Unit-5

Instrumental Methods of Analysis

B.Pharma 7 Sem Notes

Unit: 5

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- **Ion exchange chromatography**- Introduction, classification, ion exchange resins, properties, mechanism of ion exchange process, factors affecting ion exchange, methodology and applications.
- **Gel chromatography-** Introduction, theory, instrumentation and applications.
- **Affinity chromatography-** Introduction, theory, instrumentation and applications.

ION EXCHANGE CHROMATOGRAPHY (IEC)

Ion Exchange Chromatography (IEC)

Introduction

Ion Exchange Chromatography (IEC) is a separation technique that exploits the ability of ions to exchange between a solution and a solid material carrying fixed ionic charges. The stationary phase is an ion exchange resin — an insoluble matrix bearing charged functional groups that can reversibly attract and hold oppositely charged ions from the mobile phase.

Simple Analogy — Understanding Ion Exchange
Imagine a magnetic notice board covered with positively-charged pins (— the resin).
You throw a handful of differently-coloured balls (positively and negatively charged ions).
Only the NEGATIVELY charged balls stick to the pins; positively charged balls bounce off.
If you now spray a strong salt solution over the board, some stuck balls are displaced
by the salt ions and fall off — that is elution! This is exactly how an anion exchange
resin works. Cation exchangers do the reverse — they attract positive ions.

Ion exchange is found everywhere in nature — in soil (holding mineral nutrients), in kidneys (regulating blood electrolytes), and in living cells. Analytically, IEC was first described by Thompson and Way in 1850, and resins were developed commercially from the 1940s onwards.

Historical Development

Year	Milestone
1850	Thompson & Way discover natural zeolite (aluminosilicate) ion exchange in soil
1935	Adams & Holmes synthesise first synthetic organic ion exchange resins (phenol-formaldehyde)
1944	D'Alelio patents sulfonated polystyrene cation exchange resin — foundation of modern resins
1950s	Ion exchange used in nuclear industry (uranium purification); sugar decolourisation
1975+	HPIC (High-Performance Ion Chromatography) developed by Small, Stevens & Bauman
Today	IEC is the primary method for amino acids, proteins, nucleotides, water purification, pharma

Classification of Ion Exchange Resins

Ion exchange resins are classified in two main ways: by the TYPE of ion they exchange (cation vs anion) and by the STRENGTH of their ionisation (strong vs weak).

A. Based on the Type of Ion Exchanged

1. Cation Exchange Resins

Cation exchangers carry NEGATIVE fixed charges on the resin matrix and therefore attract and hold POSITIVE ions (cations) from the solution. The resin releases its own exchangeable cation (usually H^+ or Na^+) into the solution in exchange.

Type	Fixed Group on Resin	Exchangeable Counter-ion	Example Trade Name
Strong Acid Cation (SAC)	Sulphonic acid (—SO_3H)	H^+ (H-form) or Na^+ (Na-form)	Dowex 50, Amberlite IR-120, Zeo-Karb 225
Weak Acid Cation (WAC)	Carboxylic acid (—COOH)	H^+	Amberlite IRC-50, Zeo-Karb 226
Phosphonic acid cation	Phosphonic acid (—PO_3H_2)	H^+	Specialised resins for metal removal

2. Anion Exchange Resins

Anion exchangers carry POSITIVE fixed charges on the resin matrix and therefore attract and hold NEGATIVE ions (anions) from the solution. They release their own exchangeable anion (usually OH^- or Cl^-) in exchange.

Type	Fixed Group on Resin	Exchangeable Counter-ion	Example Trade Name
Strong Base Anion (SBA) — Type I	Quaternary ammonium — $N^+(CH_3)_3$	OH^- or Cl^-	Dowex 1, Amberlite IRA-400
Strong Base Anion (SBA) — Type II	Quaternary ammonium — $N^+(CH_3)_2(C_2H_4OH)$	OH^-	Dowex 2, Amberlite IRA-410
Weak Base Anion (WBA)	Primary/secondary/tertiary amine (—NH_2, —NHR, —NR_2)	OH^- (only when protonated at low pH)	Amberlite IR-45, Duolite A-2

B. Based on Strength of Ionisation

Property	Strong Acid/Base Resins	Weak Acid/Base Resins
Ionisation	Completely ionised over the FULL pH range (pH 0–14)	Only ionised in a LIMITED pH range

Property	Strong Acid/Base Resins	Weak Acid/Base Resins
Capacity	Constant capacity regardless of pH	Capacity varies with pH (max capacity in favourable pH)
Best used at	Any pH	Specific pH ranges only
Example functional group	SAC: —SO_3H; SBA: —$N^+(CH_3)_3$	WAC: —COOH (effective pH >6); WBA: —NH_2 (effective pH <6)
Elution	Requires strong salt or acid/base	Milder conditions sufficient
Regeneration	Requires strong HCl (for SAC) or NaOH (for SBA)	Milder regenerants; easier to regenerate

Ion Exchange Resins — Structure and Properties

A. Physical Structure of an Ion Exchange Resin

Most ion exchange resins are made from a CROSS-LINKED POLYMER MATRIX (usually polystyrene cross-linked with divinylbenzene, DVB) onto which charged functional groups are chemically attached.

Structure of a Typical Cation Exchange Resin (SAC)
1. POLYMER MATRIX: Polystyrene chains cross-linked with divinylbenzene (DVB).
— DVB percentage determines cross-linking: More DVB → more rigid, smaller pores, less swelling.
— Typical cross-linking: 8% DVB (denoted X8, e.g., Dowex 50–X8).
2. FIXED IONIC GROUPS: Sulphonic acid groups (—SO_3^-) chemically bonded to the polymer.
— These groups are FIXED and cannot leave the resin.
3. COUNTER-IONS (exchangeable): H^+ ions that are attracted to the —SO_3^- groups.
— These H^+ ions CAN be exchanged for other cations from the solution.
When Na^+ solution passes through: Na^+ displaces H^+ from the resin.
Resin–SO_3^- H^+ + Na^+ ⇌ Resin–SO_3^- Na^+ + H^+

B. Important Properties of Ion Exchange Resins

Property	Definition	Typical Values & Significance
1. Ion Exchange Capacity	The number of milliequivalents of ions that 1 gram (dry) or 1 mL (wet) of resin can exchange.	Expressed as meq/g or meq/mL. SAC resins: 1.7–2.0 meq/mL. Higher capacity = can process more sample before exhaustion.
2. Cross-linking Degree	Percentage of DVB (divinylbenzene) used in synthesis.	Common: 4%, 8% (X4, X8), 12% DVB. Higher cross-

Property	Definition	Typical Values & Significance
	Controls pore size and mechanical strength.	linking: more rigid, smaller pores, slower kinetics but better selectivity.
3. Mesh Size (Particle Size)	Particle size of the resin beads, expressed as US mesh number.	50–100 mesh (149–297 µm) for column use. 100–200 mesh for analytical/HPIC. Smaller beads = better resolution but slower flow.
4. Swelling	Resins swell when equilibrated with water or buffer. Degree depends on cross-linking and ion form.	Low DVB resins swell more. Must equilibrate resin in the target buffer BEFORE packing the column.
5. Selectivity / Affinity	The relative preference of the resin for one ion over another. Governs the order of elution.	Determines which ions are retained longer on the resin (see selectivity sequences below).
6. Chemical Stability	Resistance to degradation by acid, base, oxidising agents, and organic solvents.	SAC/SBA resins are stable over wide pH (1–14). WAC/WBA resins have narrower pH stability.
7. Physical Form	Spherical beads (preferred for columns) or granules.	Spherical beads give more uniform packing, lower backpressure, and better reproducibility.

C. Selectivity Series for Ion Exchange

The selectivity of a resin for different ions follows a general order based on the charge, hydrated ionic radius, and polarisability of the ion:

Selectivity Order for Strong Acid Cation Resins (Cation Exchange Affinity)
ORDER OF INCREASING AFFINITY (weakest → strongest retention):
$Li^+ < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+ < Ag^+ < Tl^+$
(monovalent cations → larger ions retained more strongly)
$Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$
(divalent cations retained MORE strongly than monovalent)
KEY RULE: Higher charge → stronger retention. For same charge: larger hydrated radius → stronger retention.

Selectivity Order for Strong Base Anion Resins (Anion Exchange Affinity)

ORDER OF INCREASING AFFINITY (weakest → strongest retention):
$F^- < OH^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < SO_4^{2-}$
KEY RULES: Fluoride is the weakest; sulphate (divalent) is the strongest.
Citrate, EDTA, and large organic anions can also be used to displace tightly held anions (chelation elution).

Mechanism of Ion Exchange Process

The ion exchange process is an equilibrium reaction. When a sample solution containing ions is passed through an ion exchange column, the following steps occur:

Step	Process	Description
1	Sample application	Sample ions dissolved in mobile phase enter the top of the column; mobile phase is usually a dilute buffer of low ionic strength.
2	Diffusion to resin surface	Sample ions diffuse through the bulk solution to the surface of the resin bead (film diffusion).
3	Diffusion into resin pores	Ions diffuse through the pores of the resin bead to reach the fixed ionic sites inside (particle diffusion — often rate-limiting step).
4	Ion exchange reaction	Sample ions displace the exchangeable counter-ions from the fixed sites on the resin. The exchange is REVERSIBLE and governed by the equilibrium constant (selectivity coefficient).
5	Diffusion of displaced ions outward	The displaced counter-ions (H^+ or Na^+) diffuse out of the resin and into the bulk mobile phase.
6	Elution	An eluting solution (usually a buffer with higher ionic strength or different pH) is passed through. Eluent ions compete with the adsorbed sample ions for the fixed sites and displace them in order of their affinity (weakest affinity → elutes first).
7	Regeneration	After the run, the resin is regenerated back to its original ionic form (H-form or OH-form) by passing a strong acid or base through the column.

The ion exchange reaction for a cation exchange resin can be represented as:

$$\text{Resin–A}^+ + \text{B}^+(\text{solution}) \rightleftharpoons \text{Resin–B}^+ + \text{A}^+(\text{solution})$$

The equilibrium constant for this exchange is called the SELECTIVITY COEFFICIENT (K_A/B):

$$K(A/B) = [\text{Resin–B}^+][\text{A}^+(aq)] / [\text{Resin–A}^+][\text{B}^+(aq)]$$

If K > 1, the resin prefers B over A — B is more tightly retained.

Factors Affecting Ion Exchange

Factor	Effect on Ion Exchange	Practical Implication
1. Nature of the Ion (Charge)	Higher ionic charge → STRONGER retention on resin (more electrostatic attraction)	Divalent ions (Ca^{2+}, Mg^{2+}) are held much more tightly than monovalent (Na^{+}, K^{+}); need stronger eluent to displace them
2. Hydrated Ionic Radius	Smaller hydrated radius → closer approach to fixed site → STRONGER retention	Among monovalent cations: $Cs^{+} > K^{+} > Na^{+} > Li^{+}$ in affinity (despite Li^{+} being smallest bare ion, its large hydration shell keeps it away from the resin)
3. pH of the Mobile Phase	Controls ionisation of both the resin functional groups and the analyte molecules	WAC resins: only effective above pH 6 (COOH must be deprotonated). WBA resins: only effective below pH 6 (amine must be protonated). pH controls charge on amino acids, peptides, proteins — crucial for biomolecule separations
4. Ionic Strength of Eluent	Higher ionic strength (more salt) → STRONGER competition with bound ions → FASTER elution	Low ionic strength: weak elution, good retention. High ionic strength: strong elution, displaces retained ions. Gradient from low to high ionic strength is used for complex samples.
5. Temperature	Higher temperature → faster exchange kinetics → sharper peaks; also shifts equilibrium	Amino acid analysers typically operate at 50–60°C for better resolution and speed.
6. Cross-linking of Resin	More cross-linking (more DVB) → smaller pores → SLOWER diffusion → better selectivity but slower kinetics	Low cross-linking used for large molecules (proteins); high cross-linking for small ions and better selectivity.
7. Flow Rate	Lower flow rate → more time for equilibration → better resolution (up to optimum)	Excessively slow flow rate wastes time; too fast causes peak broadening (kinetic effects). Optimal flow rate must be determined for each system.
8. Nature of Counterion	The initial exchangeable ion on the resin (H^{+}, Na^{+}, OH^{-}, Cl^{-}) affects initial selectivity and capacity	Resins are converted to the desired counter-ion form before use (conditioning).
9. Polarity & Hydrophobicity of Analyte	Large organic ions may interact with resin matrix by hydrophobic interactions in addition to ion exchange	Can improve or worsen selectivity depending on the compound; organic solvents in mobile phase can modulate this.

Methodology of Ion Exchange Chromatography

Step 1: Selection of Resin

- Choose based on the analyte charge:

- Cation exchange resin (SAC or WAC) for positively charged analytes (Na^+, K^+, Ca^{2+}, amino acids at low pH, basic drugs, proteins with pI below running pH).
- Anion exchange resin (SBA or WBA) for negatively charged analytes (Cl^-, NO_3^-, SO_4^{2-}, amino acids at high pH, acidic proteins at pH above their pI).

- Select resin capacity, particle size, and cross-linking based on scale and resolution required.

Step 2: Resin Pretreatment and Conditioning

- Wash the dry resin with distilled water to remove fines. Allow to swell completely (30–60 min). Fine particles are removed by decanting.
- Convert to desired ionic form: For SAC resin in H-form — wash with 2M HCl (3–5 column volumes). For SAC in Na-form — wash with 2M NaCl after HCl treatment.
- Equilibrate with starting buffer (3–5 column volumes) until pH and conductivity of the effluent match the inlet buffer. The resin is now ready.

Step 3: Column Packing

- Use a glass or plastic column with an inlet (top) and a frit/mesh at the bottom. Prepare a slurry of the conditioned resin in starting buffer.
- Pour the slurry into the column in one continuous addition while allowing buffer to drain slowly. Avoid trapping air bubbles.
- Apply a steady flow of buffer until the resin bed is stable (not settling further). The bed must be uniform and free of channels.
- Typical column size for analytical work: 1 cm diameter × 10–20 cm long.

Step 4: Sample Application

- Dissolve or dilute sample in the STARTING BUFFER (low ionic strength). This ensures the sample is in the correct ionic form and will bind to the resin.
- Load sample slowly onto the top of the column. Allow it to absorb into the resin.
- Wash the column with starting buffer (2–3 column volumes) to remove unretained components (those that did not bind to the resin).

Step 5: Elution

- Elute retained ions using:
  - Stepwise gradient: Increase salt concentration in a series of steps (e.g., 0.1 M → 0.5 M → 1.0 M NaCl).
  - Linear gradient: Continuously increase salt concentration using a gradient mixer.
  - pH gradient: Change the pH to alter the charge on the analyte and decrease its affinity for the resin.
- Collect the eluate in fractions (using a fraction collector). Each fraction is analysed for the compound of interest.

Step 6: Detection

- UV Detector (most common for pharmaceutical compounds): Measure absorbance at 254 nm or 280 nm (for aromatic compounds, proteins).
- Conductivity Detector: Measures ionic conductivity of the eluate; universal for inorganic ions; used in High-Performance Ion Chromatography (HPIC).

- Post-column derivatization and colorimetric detection: Used in amino acid analysers (ninhydrin at 570/440 nm for all amino acids).
- Refractive Index, Fluorescence, or Mass Spectrometry detectors can also be used.

Step 7: Regeneration and Reuse

- After the run, wash the column with a strong eluent (1–2 M HCl for SAC; 1 M NaOH for SBA) to remove all retained components.
- Re-equilibrate with starting buffer. Ion exchange resins can be reused hundreds of times if maintained properly.

Types of Elution Techniques in IEC

Elution Mode	Description	Advantage	Best For
Isocratic Elution	Single, constant buffer composition throughout the run	Simple; reproducible; no re-equilibration needed	Simple mixtures; routine QC
Step Gradient	Salt or pH increased in discrete steps	Good selectivity control; easy to implement manually	Batch elution of distinct groups of ions
Linear Gradient	Continuous, smooth increase in ionic strength or pH change	Best resolution; all peaks elute with similar widths; quantitative	Complex mixtures: proteins, amino acids, nucleotides
Displacement Chromatography	A displacer ion (with higher affinity than analytes) is loaded after sample	Very high loading capacity; all analytes eluted as pure, concentrated bands	Preparative purification of large amounts

Applications of Ion Exchange Chromatography

Application Area	Specific Example
Amino Acid Analysis	Amino acid analysers use sulphonated polystyrene cation exchange resin with lithium citrate buffer gradient + ninhydrin detection. Separates all 20 amino acids from protein hydrolysates.
Protein Purification	IEC is a primary step in purification of therapeutic proteins (insulin, erythropoietin, monoclonal antibodies) and enzymes. DEAE-cellulose (anion exchanger) widely used.
Water Purification (Deionisation)	SAC + SBA resins in series remove ALL ions from water: Resin-H replaces cations with H^+; Resin-OH replaces anions with OH^-. $H^+ + OH^- \rightarrow H_2O$. Produces ultra-pure deionised water (conductivity < 0.1 µS/cm).
Pharmaceutical Drug Formulations	Ion exchange resins used in extended-release formulations: drug ion bound to resin → releases slowly in GI tract as ions compete. Example: Amberlite IRP-69 for cationic drugs like amphetamine, chlorpheniramine.

Application Area	Specific Example
Inorganic Ion Analysis (HPIC)	Simultaneous determination of anions (F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}, PO_4^{3-}) and cations (Li^+, Na^+, K^+, Ca^{2+}, Mg^{2+}) in water samples, food, biological fluids.
Sugar and Carbohydrate Separation	Strong acid cation resins in Ca^{2+} form separate mono- and disaccharides by size and ligand interaction. Used in food, pharmaceutical, and bioethanol industries.
Biopharmaceutical QC	Analysis of charge variants, glycoforms, and post-translational modifications of protein drugs. Critical in the characterisation of biosimilars.
Uranium & Metal Recovery	Selective extraction and concentration of uranium, lanthanides, and other metal ions from solutions using chelating ion exchange resins.

GEL CHROMATOGRAPHY (SIZE EXCLUSION CHROMATOGRAPHY)

Gel Chromatography

Introduction

Gel Chromatography — also known as Size Exclusion Chromatography (SEC), Gel Filtration Chromatography (GFC), or Gel Permeation Chromatography (GPC) — is a separation technique that separates molecules entirely based on their SIZE (hydrodynamic volume) as they pass through a column packed with porous gel beads. There is NO chemical interaction between the molecules and the stationary phase; separation is purely physical.

Simple Analogy — The Sponge in a River
Imagine placing sponges (gel beads) in a flowing river (mobile phase). Small fish (small molecules)
can swim INTO the sponge pores and get temporarily trapped inside, delaying their journey.
Big fish (large molecules) are TOO BIG to enter the sponge pores — they flow freely around the
outside and come out at the other end FIRST (shortest path).
Medium fish enter some pores but not all — they come out in BETWEEN.
Result: Large molecules elute FIRST, small molecules elute LAST.

Gel Filtration Chromatography was first developed by Porath and Flodin in 1959 using dextran-based Sephadex gels for proteins and other water-soluble biomolecules. Gel Permeation Chromatography (for organic-soluble polymers) was developed by Moore in 1964.

Theory of Gel Chromatography

A. The Separation Principle

The gel beads used as stationary phase are porous spheres with a specific, defined pore size distribution. When the sample mixture is passed through the column:

Molecule Size (Relative to Pore Size)	Behaviour	Elution Order	Example
LARGER than ALL pores (excluded)	Cannot enter any pores. Flows only through the VOID VOLUME (space between beads). Shortest path.	Elutes FIRST (earliest)	Very large proteins, virus particles, aggregates
SMALLER than ALL pores (totally included)	Enters ALL pores freely. Travels through	Elutes LAST (latest)	Small molecules: salts, amino acids, glucose, buffer

Molecule Size (Relative to Pore Size)	Behaviour	Elution Order	Example
	maximum volume of gel.		
INTERMEDIATE size	Partially included — can enter some pores but not the smallest ones.	Elutes between the two extremes — separated by size	Most proteins (20–200 kDa), oligosaccharides, DNA fragments

B. Key Volume Parameters in Gel Chromatography

Volume Terms You MUST Know
V_0 = VOID VOLUME: Volume of mobile phase OUTSIDE the gel beads (between particles).
— Determined by running Blue Dextran 2000 (MW ~2,000,000 Da; totally excluded).
— Large molecules elute at V_0.
Vi = INTERNAL VOLUME: Volume of solvent INSIDE the gel pores.
— Determined by running a small molecule (D_2O, acetone, salt).
Vt = TOTAL VOLUME of column = V_0 + Vi + Vs (Vs = volume of dry gel matrix itself).
Ve = ELUTION VOLUME of a compound: Volume of mobile phase needed to elute it.
— For totally excluded molecules: Ve = V_0
— For totally included molecules: Ve = V_0 + Vi
— For partially included: V_0 < Ve < (V_0 + Vi)

C. Distribution Coefficient (Kav or Kd)

The distribution coefficient describes how much of the molecule's time is spent inside the gel pores:

$$Kav = (Ve - V_0) / (Vt - V_0)$$

Kav Value	Meaning
Kav = 0	Molecule is TOTALLY EXCLUDED (too large for any pore); elutes first at V_0
0 < Kav < 1	Molecule is PARTIALLY included; separated based on size; linear calibration range

Kav Value	Meaning
Kav = 1	Molecule is TOTALLY INCLUDED in ALL pores; elutes last
Kav > 1	Molecule has some affinity for gel matrix (non-ideal behaviour); adsorption occurring

D. Molecular Weight Calibration Curve

The most important application of gel chromatography is molecular weight determination of unknown proteins. By running known molecular weight standards, a calibration curve is constructed:

- **Plot: log (Molecular Weight) on Y-axis vs Kav (or Ve) on X-axis.**
- **A LINEAR relationship exists in the FRACTIONATION RANGE of the gel.**
- **From the calibration curve, the molecular weight of an unknown protein can be read off from its measured Kav.**
- **This is the most widely used method for estimating protein molecular weight in solution (native conditions, unlike SDS-PAGE which denatures the protein).**

Types of Gel Media (Stationary Phases)

Different gel materials are used for aqueous (GFC) and organic solvent (GPC) separations:

A. Hydrophilic Gels (for Gel Filtration Chromatography — aqueous systems)

Gel Name	Base Material	Fractionation Range	Features
Sephadex G-series	Cross-linked dextran (polysaccharide)	G-10: 700–1,000 Da up to G-200: 5,000–600,000 Da	Original gel filtration medium; stable, reproducible; G-25 most used for desalting
Sepharose (Agarose gels)	Cross-linked agarose	4B: 60,000–0.2×10⁶ Da; 2B: 70,000–40×10⁶ Da	For very large proteins, nucleic acids, viruses; large pore size
Sephacryl S-series	Allyl dextran + bisacrylamide	S-100 to S-500: 1,000–250,000 Da	Rigid; withstands higher flow rates; good mechanical stability
Bio-Gel P-series	Polyacrylamide	P-2: 100–1,800 Da to P-300: 60,000–400,000 Da	Excellent for peptides and small proteins; narrow fractionation range
Superdex (Composite)	Cross-linked dextran + agarose composite	Superdex 75: 3,000–75,000; Superdex 200: 10,000–600,000 Da	High resolution; rigid beads; used in modern FPLC/HPLC-SEC systems

B. Non-hydrophilic Gels (for Gel Permeation Chromatography — organic solvents)

Gel Name	Base Material	Solvent Compatibility	Used For
Styragel	Cross-linked polystyrene-DVB	Organic (THF, toluene, DMF)	Synthetic polymer MW distribution; rubber, plastics industry
Bio-Beads S-X series	Polystyrene-DVB	Organic solvents	Removal of small molecules from polymers; polymer fractionation
Sephadex LH-20	Hydroxypropyl dextran	Both aqueous AND organic	Lipids, natural products, steroids; versatile

Sephadex Nomenclature Explained
The 'G' number in Sephadex G-series represents the WATER REGAIN value × 10.
Sephadex G-25: Absorbs 2.5 mL water per gram of dry gel.
Sephadex G-200: Absorbs 20 mL water per gram of dry gel.
Higher G number → MORE water absorbed → LARGER pores → higher molecular weight fractionation range.
Most Common Uses:
G-25 and G-50: DESALTING (remove small MW salt from protein solution); group separation.
G-75, G-100, G-150, G-200: Protein separation and molecular weight determination.

Instrumentation of Gel Chromatography

Gel chromatography equipment ranges from simple gravity-flow glass columns to sophisticated FPLC (Fast Protein Liquid Chromatography) and HPLC-SEC systems. The key components are:

Component	Description	Specifications
1. Column	Glass or acrylic column with end fittings; must be vertical, uniform, and free of air bubbles	Analytical: 1–1.6 cm × 30–60 cm; Preparative: 2.6–26 cm diameter; Gel columns should be 10–20× longer than wide for best resolution
2. Mobile Phase (Eluent)	Aqueous buffer for GFC; organic solvent for GPC; must be degassed and filtered (0.2–0.45 µm)	GFC: Phosphate, Tris, acetate buffer pH 5–8; NaCl (0.1–0.5 M) to suppress non-specific ionic interactions

Component	Description	Specifications
3. Pump (for HPLC-SEC/FPLC)	Peristaltic pump (low flow, gravity systems) or high-pressure HPLC pump for rigid packings	Flow rate: 0.5–3 mL/min for analytical; HPLC-SEC can use 0.5–1.5 mL/min at moderate pressure
4. Sample Injector	Manual syringe application (gravity) or FPLC/HPLC injector with sample loop	Sample volume: 1–5% of total column volume for best resolution. Overloading causes peak broadening.
5. UV Detector	Continuous UV absorbance monitoring of column effluent at 280 nm (proteins), 260 nm (nucleic acids), or 214 nm (peptide bonds)	Most common: Variable wavelength UV or photodiode array; RI detector for carbohydrates and polymers
6. Fraction Collector	Automatically collects the column eluate into numbered tubes or vials at set time or volume intervals	Allows downstream processing, assay, or further purification of each fraction
7. Chart Recorder / Data System	Records UV signal over time as the chromatogram (elution profile)	Modern systems use computer workstations (UNICORN for FPLC, Empower for HPLC)

Gel Preparation and Column Packing

- Swell the dry gel: Add excess buffer (5–10 mL buffer per gram of dry gel). Sephadex gels: let swell in warm water (2 h at room temperature or 1 h at 90°C for faster swelling). DO NOT use magnetic stirring (damages beads).
- Remove fines: After swelling, let beads settle and decant the cloudy supernatant (fine particles) 2–3 times. Fines cause uneven flow and high backpressure.
- Degas the gel slurry under vacuum with gentle swirling to remove trapped air bubbles.
- Pack the column: Close the bottom outlet. Pour gel slurry in a single continuous addition (do not pour in batches — causes uneven layers). Open outlet and let gel settle under buffer flow. Fill to the desired bed height.
- Check packing uniformity: Run Blue Dextran 2000 (1 mg/mL solution) through the column. The blue band should be sharp, symmetrical, and move uniformly through the column. A distorted band indicates poor packing.

Special Technique: Desalting / Group Separation

Desalting — The Most Practical Application of Sephadex G-25
Problem: Protein solution contains high concentration of salt (ammonium sulphate from precipitation,
buffer salts, etc.) that interferes with downstream steps.
Solution: Pass sample through a Sephadex G-25 column equilibrated with the new buffer.
Result: The PROTEIN (large, excluded from pores) elutes rapidly in the VOID VOLUME (Kav = 0).

The SALT (small, totally included) elutes much later.
The two are completely separated in a single fast step (2–5 min on a small column).
This is called a GROUP SEPARATION — two groups (large vs small) separated, not individual molecules.
Also used to change buffer (buffer exchange) of protein samples.

Applications of Gel Chromatography

Application	Details
Molecular Weight Determination	Run protein sample with MW standards; plot log(MW) vs Kav; read off unknown MW from calibration line. Gives MW in native (non-denatured) solution state.
Protein Purification	Separates proteins by size as part of a multi-step purification protocol. Useful when proteins differ significantly in size. Resolution improves with longer columns.
Desalting & Buffer Exchange	Sephadex G-25 or G-50 rapidly removes small molecules (salts, dyes, free drug) from macromolecule solutions. Column equilibration buffer becomes the new buffer for the protein.
Polymer Molecular Weight Distribution (GPC)	Styragel columns in THF separate synthetic polymers by size. Compare with polystyrene standards to get Mn, Mw, and PDI (polydispersity index) for quality control of polymers.
Separation of Oligomers / Aggregates	Detect and remove protein aggregates, oligomers, and fragments from monomer preparations. Critical QC step for biopharmaceuticals (monoclonal antibodies, vaccines).
Liposome Characterisation	Assess size distribution of liposomal drug formulations. Separate free drug from encapsulated drug.
Removal of Low MW Interferents	Remove unconjugated small molecule drugs or fluorescent labels from antibody-drug conjugates (ADCs) or antibody-fluorophore conjugates.
Nucleic Acid Purification	Separate large DNA/RNA from free nucleotides, primers, and small RNA using agarose gels (Sepharose 2B, 4B).

AFFINITY CHROMATOGRAPHY

Affinity Chromatography

Introduction

Affinity Chromatography is the most SELECTIVE of all chromatographic techniques. It exploits the highly specific, reversible binding interaction that exists naturally between biological molecules — for example, an enzyme and its substrate, an antibody and its antigen, or a receptor and its ligand. This biological specificity is harnessed to separate a target molecule from a complex mixture in a single step with extraordinary purity.

Simple Analogy — The Lock and Key
Imagine a wall covered with millions of specific 'locks' (the immobilised ligand on the column).
When you pour a complex mixture of different molecules through it, ONLY the molecule with the
correct 'key' (the target molecule with complementary structure) fits the lock and is held back.
Everything else flows straight through and is washed away.
To recover your target molecule (unlock it), you simply change the conditions
(pH, ionic strength, add competitor) to weaken the lock-key interaction — and the target elutes
in a pure, concentrated form. This is the extraordinary power of affinity chromatography.

Affinity chromatography was first described by Cuatrecasas, Wilchek, and Anfinsen in 1968. A single affinity chromatography step can often achieve what might take 5–10 conventional chromatography steps to accomplish, and with purity exceeding 90–95%.

Theory and Principles of Affinity Chromatography

A. Components of an Affinity Column

Component	Role	Examples
1. Matrix (Support)	Insoluble, porous material to which the ligand is attached. Must be non-specific (does not adsorb other proteins), rigid, stable, and have functional groups for ligand attachment.	Agarose (Sepharose CL-4B, CL-6B) — most common; also silica, cellulose, polyacrylamide, controlled-pore glass
2. Spacer Arm	A flexible chain (6–12 atoms long) that separates the ligand from the matrix surface, reducing steric hindrance and	Hexanediamine (6-carbon), ε-aminocaproic acid; without spacer arm, ligand may be too close to the matrix and inaccessible to the target

Component	Role	Examples
	allowing better access for the target molecule.	
3. Ligand	The biospecific molecule immobilised on the matrix. Must retain its activity and binding specificity after immobilisation. Chosen based on the target molecule.	Enzyme substrates/inhibitors, antibodies, lectins, protein A/G, dyes (Cibacron Blue), metal ions, biotin, vitamins
4. Target Molecule (Ligate)	The molecule to be purified. Must bind specifically and reversibly to the immobilised ligand.	Enzymes (bind substrate/inhibitor ligand); antibodies (bind protein A/G); glycoproteins (bind lectin); His-tagged proteins (bind Ni^{2+}-NTA)

B. The Four Phases of Affinity Chromatography

Phase	Conditions	What Happens
1. Equilibration	Column washed with binding buffer (pH and ionic strength optimised for strong binding)	Ligand on matrix is in the correct conformation and environment for specific binding
2. Sample Application (Adsorption)	Sample in binding buffer applied at slow flow rate (allow sufficient contact time)	Target molecule binds SPECIFICALLY to the ligand; all other molecules pass straight through (flow-through fraction contains unwanted proteins)
3. Washing	Wash with several column volumes of binding buffer; may use slightly stringent conditions	Removes non-specifically bound proteins and impurities while keeping the target bound
4. Elution	Change conditions to WEAKEN or BREAK the target–ligand interaction; collect pure target	Specific or non-specific conditions used to release the pure target molecule in a concentrated, pure form

C. Elution Strategies

The choice of elution method depends on the strength of the interaction and the stability of the target molecule:

Elution Method	Principle	Example	Advantage
Specific (Biospecific) Elution	Add a soluble competitor that competes with the ligand for the same binding site on the	Elute glucose oxidase with free glucose; elute enzyme with free substrate or inhibitor	Mild conditions; target retains native structure and activity

Elution Method	Principle	Example	Advantage
	target molecule. Target is displaced.		
Non-Specific (pH Change)	Lower pH (e.g., to 2–3) disrupts ionic, hydrogen bond, and hydrophobic interactions	Protein A/G columns eluted with 0.1 M glycine pH 2.7 to release IgG	Simple; universally applicable
Non-Specific (High Salt)	Very high salt concentration (1–3 M NaCl or KCl) disrupts electrostatic and some hydrophobic interactions	Elution from ion-based affinity resins	Milder than low pH; good for acid-sensitive proteins
Non-Specific (Denaturing)	Chaotropic agents (urea, guanidine HCl) unfold the target protein, disrupting all non-covalent interactions	Used when target is otherwise not releasable	Complete elution; but protein is denatured (must renature)
Non-Specific (Polarity Change)	Organic solvents (10–50% ethylene glycol, dioxane) disrupt hydrophobic interactions	Hydrophobic affinity resins	Good for hydrophobic targets

D. Important Quantitative Concept — Dissociation Constant (Kd)

The strength of the interaction between the target molecule (T) and ligand (L) is described by the dissociation constant Kd:

$$T + L \rightleftharpoons TL \qquad Kd = [T][L] / [TL]$$

Kd Value	Affinity Strength	Suitability for Affinity Chromatography	Example Interaction
Kd = 10^{-4} to 10^{-6} M	Weak–moderate	Borderline — may not retain target well enough	Enzyme–substrate interactions (weak)
Kd = 10^{-7} to 10^{-9} M	Strong	IDEAL for affinity chromatography	Most enzyme–inhibitor interactions; protein A–IgG
Kd = 10^{-10} to 10^{-12} M	Very strong (near-irreversible)	Very tight — requires harsh conditions for elution	Biotin–streptavidin (Kd ~ 10^{-13} M); used in capture only, not purification

Types / Classification of Affinity Chromatography

Type	Immobilised Ligand	Target Molecule	Application
Immunoaffinity	Antibody (monoclonal or polyclonal)	Corresponding antigen	Purification of specific proteins, hormones, virus particles; immunoprecipitation
Protein A / Protein G Affinity	Protein A (from S. aureus) or Protein G (from Streptococcus)	IgG antibodies (all species)	Purification of monoclonal antibodies — MOST WIDELY USED in biopharmaceuticals
Lectin Affinity	Concanavalin A (Con A), wheat germ agglutinin (WGA)	Glycoproteins and glycolipids (sugar residues)	Isolation of glycoproteins, cell membrane proteins, polysaccharides; blood group typing
Enzyme Substrate / Inhibitor	Substrate analogue, inhibitor, or cofactor	Specific enzyme	Purification of enzymes from crude tissue extracts
Dye Affinity	Cibacron Blue F3G-A (reactive textile dye)	Kinases, dehydrogenases, albumin, coagulation factors (enzymes with NAD/NADP cofactors)	Inexpensive; high capacity; group-specific purification of many enzymes; albumin removal from plasma
Metal Chelate (IMAC)	Metal ion (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}) chelated to NTA or IDA on matrix	His-tagged recombinant proteins (6×His-tag)	MOST COMMON in molecular biology research; purification of recombinant proteins expressed in E. coli or yeast
Biotin–Streptavidin	Streptavidin on matrix	Biotinylated proteins, DNA, antibodies	Capture of biotin-labelled molecules; extremely high affinity; used in many assay formats
Hormone / Receptor Affinity	Hormone (e.g., insulin, oestrogen)	Corresponding hormone receptor	Isolation of cell surface receptors; drug–receptor binding studies
Nucleotide / Coenzyme Affinity	NAD, NADP, ATP, ADP	Nucleotide-binding enzymes (oxidoreductases, kinases, ATPases)	Group-specific purification of many metabolic enzymes

Special Focus: IMAC (Immobilised Metal Affinity Chromatography)

IMAC is the most widely used affinity technique in modern biotechnology and molecular biology.

IMAC — His-Tag Protein Purification (Step by Step)
1. DESIGN: The gene for the protein of interest is cloned with a sequence encoding 6×Histidine (His-tag)
at the N- or C-terminus. The protein is expressed in E. coli or another host.
2. COLUMN: Nickel (Ni^{2+}) ions chelated to NTA (nitrilotriacetic acid) on agarose or silica matrix.
3. BINDING: At neutral pH (binding buffer: 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole pH 8.0),
the His-tag coordinates strongly with the Ni^{2+} atoms. All other E. coli proteins flow through.
4. WASHING: Wash with low imidazole (20–50 mM) to remove weakly bound contaminants.
5. ELUTION: Elute with high imidazole (250–500 mM) which competes with the His-tag for Ni^{2+} binding.
The His-tagged protein is released in a pure, concentrated band.
6. RESULT: One step purification often gives >90% purity from a crude cell lysate!

Instrumentation of Affinity Chromatography

Component	Description	Practical Notes
1. Matrix with Ligand	Pre-made affinity column (protein A, Ni-NTA, etc.) or custom-made by ligand immobilisation to activated agarose	Pre-packed columns (GE/Cytiva, Qiagen, Thermo) available for common applications; custom columns for unique ligands
2. Column Housing	Glass or plastic column with end fittings; pre-packed columns in cartridge format	Column size chosen based on amount of target protein; 1–5 mL columns for analytical/small prep; 25–500 mL for large-scale
3. Peristaltic Pump / FPLC System	Delivers mobile phase at controlled, low flow rates. FPLC (AKTA Pure, AKTA FPLC) provides precise gradient control, UV monitoring, fraction collection, and automated elution.	Low flow rates critical to allow sufficient contact time for binding (usually 0.5–2 mL/min). Slower = better binding but longer run.
4. Sample Loop / Injector	Large sample loops (1–500 mL) to accommodate large volumes of dilute sample	Sample is applied in binding buffer; crude lysate or partially purified sample acceptable

Component	Description	Practical Notes
5. UV Detector	Continuous monitoring at 280 nm (protein aromatic amino acids) allows real-time tracking of protein elution	UV baseline rises during washing; sharp UV peak = target protein eluting
6. Fraction Collector	Automatically collects timed or volume-based fractions into numbered tubes	Pool fractions from the elution peak; check each fraction by SDS-PAGE or activity assay
7. Conductivity Monitor	Detects ionic strength changes in gradient (salt or imidazole gradients)	Confirms that the elution gradient is being delivered correctly

Ligand Immobilisation Methods

When using a custom ligand, it must be covalently attached to the activated matrix. Common coupling chemistries include:

Coupling Chemistry	Activated Matrix	Reactive Group on Ligand	Notes
Cyanogen Bromide (CNBr) coupling	CNBr-activated Sepharose	Primary amines (—NH_2) on ligand	Classic method; simple; but isourea linkage may be positively charged — can cause non-specific ionic binding
N-Hydroxysuccinimide (NHS) coupling	NHS-activated Sepharose or silica	Primary amines (—NH_2)	Stable amide bond; mild conditions; widely used; NHS-Sepharose ready-made available
Epoxy coupling	Epoxy-activated Sepharose 6B	NH_2, OH, or SH groups on ligand	Very stable ether bond; good for stable ligand; ligand can be coupled at different groups
Aldehyde coupling (reductive amination)	Aldehyde-activated matrix + $NaBH_3CN$	Primary amines (—NH_2)	Very stable secondary amine bond; good for ligands that need orientation control
Maleimide coupling	Maleimide-activated matrix	Thiol (—SH) groups on ligand	Thiol-specific; allows oriented coupling if ligand has a specific SH group
Streptavidin-Biotin	Streptavidin-coated beads	Biotinylated ligand	Easy; no chemistry needed; ligand simply biotinylated; very high affinity capture

Applications of Affinity Chromatography

Application	Ligand Used	Target Purified	Scale
Monoclonal Antibody Purification	Protein A or Protein G on agarose	IgG monoclonal antibodies (mAbs)	Industrial scale; the core step in biopharmaceutical manufacturing (insulin, Herceptin, Humira)
Recombinant Protein Purification (IMAC)	Ni^{2+}-NTA or Co^{2+}-NTA	6×His-tagged recombinant proteins	Lab scale; used in thousands of research labs daily
Enzyme Purification from Tissues	Substrate analogue / inhibitor of target enzyme	Specific enzyme (e.g., trypsin on BPTI-Sepharose)	Lab/pilot scale; high purity in one step
Albumin Removal from Plasma	Cibacron Blue F3G-A agarose	Serum albumin (and other blue dye-binding proteins)	Analytical/clinical; depletes albumin to reveal low-abundance proteins in proteomics
Glycoprotein Isolation (Lectins)	Con A-Sepharose (for mannose/glucose residues); WGA (for GlcNAc)	Glycoproteins: hormone receptors, viral envelope proteins, IgG	Research; purification of membrane proteins, virus characterisation
Steroid Hormone Receptor Isolation	Steroid hormone (oestradiol, cortisol) on matrix	Nuclear steroid hormone receptors	Research; drug binding studies
Nucleic Acid Purification	Oligo(dT) on cellulose / magnetic beads	Messenger RNA (mRNA) with poly-A tail	Universal; used in all molecular biology labs to isolate mRNA from total RNA
GST-fusion protein purification	Glutathione on Sepharose	GST-tagged recombinant proteins	Alternative to His-tag system; eluted with reduced glutathione
DNA-binding Protein Isolation	Specific DNA sequence on matrix	Transcription factors; DNA-binding proteins	Research; gene regulation studies
Vitamin and Coenzyme Binding Proteins	Biotin, folate, or vitamin B12 on matrix	Avidin/streptavidin; folate-binding proteins	Research and diagnostics

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