200 *ml* of binding buffer (20 *mM* phosphate buffer, pH 7.0 in this case) containing 1*M*NaCl.
    b. Allow swelling of the matrix for at least 24 hours.
    c. Remove the fines by decanting the buffered saline.
    d. Wash the swollen matrix with the buffered saline 2-3 times.
    e. Wash the matrix with distilled water to remove the salts (if the binding buffer does not have a neutral pH, achieving a neutral pH is an indicator of removal of salts).
    f. Add 200 ml of 0.1 *N*NaOH in the matrix and leave it for 30 minutes. This ensures that the matrix functional groups, carboxylate groups in this case, are completely ionized.

g. Wash the matrix with the binding buffer 3-4 times. This is to remove the excess NaOH present in the matrix gel.

82. Mount the glass column on a laboratory stand.
83. Place a cotton plug or glass wool plug at the bottom (Figure 24.1A). This is done to prevent the loss of the matrix gel from the column. Alternatively, fritted columns (Figure 24.1B) can be used.

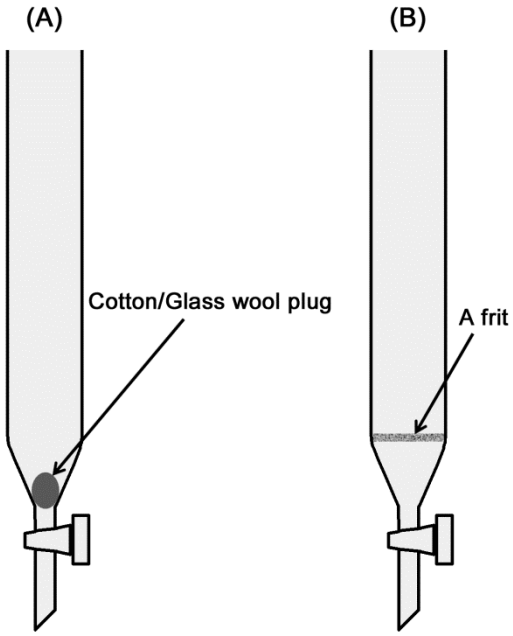

Figure 24.1: A column with cotton/glass wool plug (A); a fritted column (B)

84. Close the stop-cock and add 20 *ml* of binding buffer.
85. Open the stop cock to a have a flow rate of not more than 0.5 *ml/min*.
86. Gently pour the swollen ion exchange matrix to obtain a column bed of 20 *cm* height.
87. Equilibrate the column with 3 bed volumes of the binding buffer.
88. Load 2-3 *ml* of the protein solution on the column.
89. Allow one column volume of binder buffer to pass through the column.
90. Set up the gradient mixer as shown in figure 24.2.

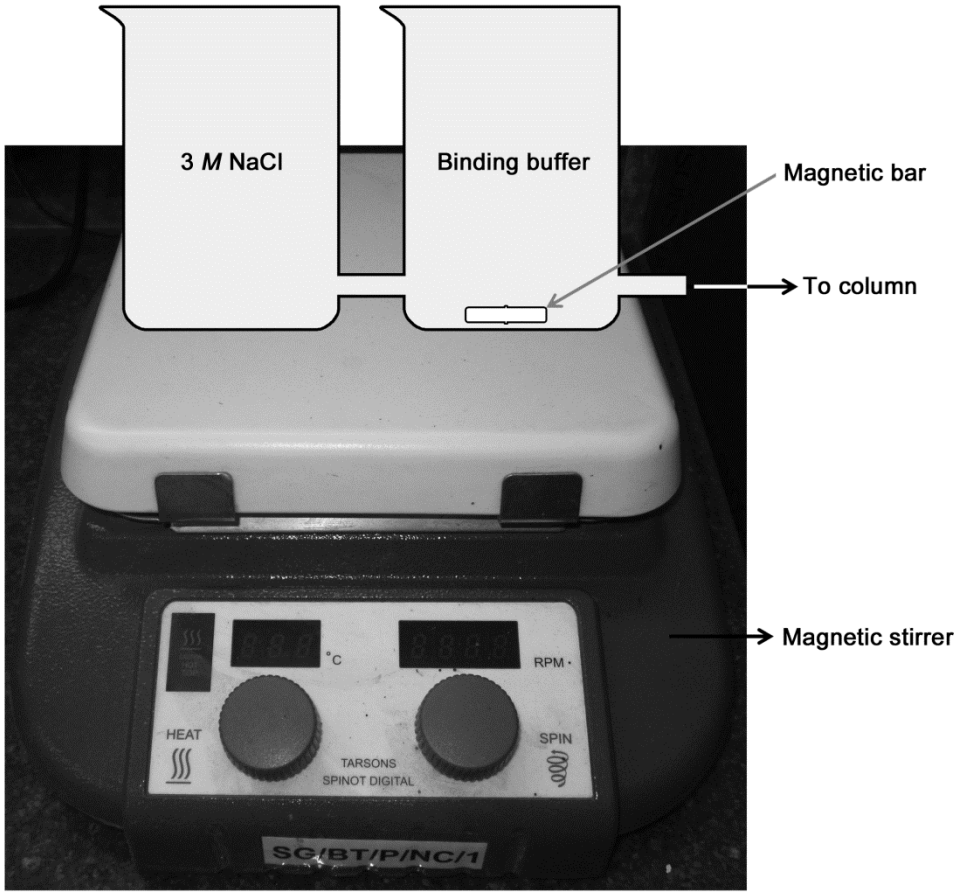

Figure 24.2: A gradient mixer kept on the magnetic stirrer

91. Connect the gradient mixture outlet to the column and try to achieve a flow rate of ~0.5 – 0.6 *ml/min*.
92. Start collecting fractions of 5 *ml* each as soon as the NaCl gradient is set up.
93. If the column is to be reused, regenerate it as follows:
    a. Wash the column with 2 volumes of 3*M*NaCl to remove any substance that might still be bound to the column.
    b. Wash the column with 2-3 volumes of binding buffer containing 200 *ppm* sodium azide. Sodium azide prevents microbial growth in the column.
    c. Store the column at 4 °C.

Results and analysis:

1. Switch ON the UV/Visible spectrophotometer and allow it 30 minutes warm up.
2. Set the instrument to absorbance mode and wavelength to 280 nm.

3. Zero the reading taking first fraction as a blank.
4. Measure the absorbance of all the fractions (do not wash the cuvette in-between the readings).
5. Record the absorbance in an observation table (Table 24.2).

Table 24.2: Table for recording absorbance of collected fractions

Fraction number	A_{280}
1	0.000
2	
3	
⋮	
⋮	
n	

6. Plot the absorbance values against the fraction number to obtain the chromatogram.
7. The peak corresponding to hen egg white lysozyme can be identified by checking the mass of the collected fractions using mass spectrometry (***Note 2***).

Notes:

12. The molecules in ion exchange chromatography are also eluted by varying the pH of the buffer. This, however, may not work well for proteins as variation in pH may denature the proteins.
13. Ion exchange chromatography may not be able to provide 100% pure protein from a complex mixture of proteins. The proteins having same or very similar isoelectric points may not get resolved, thereby eluting in the same fraction. The fractioncontaining hen egg white lysozyme therefore may have other proteins of very similar isoelectric points.

Lecture 25 Protein purification using metal chelate affinity (Ni-NTA)chromatography

Aim:

To purify an overexpressed protein (with 6×His tag) using Ni-NTA affinity chromatography

Introduction:

Affinity chromatography encompasses the techniques wherein separation is achieved based on specific binding of the protein to their ligands or receptors. Some of the receptors or ligands, such as antigenic epitopes and the active sites of enzymes, are highly specific to the protein of interest and affinity chromatography takes advantage of these biospecific interactions for purifying a protein. This approach of purification should, in principle, purify a protein in a single step.

Although receptor-ligand interactions are natural properties of the proteins and enzymes, it is sometimes convenient to engineer the proteins making them amenable for specific interactions. This has become popular for the purification of the recombinant proteins. The cDNA sequences are engineered so that the expressed proteins have the desired binding site at one of its termini. All forms of affinity chromatography usually include the following steps:

1. Choosing an appropriate ligand.
2. Immobilization of the ligand onto the matrix gel.
3. Loading the sample (protein mixture) on the matrix gel.
4. Washing to remove the substances that are bound non-specifically.
5. Elution of the protein of interest.

A variety of protein interactions are utilized for purifying a protein using affinity chromatography; some of them are listed in table 25.1.

Table 25.1 Some of the routinely using affinity chromatographic techniques

Chromatographic technique	**Interaction utilized**	**Application/ suitability**	**Remarks**
Immunoaffinity chromatography	Antibody–antigenic epitope interaction	For any protein	Very high specificity
Lectin affinity chromatography	Lectin–sugar interactions	Purification of glycoproteins	Many glycoproteins have very similar sugar chains; specificities are, therefore, not absolute.
Natural-ligand affinity chromatography	Protein–natural ligand	For any protein whose ligand is known and available	High specificity
Dye affinity chromatography	Protein-dye interactions	For the proteins that are known to bind any dye	-
Metal-chelate affinity chromatography	Protein-metal ion	For proteins having metal binding sites	Routinely used for purification of recombinant proteins that areengineered to have metal chelating sites.

The physiological concentrations of most proteins in the host are too low. To obtain large amounts of the proteins, their cDNAs are usually cloned in a suitable expression system. For purification using nickel affinity chromatography, a stretch of 6 histidine residues, known as 6×Histag, is incorporated at one of the protein termini. For nickel affinity chromatography, agarose or sepharose beads are functionalized with nitrilotriacetic acid (NTA). The NTA has four chelating sites for nickel ions thereby binding the ions very tightly. The Ni-NTA complex has very high affinity of the proteins having 6 consecutive histidine residues (Figure 25.1). The bound protein can be eluted using a high concentration of imidazole that competes with the histidine residues for the nickel ions. Ready to use Ni-NTA agarose and sapharosebeads are commercially available.

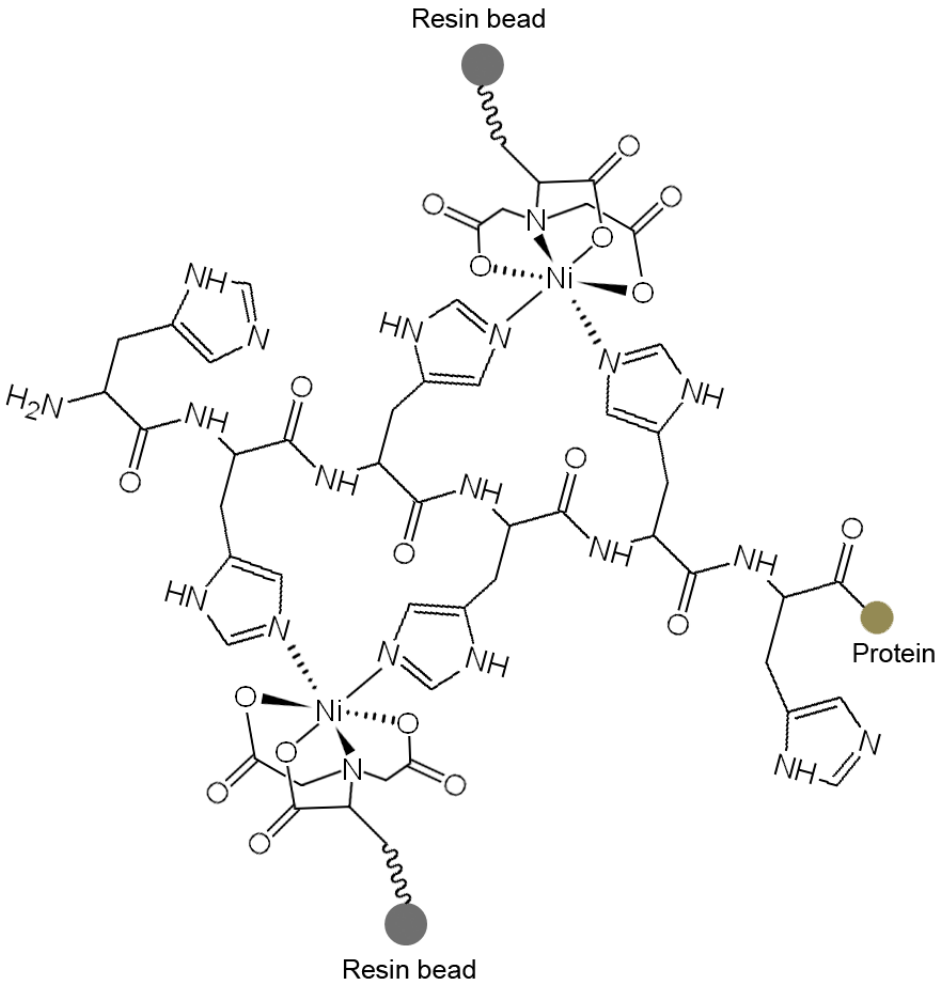

Figure 25.1: Interaction of histidine residues in 6×His tag with Ni-NTA beads

Ni-NTA affinity chromatography can be performed as described in the protocol below wherein a Ni-NTA resin is packed into a column and cell lysate is loaded into the packed column. Alternatively, the Ni-NTA resin is mixed with the cell lysate and then column is packed with lysate-containing Ni-NTA resin (Batch purification, see ***Note 1***).

Materials:

Equipments:

9. Refrigerated centrifuge
10. Sonicator equipped with microtip
11. UV/visible spectrophotometer

Reagents and samples:

1. Ni-NTA agarose resin slurry (commercially available)
2. The bacterial culture having over-expressed 6×Histidine-tag fusion protein
3. Hen egg white lysozyme
4. RNase A
5. Dnase I
6. 100 *mM* phosphate buffer, pH 7.4
7. 100 *mM* $NiSO_4 \cdot 6H_2O$ solution
8. 1 *M* imidazole solution
9. 1 *M*NaCl solution
10. 0.1 *M*EDTA, pH 8.0
11. 2% (w/v) SDS
12. Ethanol
13. 1 *M* acetic acid
14. 8 *M*guanidine·HCl

Glassware, plasticware, and other materials:

1. A 10 *ml* capacity polypropylenecolumn
2. Laboratory stand
3. Pipettes
4. Pipette tips

5. 200 *ml* volumetric flasks
6. 50 *ml* and 100 *ml* measuring cylinders
7. 1 *ml* and 5 *ml* glass pipettes

Preparation of reagents and matrix:

Binding buffer (20 mM phosphate buffer, pH 7.4 + 300 mMNaCl + 10 mM imidazole):

1. Take 40 *ml* of 100 *mM* phosphate buffer, add 60 *ml* of 1 *M*NaCl and 2 *ml* of 1 *M* imidazole solution.
2. Add water to make the final volume to 200 *ml*.

Wash buffer (20 mM phosphate buffer, pH 7.4 + 300 mMNaCl + 25 mM imidazole):

1. Take 40 *ml* of 100 *mM* phosphate buffer, add 60 *ml* of 1 *M*NaCl and 5*ml* of 1 *M* imidazole solution.
2. Add water to make the final volume to 200 *ml*.

Elution buffer (20 mM phosphate buffer, pH 7.4 + 300 mMNaCl + 250 mM imidazole):

1. Take 40 *ml* of 100 *mM* phosphate buffer, add 60 *ml* of 1 *M*NaCl and 50*ml* of 1 *M* imidazole solution.
2. Add water to make the final volume to 200 *ml.*

Stripping solution (0.2 M acetic acid in 6 M guanidine·HCl):

1. Take 15 *ml* of 8 *M*guanidine·HCl solution and add 4*ml*of 1 *M*acetic acid solution.
2. Add 1 *ml*water to make the final volume to 20*ml.*

Procedure:

Packing of the column (all the steps are to be carried out at 4 ºC)

94. Prepare all the reagents and solutions and keep them at 4 ºC.
95. Take out the bottle of the Ni-NTA resin and resuspend the resin by inverting the bottle and gently tapping it.

96. Mount the polypropylene column on the laboratory stand in the cold room.
97. Gently pour the Ni-NTA resin to obtain a packed bed volume of ~5*ml*.
98. Allow the storage buffer to drain down the column.
99. Equilibrate the column with 3 bed volumes of the binding buffer, keep sufficient binding buffer above the resin bed andstop the flow.

Preparation of cell lysate(all the steps are to be carried out at 4 ºC)

100. Collect the bacterial culture (after His-tag fusion protein expression) in a centrifuge tube.
101. Harvest the cells by centrifuging at 4,500 × g for 10 minutes at 4 ºC.
102. Resuspend the cell pellet in 10 *ml* of binding buffer.
103. Add 10*mg* of hen egg white lysozyme (final concentration: 1 *mg/ml*) and incubate in ice for 30 *min*.
104. Sonicate the lysozyme treated bacterial suspension with six 10-seconds bursts of 200-300 W each with a 10 second cooling after each burst. If the bacterial lysate appears to be viscous, add Rnase A (*10 μg/ml*) and Dnase I (*5 μg/ml*) and incubate on ice for 15 minutes.
105. Centrifuge the lysate at 10,000 × g for 20 minutes to pellet down the cellular debris.
106. Transfer the supernatant to a fresh tube (***Notes2 and 3***).
107. Centrifuge the supernatant collected in step 13 at 25,000 × g for 5 minutes to remove the insoluble aggregates and obtain the clear lysate.

Protein purification(all the steps are to be carried out at 4 ºC)

108. Start the flow of the column and load the entire clear lysate to the column.
109. Allow the cell lysate to enter the column completely.
110. Wash the column 4 times with 2 bed volumes of wash buffer(*i.e.* a total of 8 bed volumes).
111. Add 0.5 bed volumes of the elution buffer 10 times collecting the 0.5 *ml* fractions each time.

NTA resin regeneration

112. Wash the resin with the following solutions without allowing the resin to dry:
    a. 2 bed volumes of stripping solution
    b. 2 bed volumes of water
    c. 3 bed volumes of 2% SDS
    d. 1 bed volume each of 25% ethanol, 50% ethanol, and 75% ethanol
    e. 5 bed volumes of ethanol
    f. 1 bed volume each of 75% ethanol, 50% ethanol, and 25% ethanol
    g. 1 bed volume of water
    h. 5 bed volumes of 0.1 *M* EDTA, pH 8.0
113. After step 1, the NTA resin is completely stripped of the nickel ions (***Note 4***).
    i. For long-term storage, wash the uncharged resin with 5 bed volumes of 20% ethanol and store at 4 °C.
114. For immediate use, recharge the column by adding 5 bed volumes of 100 *mM* $NiSO_4 \cdot 6H_2O$. andwashing with 10 bed volumes of binding buffer. The column is charged again.

Results and analysis:

8. Switch ON the UV/Visible spectrophotometer and allow it 30 *min* warm up.
9. Set the instrument to absorbance mode and wavelength to 280 nm.
10. Zero the reading taking wash buffer as a blank.
11. Measure the absorbance of all the fractions (do not wash the cuvette in-between the readings).
12. Record the absorbance in an observation table (Table 25.2).

Table 25.2: Table for recording absorbance of collected fractions

Fraction number	A_{280}
1	0.000
2	
3	
⋮	
n	

13. Plot the absorbance values against the fraction number to obtain the chromatogram.
14. The purified His-tag fusion protein usually elutes in fraction no. 2 – 5.

Notes:

14. Batch purification:
    a. Take out the bottle of the Ni-NTA resin and resuspend the resin by inverting the bottle and gently tapping it.
    b. Pipette out the required volume of the slurry (5 ml packed volume) in a polypropylene centrifuge tube.
    c. Centrifuge at 750 × g for 2 minutes and gently aspirate the supernatant.
    d. Add three bed-volumes of the binding buffer, resuspend the resin by inverting the tube and gently tapping it, centrifuge at 750 × g for 2 minutes and aspirate the supernatant.
    e. Repeat step‘d’ two more times.
    f. Add the clear lysate (obtained in step 14 of ‘Procedure’ section) to the Ni-NTA resin and mix gently by shaking the tube on a rotary shaker at 4 °C for 60 minutes.
    g. Mount the polypropylene column on the laboratory stand in the cold room.
    h. Gently pour the lysate-containing Ni-NTA resin to obtain a packed bed volume of ~5 *ml*.

i. Equilibrate the column with 3 bed volumes of the binding buffer, keep sufficient binding buffer above the resin bed and stop the flow.
j. Wash the column 4 times with 2 bed volumes of wash buffer.
k. Add 0.5 bed volumes of the elution buffer 10 times collecting the 0.5 *ml* fractions each time.
l. Perform analysis as discussed in 'Results and Analysis' section.

15. Some amount of the protein of interest may be present in insoluble form in the pellet. For recovery of this fraction, the protein can be solubilized using denaturing conditions.
16. Before starting purification, it is recommend to run an SDS-PAGE with the lysate to ensure that the protein was expressed by the bacteria (or whatever expression system was used) in good amount and is present in the lysate.
17. The NTA resin appears light blue-green when charged with nickel ions and turns white when stripped of the ions.

Lecture 26 HPLC of proteins and peptides

Aim:

To check the purity of a protein or peptide using reversed-phase HPLC

Introduction:

HPLC is an acronym for high performance liquid chromatography or high pressure liquid chromatography. The columns that are used in HPLC are made up of very homogeneous small particles. Such columns show improved physical and chemical stability, faster separation, and better reproducibility compared to the traditional columns having soft gels. Very small, tightly packed particles offer a high resistance to the mobile phase flow; the mobile phase therefore needs to be pushed through the column at very high pressures (~500 – 5000 psi). The basic components of an HPLC system are shown in figure 26.1.

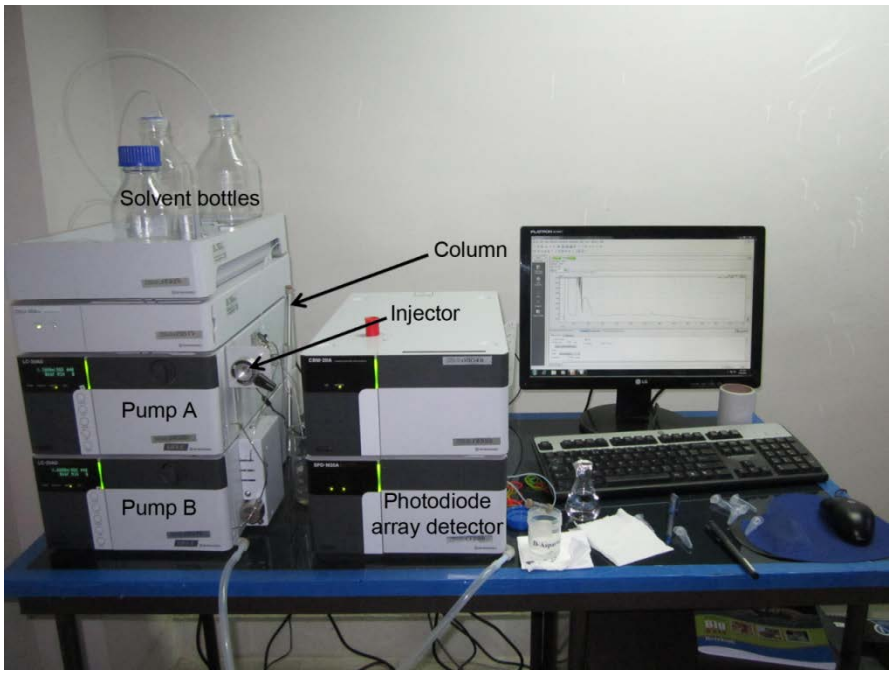

Figure 26.1: An HPLC instrument

1. Pumps:
    a. The role of the pumps is to force the liquids into the column at desired flow rates, expressed as *ml/min*.
    b. Flow rates between 0.5 – 2.0 *ml* are usually sufficient for most experiments.
    c. Pumps in modern instruments can reach pressures in the range of 6000 – 9000 psi.
    d. The pumps can be used for delivering either a constant (isocratic) or changing (gradient) composition of the mobile phase.
2. Injector:
    a. Injector is used for introducing the sample into the mobile phase flow stream just before the column.
    b. The injector is connected with a loop that determines the maximum volume of the sample that can be injected.
    c. The samples are injected using a syringe; the samples can also be introduced into the system using an automatic injector, called an autosampler.
3. Column:
    a. Column is made up of small particles with very high homogeneity. Smaller particles provide more surface area and higher resolution.
    b. As the particle size decreases, the pressure required for achieving optimum mobile phase flow increases.
4. Guard column (Optional):
    a. Guard columns or column guards are the small versions of the analytical columns that are packed with the exactly same material.
    b. They are placed between the injector and the column and collect any insoluble or particulate material present in the sample thereby increasing the column's life.
5. Detector:
    a. An inline detector allows monitoring the chromatographic run in real-time.
    b. Absorbance of light, fluorescence, and change in the refractive index caused by the analyte molecules are the most commonly used detection methods.

6. Fraction collector (Optional)
    a. It collects the fractions of defined volume automatically.
    b. It is completely an optional module as fractions can be collected manually.
7. Computer:
    a. A computer controls all the modules of the HPLC system.
    b. It processes the data, displays it in real-time, and stores it.

Although can be used with any type of chromatographic method, the major separation modes of HPLC are:

1. Size exclusion chromatography (discussed in lectures 22 and 23)
2. Ion exchange chromatography (discussed in lecture 24)
3. Normal phase and adsorption chromatography: In principle, this mode is the column version of thin layer chromatography. The stationary phase is polar (such as silica gel, with or without functionalization with the polar groups) while the mobile phase is non-polar.
4. Reversed-phase chromatography: The name ‘reversed-phase’ has historical significance. In 1970s, liquid chromatography was usually performed with polar stationary phases, usually unmodified silica or alumina. Polar molecules will preferentially bind to the polar stationary phase. This method is now termed as the normal phase chromatography. If the stationary phase is made hydrophobic through covalent attachment of long alkyl chains, it would preferentially bind the hydrophobic molecules. This can be considered the opposite or reverse of the normal phase, hence the term reversed-phase chromatography.

Reversed-phase chromatography finds applications in both analytical and preparative biochemical separations. Since its introduction, reversed-phase chromatography has come a long way to become the method of choice for analytical purposes. The solute molecules that possess some hydrophobic character bind to the hydrophobic stationary phase; reversed-phase chromatography, therefore, is a type of adsorption chromatography. The actual mechanism of binding of the molecules is not very clear but is believed to happen due to favorable entropic effect. The initial mobile phase in reversed-phase chromatography is primarily aqueous. Binding of the solute’s

hydrophobic regions to the stationary phase reduces the exposed hydrophobic area thereby diminishing the degree of ordered water structure and increasing the entropy of the system (Figure 26.2). The bound analyte molecules can then be eluted using a gradient of a non-polar solvent.

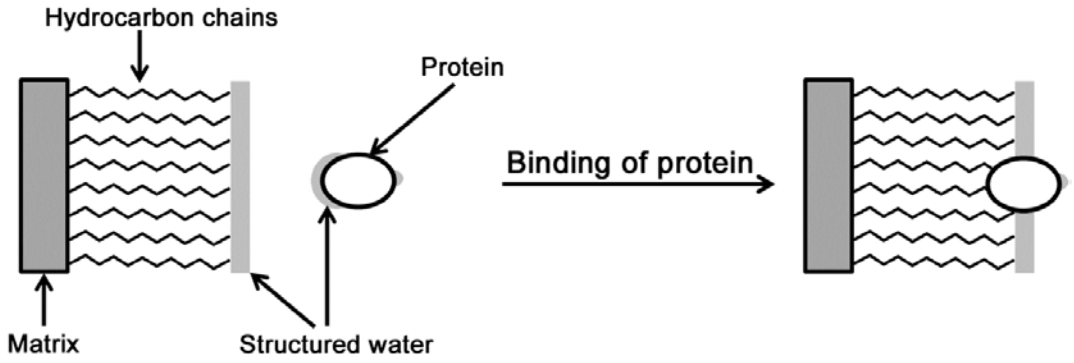

Figure 26.2: Interaction of a protein to a reversed-phase column; binding of hydrophobic regions of the protein to the hydrophobic stationary phase would displace the structured water molecules thereby increasing the entropy of the system.

Materials:

Equipments:

12. HPLC instrument with binary pump and spectrophotometric detection
13. Vacuum pump
14. Centrifuge

Reagents and chemicals:

1. Acetonitrile (HPLC grade)
2. Methanol (HPLC grade)
3. Deionized water
4. Trifluoroacetic acid (TFA)

Glassware, plasticware, and other items:

1. Two 1 liter bottles
2. Two 1 liter measuring cylinders
3. Filter assembly
4. 47 *mm* PTFE membrane filter (0.45μm)
5. Pipettes

6. Pipette tips
7. Reversed-phase C18 column
8. Guard column
9. 13 *mm* syringe filter (0.22 μm)
10. 100 μl injection syringe
11. 1.5 *ml* microfuge tubes
12. Spanners of appropriates sizes (for fixing the column and the guard column)

Reagent preparation

Solvent A (0.1% TFA in deionized water)

1. Measure 1 litre of deionized water using a measuring cylinder.
2. Add ~400 – 500 *ml* of this in the bottle labeled 'Solvent A'.
3. Add 1 ml TFA to the bottle and shake the bottle slowly to achieve mixing.
4. Add rest of the water into the bottle and cover the bottle.
5. Filter the solvent through 47 mm PTFE membrane filter (0.45μm).

Solvent B (0.1% TFA in acetonitrile)

1. Measure 1 litre of HPLC grade acetonitrile using a measuring cylinder.
2. Add ~400 – 500 *ml* of this in the bottle labeled 'Solvent B'.
3. Add 1 *ml* TFA to the bottle and shake the bottle slowly to achieve mixing.
4. Add rest of the acetonitrile into the bottle and cover the bottle.
5. Filter the solvent through 47 mm PTFE membrane filter (0.45μm).

Protein/peptide solution

1. Weigh 1 *mg* of the given protein or peptide and transfer it into a 1.5 *ml* microfuge tube.
2. Add 1 *ml* deionized water to dissolve the protein/peptide.
3. If dissolution is not complete, add 25 μl acetonitrile and vortex the sample.
4. Filter the sample through a 0.22 μm filter to remove any particular material or insoluble protein/peptide. Alternatively, centrifuge the sample at 16,000 × g for 5 minutes and collect the supernatant.

Procedure (*Note 1*):

115. Turn on the HPLC instrument and the software as per the manufacturer's instructions.
116. Wash the inlet filter of the tube connected to pump A (labeled as line 'A') with solvent A and place the filter in the bottle having solvent A.
117. Similarly, wash the inlet filter of the tube connected to pump B (labeled as line 'B') with solvent B and place the filter in the bottle having solvent B.
118. Open the purge valve. Opening the purge valve causes the solvent to bypass the column and travel directly to the waste liquid container.
119. Set the pump at 100% solvent A with a flow rate of 1 *ml/min*.
120. Allow the solvent A to run for 10 minutes, ensure that there are no bubbles in the solvent line.
121. Set the pump at 100% solvent B keeping the flow rate at 1 *ml/min*.
122. Allow the solvent B to run for 10 minutes, ensure that there are no bubbles in the solvent line.
123. Stop the flow by setting a flow rate of 0 *ml/min*.
124. Close the purge valve.
125. Set the pump at 50% solvent A with a flow rate of 0.5 ml/min. Now the eluant will enter the injector and come out of its outlet. You may see some bubbles coming out if injection loop were dry.
126. Allow the eluant to run till the eluant starts flowing without any air bubble.
127. Stop the flow by setting a flow rate of 0 *ml/min*.
128. Open both the caps of the column and connect it with the injector outlet in the proper orientation (Reversed-phase columns come with flow direction indicators).
129. Set the pump at 100% solvent B keeping the flow rate at 0.5 *ml/min*.
130. When the solvent starts coming out of the column, attach the column with the detector inlet.
131. Wash the column with 100% solvent B at a flow rate of 0.5 *ml/min*for at least 20 minutes.

132. Meanwhile, wash the injection loop with solvent B at least 5 times followed by washing with solvent A at least three times.

133. Set the pump at 100% solvent A with a flow rate of 0.5 *ml/min* and allow column equilibration.

134. Set up the instrument parameters:

a. Set the flow rate to 0.5 *ml/min* for the HPLC runs.

b. Select the UV absorption for detection.

c. Set the wavelength to 214 nm. If instrument can record absorbance at multiple wavelengths, as is the case with Photodiode Array Detectors, select two more wavelengths: 254 nm and 280 nm.

d. Set up the gradient as shown in figure 26.3.

(A)

Run time (min)	Percentage of solvent B
0	0
10	0
40	100
50	100
55	0
65	0

(B)

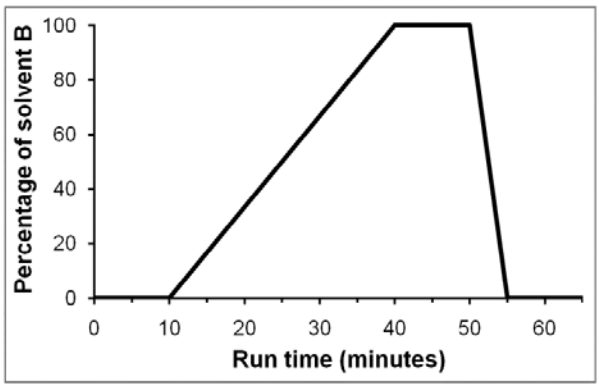

Figure 26.3: A linear gradient of acetonitrile. 0 – 10 minutes is 0% B that allows binding of the analytes to the column; from 10 – 40 minutes, the acetonitrile concentration linearly increases at 3.33% per minute causing elution of the analytes; during 40 – 50 minutes, the column is washed with 100% B causing elution of the molecules that might have not eluted during the linear gradient; during 50 – 55 minutes, the solvent composition returns to 0% B; and during 55 – 65 minutes, the column is equilibrated with 100% A making it ready for next injection.

135. Once the column is equilibrated with solvent A *i.e.* once you see a very stable baseline, set the absorbance to ZERO (for PDA detector, it becomes zero for all the wavelengths).

136. In the appropriate software window, include the sample information, the file name for the chromatogram, and the directory where the chromatogram is to be stored.

137. Turn the injector knob to the 'Load' mode and inject 100 μl of the solvent used for sample dissolution using a clean injection syringe.

138. Turn the injector knob to 'Inject' mode and take out the injection syringe. Turning the injector knob starts the HPLC run. It is, however, possible to start the run at desired time following sample injection.

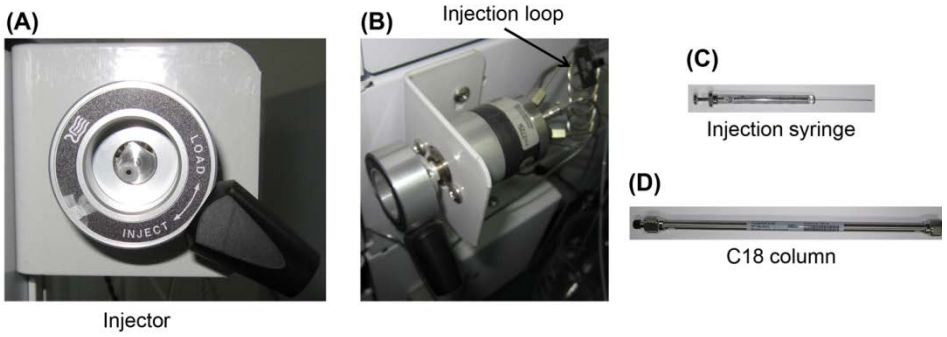

Figure 26.4: Injector in 'Inject' mode (panel A), an injection loop (panel B), an injection syringe (panel C), and a C18 column (panel D)

139. The HPLC run will be complete after 65 minutes (Figure 26.3).

140. As the equilibration step for the next run is already incorporated in the gradient program, the column is ready for next injection.

141. If the 'Blank' run is clean, inject your sample as discussed in the subsequent steps. (If the blank run is not clean, wash the column with 100% B at a flow rate of 1 *ml/min* for 30 minutes and wash injection loop 10 times with solvent B followed by 3 times washing with solvent A. Repeat the blank run).

142. Include the sample information and the file name for the next run.

143. Turn the injector knobto 'Load' mode, inject 100 μl of the protein/peptide sample, and turn the knob to 'Inject mode'.

144. Monitor the HPLC run and collect the peaks, if the protein/peptide is to be analyzed/used further.

145. After the completion of HPLC run, set the flow rate to 0 *ml/min*.

146. Once the instrument displays 0 *ml/min* flow rate, take out line B from the solvent bottle.

147. Rinse the inlet filter of line B with methanol and put it in methanol bottle.

148. Open the purge valve.

149. Set the pump at 100% solvent B (which is methanol now) with a flow rate of 1 *ml/min*.

150. Allow the methanol to run for 10 minutes.

151. Close the purge valve.

152. Set the pump at 100% B with a flow rate of 1 *ml/min* and let it run for 20 minutes.

153. Set the flow rate to 0 *ml/min*.

154. Once the instrument displays zero pressure,switch ‘off’ the instrument as per manufacturer’s instructions.

155. Remove the column, cap both the ends and store it at room temperature till further use.

156. Connect the outlet of the injector and the inlet of the detector with an adapter provided with the instrument; this slows down drying of the solvent lines.

Results and analysis:

1. Go to ‘Data Analysis’ after the run is complete; in modern instruments, the software automatically calculates the area under the peaks.
2. Calculate the percentage purity of the peaks as follows:

$$\text{Percentage purity of peak } n = \frac{\text{Area under peak } n}{\text{Total area under all the peaks}} \times 100$$

3. The peak that corresponds to the protein/peptide of interest is identified by determining the mass of the collected fractions using mass spectrometry.

Notes:

1. The exact procedure may vary from instrument to instrument but the procedure discussed here should give the user an idea how to go ahead for performing reversed-phase chromatography on any HPLC instrument.