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PHARMACOGNOSY & PHYTOCHEMISTRY

BASICS OF PHYTOCHEMISTRY & MODERN ANALYTICAL TECHNIQUES

B. Pharmacy | 5th Semester | Unit-5

- ★ Basics of Phytochemistry – Introduction, Scope & Importance
- ★ Modern Methods of Extraction (Classical & Advanced)
- ★ Spectroscopy – UV-Vis, IR/FTIR, NMR, Mass Spectrometry
- ★ Chromatography – TLC, CC, HPLC, GC, GC-MS, LC-MS
- ★ Electrophoresis – PAGE, Capillary, 2D-PAGE
- ★ Applications in Isolation, Purification & Identification

BASICS OF PHYTOCHEMISTRY

Definition & Introduction

Phytochemistry is the branch of science that deals with the chemistry of plants – specifically the chemical constituents (phytochemicals/phytoconstituents) present in them, their biosynthesis, chemical nature, extraction, isolation, purification, identification, quantification, and biological activity. It bridges pharmacy, organic chemistry, biochemistry, and pharmacognosy.

ETYMOLOGY: 'Phyto' (Greek: phyton = plant) + 'Chemistry' = Science of plant chemistry. Phytochemicals are broadly divided into: (1) Primary metabolites (carbohydrates, proteins, lipids, nucleic acids – essential for life) and (2) Secondary metabolites (alkaloids, terpenoids, phenolics, flavonoids, glycosides, tannins, resins – pharmacologically significant).

Scope & Importance of Phytochemistry

SCOPE & IMPORTANCE

- **DRUG DISCOVERY:** ~25% of modern pharmaceuticals are derived from plant sources; phytochemistry enables systematic discovery of new drug leads from plants
- **QUALITY CONTROL & STANDARDISATION:** Provides methods to ensure consistent quality of herbal drugs; pharmacopoeial standards based on phytochemical content
- **UNDERSTANDING BIOSYNTHESIS:** Reveals how plants synthesise complex molecules; guides metabolic engineering for enhanced production
- **STRUCTURE-ACTIVITY RELATIONSHIPS (SAR):** Correlates chemical structure of phytoconstituents with biological activity; guides semi-synthesis of improved drugs
- **CHEMOTAXONOMY:** Classification of plants based on their chemical constituents; helps identify adulteration and authenticate crude drugs
- **FOOD & NUTRACEUTICAL INDUSTRY:** Identification and quantification of bioactive compounds in functional foods, dietary supplements
- **ENVIRONMENTAL & AGROCHEMICAL APPLICATIONS:** Biopesticides, allelopathic compounds, plant growth regulators discovered through phytochemistry
- **COSMETIC INDUSTRY:** Natural antioxidants (Vitamin E, carotenoids, polyphenols), pigments, emollients isolated via phytochemical methods

Classification of Phytoconstituents

Class	Subclasses & Examples
ALKALOIDS	Tropane (Atropine), Isoquinoline (Morphine), Indole (Vincristine), Quinoline (Quinine), Purine (Caffeine), Steroidal (Solanidine)
TERPENOIDS	Monoterpenes (Menthol, Camphor), Sesquiterpenes (Artemisinin), Diterpenes (Taxol), Triterpenes (Glycyrrhizin, Saponins), Tetraterpenes (Carotenoids)
PHENOLICS / PHENYLPROPANOIDS	Simple phenols, Cinnamic acids, Coumarins, Lignans (Podophyllotoxin), Stilbenes (Resveratrol), Lignins
FLAVONOIDS	Flavones, Flavonols (Rutin, Quercetin), Flavanones, Anthocyanins, Isoflavones, Chalcones, Catechins
GLYCOSIDES	Cardiac glycosides (Digoxin), Anthraquinone glycosides (Sennosides), Cyanogenic (Amygdalin), Saponin glycosides
TANNINS	Hydrolysable (Galloyl & Ellagitannins), Condensed (Proanthocyanidins/Catechins)
RESINS & GUMS	Oleo-resins (Ginger, Capsicum), Gum-resins (Myrrh, Guggul), True resins (Colophony)
STEROIDS	Phytosterols (β-Sitosterol), Steroidal saponins (Diosgenin), Cardiac glycoside aglycones
VOLATILE OILS	Monoterpene and phenylpropanoid mixtures; Menthol, Eugenol, Citral, Anethole, Linalool
FATTY ACIDS & POLYKETIDES	Fixed oils (Castor, Olive), Anthraquinones (Senna), Polyunsaturated fatty acids (EPA, DHA)

General Workflow in Phytochemical Investigation

Stage	Activities
1. PLANT COLLECTION & AUTHENTICATION	Botanical identification; voucher specimen preparation; chemotaxonomic verification; collection at appropriate season
2. DRYING & POWDERING	Sun drying / shade drying / forced-air drying (40–60°C); grinding to appropriate mesh; moisture content determination
3. PRELIMINARY PHYTOCHEMICAL SCREENING	Colour tests, precipitation tests for major groups (alkaloids, glycosides, tannins, saponins, flavonoids, terpenoids)
4. EXTRACTION	Selection of solvent based on polarity of target compound; classical or modern methods (Soxhlet, MAE, UAE, SFE)
5. FRACTIONATION	Liquid-liquid partitioning with solvents of increasing polarity (petroleum ether → $CHCl_3$ → EtOAc → n-BuOH → water)

6. ISOLATION & PURIFICATION	Column chromatography, preparative TLC, preparative HPLC, crystallisation
7. IDENTIFICATION	TLC, spectroscopic methods (UV, IR, NMR, MS); comparison with authentic standards
8. QUANTIFICATION (ESTIMATION)	HPLC, GC, UV-Vis spectrophotometry, titrimetry; validated analytical methods
9. BIOLOGICAL ACTIVITY	In vitro and in vivo pharmacological screening; SAR studies

MODERN METHODS OF EXTRACTION

MODERN METHODS OF EXTRACTION

Extraction is the process of separating phytoconstituents from the plant matrix using a solvent. The choice of extraction method and solvent depends on the nature of the target compound, its polarity, stability, and the purpose (research vs. industrial). Modern methods offer advantages of speed, efficiency, reduced solvent use, and better selectivity over classical methods.

Solvent Selection – Like Dissolves Like Principle

SOLVENT POLARITY SERIES (Elotropic Series)

- **NON-POLAR:** Hexane / Petroleum ether < Cyclohexane < Carbon tetrachloride < Chloroform ($CHCl_3$) < Diethyl ether
- **MODERATELY POLAR:** Ethyl acetate (EtOAc) < Acetone < Dichloromethane (DCM)
- **POLAR:** Isopropanol < n-Butanol < Ethanol (EtOH) < Methanol (MeOH) < Water (most polar)
- **RULE:** Non-polar compounds (terpenoids, steroids, chlorophyll) → extracted by non-polar solvents; Polar compounds (alkaloid salts, glycosides, tannins) → polar solvents; **Most polyphenols:** methanol or ethanol
- **SOLVENT POLARITY INDEX (P'):** Hexane P'=0; $CHCl_3$ P'=4.1; EtOAc P'=4.4; MeOH P'=5.1; Water P'=10.2

Classical Extraction Methods

Method	Principle, Process & Limitations
MACERATION	Plant powder soaked in solvent for 2–7 days at room temperature; occasionally shaken; filtered; simple but slow; incomplete extraction; used for thermolabile compounds; cold maceration for heat-sensitive compounds (e.g., volatile oil components)

PERCOLATION	Menstruum passed continuously through bed of plant powder in a percolator; fresh solvent always in contact with undepleted material; more efficient than maceration; used for tinctures, fluid extracts; IP official method
INFUSION	Plant material steeped in hot water (not boiling) for 15 minutes; used for aromatic plants; volatile components preserved; e.g., Tea infusion, digitalis infusion
DECOCTION	Plant material boiled in water for 10–15 min; used for hard plant materials (bark, roots); not suitable for volatile or heat-sensitive constituents; e.g., Dasamoola kwatham
SOXHLET EXTRACTION	Plant material in thimble; solvent heated → vapour condenses and percolates through plant material; cycles continuously; exhaustive extraction; 6–48 hours; limited to thermostable compounds; large solvent volumes; semi-automated
STEAM DISTILLATION	Volatile compounds co-distilled with steam; used for volatile oils (menthol, citral, eugenol); temperature <100°C at atmospheric pressure; preserves most volatile components; Florentine receiver separates oil from water

Advanced / Modern Extraction Techniques

Microwave-Assisted Extraction (MAE)

MICROWAVE-ASSISTED EXTRACTION (MAE)

Principle: Microwave energy (2450 MHz) absorbed by polar molecules/solvents → rapid internal heating → disruption of plant cell walls → faster release of phytoconstituents into solvent

Equipment: Closed-vessel (high-pressure, 100–300 psi) or open-vessel MAE systems; temperature range 40–150°C; power 300–1000 W; domestic microwave adapted or dedicated MAE instruments (CEM, Milestone brands)

Solvents Used: Water, methanol, ethanol, acetonitrile (polar solvents absorb microwave; non-polar solvents like hexane do NOT; use polar co-solvent if non-polar solvent needed)

Advantages: Very fast (5–30 min vs hours for Soxhlet); lower solvent consumption (50–80% reduction); higher extraction efficiency; less thermal degradation; easy automation

Disadvantages: Not suitable for thermolabile compounds; requires polar solvent; closed vessel – pressurised (safety risk); plant material must be pre-wetted

Applications: Extraction of phenolics, flavonoids, alkaloids, essential oils (with water); pharmaceutical QC; food analysis; environmental samples

Key Parameters: Power (W), Temperature (°C), Extraction time, Solvent:material ratio, Particle size

Ultrasound-Assisted Extraction (UAE) / Sonication

ULTRASOUND-ASSISTED EXTRACTION (UAE)

Principle: Ultrasonic waves (20–100 kHz) passed through solvent → cavitation (formation & collapse of micro-bubbles) → intense local pressure/temperature → disruption of cell walls → enhanced mass transfer of phytoconstituents

Equipment: Ultrasonic bath (low intensity, broad coverage) or Ultrasonic probe/horn (high intensity, localised); probe 20–25 kHz; bath 37–100 kHz; amplitude 20–100%

Solvents Used: Any solvent (polar or non-polar); ethanol, methanol, water, ethyl acetate, chloroform commonly used; no restriction on solvent polarity (unlike MAE)

Advantages: Room temperature operation (suitable for thermolabile compounds); faster than classical methods (15–60 min); modest solvent reduction; simple equipment; no solvent restriction; preserves volatile compounds

Disadvantages: Probe tip erosion (contamination risk); scale-up challenging; less efficient than MAE for some matrices; local hot spots near probe

Applications: Alkaloids (atropine, quinine), essential oils, phenolics, vitamins; food authentication; archaeological samples; dissolution testing

Optimisation Parameters: Frequency (kHz), Amplitude (%), Pulse mode, Duration, Temperature, Solvent:solid ratio

Supercritical Fluid Extraction (SFE)

SUPERCRITICAL FLUID EXTRACTION (SFE)

Principle: Supercritical fluid (above critical temperature Tc and pressure Pc) possesses density of liquid (good solvating power) AND diffusivity of gas (rapid mass transfer); used as extracting medium; CO_2 most common (Tc = 31.1°C, Pc = 73.8 atm)

Supercritical CO_2: Non-toxic, non-flammable, inexpensive, easily removed (just depressurise → CO_2 evaporates) → pure, solvent-free extract; GRAS status; operates near ambient temperature → suitable for thermolabile compounds

Modifiers (Co-solvents): Pure $scCO_2$ is non-polar (similar to hexane); adding 5–15% methanol, ethanol, or water as modifier increases polarity → extracts more polar compounds (phenolics, alkaloids)

Equipment: High-pressure pump; extraction vessel (100–10,000 mL); back-pressure regulator; collection vessel; CO_2 cylinder; automated systems (Applied Separations, Jasco, Waters)

Advantages: Selective and tunable extraction (by varying P,T,modifier); No solvent residues (pharmaceutical advantage); Fast (30–60 min); Suitable for thermolabile compounds; Green technology (CO_2 recyclable); GMP-compatible

Disadvantages: High equipment cost; batch size limitations; not suitable for very polar compounds without modifiers; polar compounds may still prefer ethanol extraction

Applications: Decaffeination of coffee/tea ($scCO_2$ at 300 bar, 70°C extracts caffeine); Hop extract for beer (humulone); Essential oils; Omega-3 fatty acids; Carotenoids (β-carotene, lycopene); Cannabis cannabinoids (CBD, THC); Artemisinin extraction

Pharmaceutical Use: USP/ICH Class 3 solvent (CO_2) – preferred for pharmaceutical extracts; no Class 1 or 2 residual solvents

Pressurised Liquid Extraction (PLE) / Accelerated Solvent Extraction (ASE)

PRESSURISED LIQUID EXTRACTION (PLE) / ASE

Principle: Extraction with conventional solvents at elevated temperature (50–200°C) and pressure (1000–3000 psi) in a closed system; increased temperature → better solubility and diffusivity; pressure maintains solvent in liquid state above boiling point

Equipment: Dedicated ASE system (Thermo Dionex ASE 150/200/350); stainless steel extraction cells (1–100 mL); automated; rapid sequential extractions

Solvents: Any conventional solvent; ethanol, methanol, water (hot pressurised water extraction = Subcritical Water Extraction); ethyl acetate, hexane

Advantages: Fast (15–20 min extraction); high extraction efficiency (comparable to Soxhlet but much faster); automated; small solvent volumes (15–40 mL per extraction); reproducible

Disadvantages: Co-extraction of unwanted co-extractables at high temperature; not for volatile or thermolabile compounds; expensive equipment

Applications: Environmental analysis (PAHs, PCBs from soil); food analysis; plant metabolite profiling; pharmaceutical raw material analysis

ENZYME-ASSISTED EXTRACTION (EAE)

Principle: Cell wall-degrading enzymes (cellulase, hemicellulase, pectinase, protease) used to break down plant cell wall polysaccharides → enhance release of intracellular phytoconstituents into extraction solvent; milder than thermal methods

Enzymes Used: Cellulase (breaks cellulose β-1,4 glycosidic bonds); Pectinase (breaks pectin); Hemicellulase; Protease; Commercial enzyme cocktails (Novozyme, Celluclast, Pectinex)

Conditions: pH 4.0–5.5 (optimal for most plant cell wall enzymes); Temperature 40–55°C; time 1–4 hours; enzyme loading 0.1–2% w/w of plant material

Advantages: Green/sustainable (no harsh chemicals); improved yields; mild conditions → preserves bioactive compounds; reduces downstream processing; applicable to thermolabile constituents

Disadvantages: Enzyme cost; optimal conditions critical; may introduce protein contamination; enzyme inactivation needed before downstream processing

Applications: Diosgenin extraction (enzyme hydrolysis of dioscin saponin – replaces acid hydrolysis); polyphenol extraction from grape pomace; essential oil extraction; pectin extraction; juice clarification

Other Advanced Methods

OTHER MODERN EXTRACTION METHODS

- PULSED ELECTRIC FIELD EXTRACTION (PEF): Short intense electric pulses (1–50 kV/cm, microsecond duration) → electroporation of cell membranes → increased permeability → better extraction without heat; Emerging technology for food and pharmaceutical applications; preserves heat-sensitive compounds
- HIGH HYDROSTATIC PRESSURE EXTRACTION (HHPE / UHPE): Plant material subjected to very high pressure (100–600 MPa) in cold aqueous solvent; pressure disrupts cell walls → rapid extraction; minimal heat → thermolabile compound preservation; used for anthocyanins, polyphenols, vitamins
- TURBO-EXTRACTION / TURBO-SOLV: High-speed mixing of plant material with solvent → mechanical disruption and rapid extraction; 10–30 min at room temperature; waring blender type; used for alkaloids, glycosides
- COLD PRESSING / EXPRESSION: Mechanical pressing of fresh plant material (citrus peel, seeds); no solvent; for volatile oils (citrus oils – bergapten, limonene) and fixed oils; GRAS process
- COUNTER-CURRENT EXTRACTION (CCE): Industrial continuous liquid-liquid extraction using immiscible solvent pair in counter-current flow; very efficient; used for alkaloids, antibiotics; scaled to tonnes.
- SUBCRITICAL WATER EXTRACTION (SWE): Hot pressurised liquid water (100–374°C) changes polarity with temperature; at 250°C, water polarity ≈ ethanol; extracts both polar and less polar compounds; green solvent

Comparison of Extraction Methods

Method	Time	Solvent Volume	Best For
Maceration	2–7 days	Large	Thermolabile; simple setup
Percolation	2–5 days	Large	Tinctures; fluid extracts (IP official)
Soxhlet	6–48 hours	Moderate-Large	Thermostable compounds; exhaustive extraction
Steam Distillation	2–4 hours	—	Volatile oils (menthol, eugenol, citral)
MAE (Microwave)	5–30 min	Small	Polar phenolics, alkaloids (fast)
UAE (Ultrasound)	15–60 min	Moderate	Thermolabile; broad applicability
SFE ($scCO_2$)	30–60 min	None (CO_2)	Lipophilic; thermolabile; solvent-free
PLE/ASE	15–20 min	Very Small	High-throughput; environmental; metabolomics
EAE (Enzyme)	1–4 hours	Moderate	Saponins, polysaccharides; green chemistry
PEF	Minutes	Moderate	Thermolabile; emerging; food applications

SPECTROSCOPIC TECHNIQUES

SPECTROSCOPY IN PHYTOCHEMICAL ANALYSIS

Spectroscopy involves the interaction of electromagnetic radiation with matter. Different types of electromagnetic radiation interact with different aspects of molecular structure, making spectroscopy the most powerful tool for structure elucidation and identification of phytoconstituents.

Spectroscopic Technique	Information Provided
UV-Visible Spectroscopy	Chromophore groups; conjugation; identification of phenolics, flavonoids; quantitation
Infrared (IR/FTIR) Spectroscopy	Functional groups (–OH, C=O, N-H, C-O, etc.); fingerprint region for identification
Nuclear Magnetic Resonance (NMR)	Carbon and hydrogen framework; connectivity; stereochemistry; complete structure elucidation
Mass Spectrometry (MS)	Molecular weight; molecular formula; fragmentation pattern; unknown structure confirmation
X-Ray Crystallography	Absolute 3D structure; definitive stereochemistry; requires single crystals
Atomic Absorption Spectroscopy (AAS)	Metal ion content; trace metal analysis in plant drugs

UV-VISIBLE SPECTROSCOPY

▶ Principle

UV-Vis spectroscopy measures the absorption of ultraviolet (200–400 nm) and visible (400–800 nm) light by molecules. Absorption occurs when a photon of the right energy promotes an electron from a ground state orbital to an excited state orbital. The energy required depends on the type of electronic transition.

Parameter	Details
Electronic Transitions	σ→σ* (very high energy; UV-opaque; C-C bonds); n→σ* (lone pair to sigma*; 150-250 nm); π→π* (conjugated systems; 200-350 nm;

	strong absorption); n→π* (lone pair to pi*; 250-350 nm; weak, carbonyl)
Beer-Lambert Law	A = εcl; A = Absorbance; ε = Molar absorptivity (L/mol/cm); c = Concentration (mol/L); l = Path length (cm); Linear relationship – basis of quantitative UV analysis
Chromophore	Group responsible for UV absorption; e.g., benzene ring (π system), C=O (carbonyl), C=C conjugated with C=O; extended conjugation → red shift (bathochromic shift)
Auxochrome	Groups that shift/intensify absorption; –OH, $–NH_2$, –OR: Hypsochromic (blue shift) or Bathochromic (red shift) effect on chromophore
Instrumentation	Deuterium lamp (UV, 200–400 nm) + Tungsten lamp (Vis, 400–800 nm); monochromator; sample cell (quartz cuvette for UV); photomultiplier detector; PC-controlled
Flavonoid UV Characterisation	Band I (300–380 nm): Cinnamoyl ring B absorption; Band II (240–280 nm): Benzoyl ring A; $AlCl_3$ shifts diagnose 3-OH, 5-OH, and catechol groups; NaOMe ionisation shifts; very characteristic for flavonoid class identification
IP Applications	Estimation of: Caffeine (272 nm), Rutin (354 nm), Curcumin (425 nm), Artemisinin (210 nm), Riboflavin (267, 373, 444 nm); standardisation of plant extracts

APPLICATIONS IN PHYTOCHEMICAL ANALYSIS

- **IDENTIFICATION:** Comparison of UV spectrum (λmax, ε) with authentic standard or published spectra; flavonoid characterisation using diagnostic reagent shifts ($AlCl_3$, NaOMe, NaOAc, H_3BO_3)
- **QUANTIFICATION (Primary method):** Official pharmacopoeial assay for caffeine (272 nm), digitalis glycosides (at 220–480 nm), codeine, quinine, rutin, curcumin (425 nm); high sensitivity
- **PURITY ASSESSMENT:** Ratio of absorbances at different wavelengths (A260/A280 for nucleic acid/protein ratio; analogous concepts for phytochemicals)
- **HPLC DETECTION:** UV-Vis detector most common in HPLC; photodiode array (PDA/DAD) captures full spectrum at each HPLC peak → peak purity confirmation
- **REACTION MONITORING:** Track progress of chemical reactions in semi-synthesis by UV spectral change
- **SCREENING:** High-throughput UV-based screening of plant extracts for presence of specific chromophore classes (flavonoids, alkaloids)

3.2 INFRARED (IR) AND FTIR SPECTROSCOPY

▶ Principle

IR spectroscopy measures absorption of infrared radiation (2.5–25 µm / 4000–400 cm^{-1}) by molecular bonds. Absorption occurs when the frequency of IR radiation matches the natural vibrational frequency of a bond (stretching or bending). FTIR (Fourier Transform IR) uses a Michelson interferometer and mathematical Fourier transformation to obtain a full spectrum rapidly and with high sensitivity.

Region / Wavenumber (cm^{-1})	Bond/Group Identified
4000–3200 cm^{-1}	O-H stretch (broad, 3200–3550: H-bonded OH; sharp 3580–3650: free OH); N-H stretch (3300–3500, sharper than OH); alcohol, phenol, amine, carboxylic acid
3000–2800 cm^{-1}	C-H stretch (alkyl); 2960: asym. CH_3; 2920: asym. CH_2; 2870: sym. CH_3; 2850: sym. CH_2; indicates aliphatic backbone
2260–2100 cm^{-1}	C≡N (nitrile, 2200–2260); C≡C (alkyne, 2100–2260); N=C=O (isocyanate); triple bonds
1870–1650 cm^{-1}	C=O stretch (carbonyl): Acid ~1710; Ester ~1735; Aldehyde ~1720; Ketone ~1715; Amide ~1650; Lactone ~1735–1770; Most important diagnostic region
1650–1500 cm^{-1}	C=C stretch (aromatic ~1600, 1500; alkene ~1620–1680); N-H bending (secondary amine ~1550); C=N (imine ~1630)
1300–1000 cm^{-1}	C-O stretch (ether ~1070–1150; C-O-C of glycosides ~1000–1200; methoxy $–OCH_3$ ~1250); complex but informative
1000–400 cm^{-1}	FINGERPRINT REGION: Unique for each compound; out-of-plane bending of C-H (aromatic substitution pattern 690–900); used for identity confirmation by comparison with reference spectrum

FTIR vs DISPERSIVE IR & APPLICATIONS

- **FTIR ADVANTAGES:** All wavelengths measured simultaneously → much faster; higher sensitivity (Fellgett advantage); better signal-to-noise; reproducible; can be coupled with microscope (IR microscopy) or ATR accessory
- **ATR-FTIR (Attenuated Total Reflectance):** No sample preparation (solid or liquid placed directly on diamond crystal); evanescent wave samples top layer; fastest method for raw material ID; used in pharmaceutical raw material ID testing (Identity test)
- **IDENTIFYING FUNCTIONAL GROUPS:** O-H broad (alcohols, phenols, carboxylic acids); N-H (alkaloids); C=O (carboxylic acids, esters, lactones in terpenoids); methylenedioxy $–O-CH_2-O–$ at 930, 1040 cm^{-1} (in podophyllotoxin, piperine); Glucoside linkage C-O-C at 1000–1200 cm^{-1}
- **PHARMACOPOEIAL USE:** IP/BP/USP include IR spectrum matching as identity test; compare spectrum of sample with reference spectrum (overlay within specified wavenumber tolerance)

- **FINGERPRINT IDENTIFICATION:** Herbal crude drug authentication; detect adulteration; cannot be confused (each molecule has unique fingerprint); species differentiation
- **NEAR-INFRARED (NIR) SPECTROSCOPY:** 12,500–4,000 cm^{-1}; overtone and combination bands; non-destructive; rapid analysis (2 min); used for moisture content, API content in tablets at-line; chemometric multivariate analysis (PLS, PCA) required

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

▶ Principle

NMR spectroscopy exploits the property of atomic nuclei with non-zero spin (^{1}H, ^{13}C, ^{15}N, ^{31}P etc.) to absorb radio-frequency radiation when placed in a strong external magnetic field (B_0). The resonance frequency (chemical shift, δ in ppm) depends on the electronic environment of the nucleus. NMR provides the most detailed structural information of any spectroscopic technique – essentially a molecular map.

NMR Parameter	Definition & Significance
CHEMICAL SHIFT (δ, ppm)	Position of NMR signal relative to TMS (tetramethylsilane, 0 ppm standard); reflects electronic environment; electron-withdrawing groups → downfield (high δ); electron-donating → upfield (low δ)
COUPLING CONSTANT (J, Hz)	Spin-spin coupling between adjacent nuclei through bonds; J value (Hz) independent of field strength; reveals connectivity: vicinal (^{3}J, 3-bond), geminal (^{2}J, 2-bond), long-range coupling
MULTIPLICITY	Number of peaks in a signal: singlet (s, no coupling); doublet (d, 1 neighbour); triplet (t, 2 neighbours); quartet (q, 3 neighbours); multiplet (m); follows n+1 rule
INTEGRATION	Area under NMR peak ∝ number of equivalent protons; used for quantitative NMR (qNMR) and structure confirmation
NOE (Nuclear Overhauser Effect)	Enhancement of signal when nearby proton (within 5 Å) is saturated; indicates spatial proximity; used for 3D structure/stereochemistry (through space, not through bonds)

◆ Types of NMR Experiments in Phytochemistry

NMR Experiment	Information Provided	Example Application
^{1}H-NMR (Proton NMR)	Number, type, connectivity of H atoms; chemical shifts,	Identify aromatic H, methylene, methyl, N-CH_3, O-CH_3 groups;

	multiplicities, coupling constants; most fundamental	sugar proton patterns (glycosides); doublet of anomeric H at δ 4–5 ppm
^{13}C-NMR	All carbon atoms; chemical shift reveals hybridization (sp3 0-50; sp2 100-150; C=O 160-220 ppm); quaternary carbons visible	Count total carbons; identify carbonyl (C=O), olefinic (C=C), aromatic, aliphatic carbons in alkaloids, terpenoids
DEPT (Distortionless Enhancement by Polarisation Transfer)	Distinguish CH, CH_2, CH_3 from quaternary C; DEPT-135: CH and CH_3 up, CH_2 down, C absent	Used with ^{13}C to assign carbon multiplicity; essential in terpenoid structure elucidation
COSY (Correlation Spectroscopy – ^{1}H-^{1}H)	2D: Off-diagonal cross-peaks show which H atoms are coupled (vicinal, geminal); maps H-H connectivity	Trace proton connectivity through molecule; identify spin systems; sequence alkaloid ring systems
HMBC (Heteronuclear Multiple-Bond Correlation)	2D: ^{1}H-^{13}C correlations over 2–4 bonds (long-range); connects protons to distant carbons; shows connectivity across quaternary carbons/heteroatoms	Connect fragments across C=O, quaternary C, O, N; essential for glycoside and alkaloid structure determination
HSQC (Heteronuclear Single Quantum Coherence)	2D: Direct one-bond ^{1}H-^{13}C correlation; assigns each H to its attached carbon	Assign all CH, CH_2, CH_3 groups; distinguish overlapping ^{1}H signals by carbon shift
NOESY/ROESY (NOE Spectroscopy)	2D: Through-space correlations (H atoms within 5 Å regardless of bond connectivity); stereochemistry	Determine relative configuration (α/β, axial/equatorial); ring conformation; diastereotopic proton assignment
qNMR (Quantitative NMR)	Absolute quantification without reference standard curve; peak area directly proportional to moles	Pharmaceutical purity determination; certified reference standard preparation (NMR replaces titration)

APPLICATIONS OF NMR IN PHYTOCHEMISTRY

- **COMPLETE STRUCTURE ELUCIDATION:** Combination of ^{1}H, ^{13}C, DEPT, COSY, HMBC, HSQC, NOESY provides complete molecular structure including relative stereochemistry of new natural products
- **DEREPLICATION:** Rapid comparison of NMR spectra with databases (SDBS, SciFinder, DNP); identify known compounds without full isolation
- **METABOLITE PROFILING (NMR Metabolomics):** ^{1}H-NMR of crude extracts; chemometric analysis (PCA, PLS-DA) gives metabolite fingerprint; useful for authentication, adulteration detection, geographic origin differentiation
- **STEREOCHEMISTRY:** NOESY/ROESY confirms relative configuration (e.g., α/β orientation of sugar in cardiac glycosides; cis/trans configuration in sesquiterpenes)

- **QUANTITATIVE ANALYSIS:** qNMR (Internal standard method) – used for reference standard characterisation by NIST, Ph. Eur.; value assignment of pharmaceutical reference standards
- **HIGH-FIELD NMR:** 400–1000 MHz instruments; higher field → better resolution and sensitivity; 600 MHz most common for complex natural products; 900–1000 MHz for large complex molecules

MASS SPECTROMETRY (MS)

▶ Principle

Mass spectrometry measures the mass-to-charge ratio (m/z) of ions produced from analyte molecules. A mass spectrometer ionises molecules, separates the ions by m/z, and detects them. The resulting mass spectrum – a plot of ion abundance vs m/z – gives molecular weight (from molecular ion M^+) and structural information from fragmentation pattern.

MS Component	Details
IONISATION SOURCES	Electron Ionisation (EI, 70 eV): Hard ionisation; extensive fragmentation; GC-MS; volatile compounds; EI gives reproducible spectra (library searchable); Chemical Ionisation (CI): Softer; $[M+H]^+$ seen; Fast Atom Bombardment (FAB): Polar, involatile compounds; Electrospray Ionisation (ESI): Very soft; $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$; suitable for thermolabile, polar, high-MW compounds; HPLC-MS interface; Matrix-Assisted Laser Desorption/Ionisation (MALDI): Large MW; proteins, polysaccharides; used with TOF analyser
MASS ANALYSERS	Quadrupole (Q): Unit mass resolution; low cost; selected ion monitoring (SIM); widely used in QC; Triple Quadrupole (QqQ): MS/MS; MRM (multiple reaction monitoring); quantitative analysis; most sensitive for known analytes; Ion Trap (IT): MS^n capability; structural elucidation; Time-of-Flight (TOF): High resolution; exact mass; molecular formula determination; Orbitrap (FT-MS): Ultra-high resolution (>100,000); sub-ppm mass accuracy; QTOF: Combination of quadrupole + TOF for sensitive structural analysis
MOLECULAR ION (M^+ or $[M+H]^+$)	Gives molecular weight; EI: M^+ (radical cation); ESI: $[M+H]^+$ (protonated molecule), $[M-H]^-$ (deprotonated, negative mode); MALDI: $[M+Na]^+$ often
FRAGMENTATION	Loss of characteristic neutral fragments reveals structure: Loss of 18 (H_2O); Loss of 15 (CH_3); Loss of 29 (CHO); Loss of 44 (CO_2, ester); Loss of 42 (acetyl, $CH_2=C=O$); Base peak = most abundant fragment

HIGH RESOLUTION MS (HRMS)	Exact mass to 4–5 decimal places; determine molecular formula by comparing with theoretical mass; error <5 ppm confirms formula; Orbitrap and QTOF provide HRMS
ISOTOPE PATTERNS	M+1 peak: ^{13}C contribution (1.1% per C atom); M+2 peak: presence of Cl (M:M+2 = 3:1), Br (M:M+2 = 1:1), S (M+2 ~4% per S); helps determine molecular formula

HYPHENATED TECHNIQUES & APPLICATIONS

- **GC-MS:** Gold standard for volatile compound identification; Menthol (m/z 71, 69, 55), Eugenol (m/z 164, 149, 77), Citral (m/z 152, 137); NIST Library matching (>300,000 EI spectra); Essential oil profiling; Residual solvent analysis (ICH Q3C)
- **LC-MS (HPLC-ESI-MS):** Analysis of non-volatile phytoconstituents; alkaloids, glycosides, phenolics; ESI-MS/MS for structural confirmation; Artesunate $[M+Na]^+$ = 407; Digoxin $[M+Na]^+$ = 803; Rutin $[M-H]^-$ = 609
- **QTOF (HPLC-QTOF-MS):** High-resolution metabolite profiling; accurate mass → molecular formula; identify hundreds of compounds in crude extract; used in metabolomics
- **MALDI-TOF:** Polysaccharide fingerprinting; protein MW determination; matrix: DHB (2,5-dihydroxybenzoic acid) or CHCA; saponin analysis
- **STRUCTURE ELUCIDATION:** MS fragmentation pathways confirm structure proposed by NMR; loss of sugar units from glycosides (Δ162 for hexose, Δ146 for deoxyhexose); loss of characteristic groups from alkaloids
- **METABOLOMICS:** Untargeted MS-based metabolite profiling; UPLC-QTOF-MS; multivariate analysis; discovery of new natural products and biomarkers

CHROMATOGRAPHIC TECHNIQUES

CHROMATOGRAPHY IN PHYTOCHEMICAL ANALYSIS

Chromatography is the separation science where components of a mixture are distributed between two phases: stationary phase (solid or liquid) and mobile phase (liquid or gas). Differential interactions (adsorption, partitioning, ion exchange, size exclusion) of different compounds with these phases result in separation. Chromatography is the most widely used tool for isolation, purification, and identification of phytoconstituents.

FUNDAMENTAL CONCEPTS: Rf (Retardation factor) = Distance travelled by compound / Distance travelled by solvent front (TLC); Retention time (Rt) – time from injection to peak maximum (HPLC/GC); Resolution (Rs) – degree of separation between adjacent peaks; Theoretical plates (N) – measure of column efficiency; Van Deemter equation – describes plate height as function of mobile phase velocity.

THIN LAYER CHROMATOGRAPHY (TLC)

THIN LAYER CHROMATOGRAPHY (TLC)

Principle: Mixture applied as spot on TLC plate (stationary phase: silica gel or alumina coated on glass/aluminium/plastic); developed in solvent system (mobile phase) in closed chamber; components separated by differential polarity interactions; Rf values characteristic

Stationary Phases: Silica gel (normal phase: polar compounds adsorb more – lower Rf); Silica gel RP-18 (reverse phase: non-polar compounds adsorb more – lower Rf); Alumina (basic/neutral/acidic); Polyamide (for polyphenols, flavonoids); Cellulose (sugars)

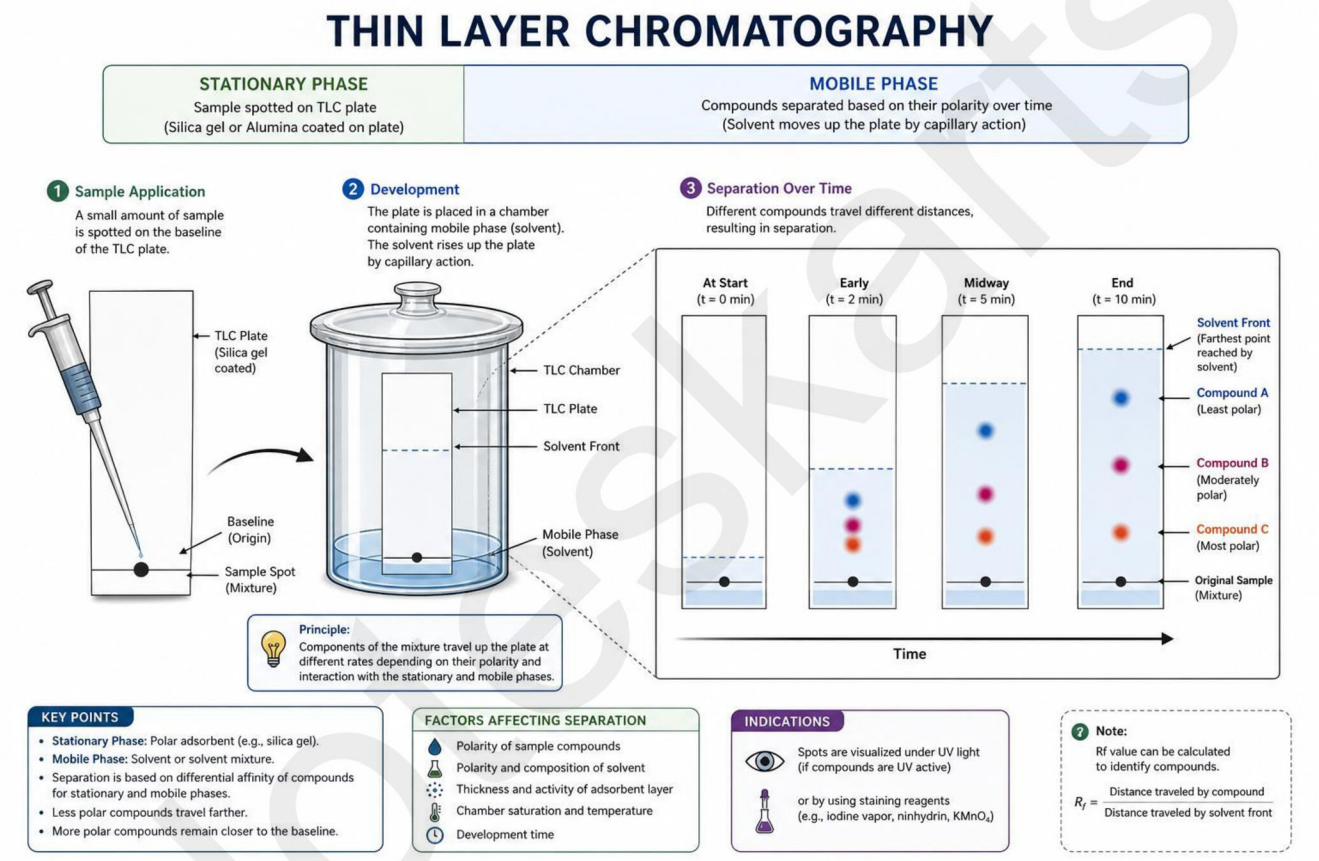
Mobile Phases (Systems): Normal phase: Non-polar solvents + modifiers: Hexane:EtOAc; $CHCl_3$:MeOH; Toluene:EtOAc:Formic acid; n-BuOH:AcOH:Water (BAW system – for flavonoid glycosides); Reverse phase: MeOH:Water; MeCN:Water

Detection Methods: UV 254 nm: Fluorescence quenching (compounds absorb UV); UV 366 nm: Fluorescence (natural – flavonoids emit yellow/green; coumarins emit blue); Chemical spray reagents: Dragendorff (alkaloids – orange), Anisaldehyde-H_2SO_4 (terpenoids – various colours), $FeCl_3$ (phenolics – dark green/blue), Ninhydrin (amino acids – purple), NaOH (anthraquinones – red), KOH (coumarins – fluorescent), Vanillin-H_2SO_4 (terpenoids, sugars); H_2SO_4 alone (chars most organics)

HPTLC (High Performance TLC): Smaller particle size (5 µm vs 10–20 µm for conventional TLC); higher resolution; automated application (Linomat/ATS instruments); densitometric scanning; quantitative; validation possible; WHO/pharmacopoeial standard for herbal authentication

Applications: Preliminary screening; Rf comparison with standards (identity); monitoring column chromatography fractions; detecting adulteration; purity check; investigating biosynthesis; HPTLC for pharmacopoeial ID tests of herbal drugs

Rf Calculation: Rf = d(spot)/d(solvent front); 0 < Rf < 1; optimal Rf 0.3–0.7; reproducibility improved by constant temperature, plate lot, and solvent freshness



COLUMN CHROMATOGRAPHY (CC)

COLUMN CHROMATOGRAPHY (CC) – Open Column

Principle: Extract loaded on top of adsorbent-packed glass column; mobile phase (eluent) allowed to flow through by gravity; components separate based on differential interaction with stationary phase; fractions collected at outlet

Stationary Phases: Silica Gel (60–200 mesh most common; normal phase; separates by polarity); Alumina (basic, neutral, acidic; for alkaloids – basic Al_2O_3; sensitive compounds); Sephadex (Dextran gel – size exclusion for polar compounds, tannins, saponins); Amberlite XAD resins (non-polar polymer resin – for polar secondary metabolites from aqueous extracts); Polyamide (flavonoids, tannins)

Gradient Elution: Stepwise or continuous gradient from non-polar → polar solvents (e.g., hexane → hexane:EtOAc 9:1 → 4:1 → 1:1 → EtOAc → EtOAc:MeOH → MeOH); each step elutes compounds of increasing polarity

Sample Loading: Sample (dissolved in minimum mobile phase or adsorbed on silica) applied as narrow band on top of column; column:sample ratio typically 30:1 to 100:1 (w/w) for good separation

Fraction Monitoring: Each fraction (10–20 mL) analysed by TLC; fractions with same Rf (same compound) pooled; evaporated; recrystallised or subjected to further CC

Flash Chromatography: Rapid CC under positive pressure (nitrogen/air); faster flow rates; shorter columns; 10–15 min per run vs hours for gravity; instruments: Isolera (Biotage), CombiFlash (Teledyne); automated gradient; UV monitoring; widely used in phytochemistry lab

Vacuum Liquid Chromatography (VLC): Silica packed in Buchner funnel; suction (vacuum) drives solvent through; very rapid; used for primary fractionation of crude extracts before CC; stepwise solvent gradient; preparative scale

Applications: Primary isolation workhorse; isolate gram-scale quantities of pure compounds; used for ALL classes of phytoconstituents

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC – High Performance Liquid Chromatography

Principle: High-pressure pump forces mobile phase through column packed with small particles (3–5 µm); analytes interact differently with stationary phase → differential retention → separation; retention time (Rt) identifies compound; peak area quantifies it

Components: (1) Solvent reservoir + degasser; (2) High-pressure pump (isocratic or gradient); (3) Injector (manual or autosampler, 5–20 µL loop); (4) Guard column (protects analytical column); (5) Analytical column (150–250 mm × 4.6 mm); (6) Detector; (7) Data acquisition system (software)

Types of HPLC: NORMAL PHASE (NP-HPLC): Polar stationary phase (bare silica), non-polar mobile phase; separates by polarity; used for lipids, terpenoids, plant pigments; REVERSE PHASE (RP-HPLC): Non-polar stationary phase (C18, C8, C4, Phenyl); polar mobile phase (water + organic modifier); most widely used; suitable for most phytoconstituents; ION-EXCHANGE: Ionic compounds; alkaloids, nucleotides; SIZE-EXCLUSION: Large molecules, polysaccharides, proteins

Stationary Phases: C18 (ODS – Octadecylsilyl): Most common; for phenolics, alkaloids, terpenoids, glycosides; C8: Slightly less retentive; faster; Phenyl column: π-π interactions; for aromatic compounds; Amino column: For carbohydrates, flavonoids; Cyano (CN): Intermediate polarity

Mobile Phases: Water + MeOH / MeCN (gradient); buffer for ionisable compounds; phosphate buffer pH 2–7 (for alkaloids, phenolic acids); ammonium acetate (compatible with MS); TFA (0.1%) for sharpening peaks

Detectors: UV (Fixed wavelength, 254/280 nm); PDA/DAD (Photodiode Array – captures full spectrum 200–800 nm simultaneously, peak purity); Fluorescence (very sensitive, specific – alkaloids, aflatoxins); RI (Refractive Index – sugars, fats; no UV chromophore); ELSD (Evaporative Light Scattering – universal; for artemisinin, diosgenin); MS (most specific – LC-MS)

UPLC/UHPLC: Sub-2 µm particles (1.7 µm BEH C18); operating pressure up to 15,000 psi (1000 bar); 3–5× faster than HPLC; better resolution; less solvent consumption; Waters Acquity UPLC is the pioneer instrument

Validation Parameters: Specificity, Linearity, Accuracy (% recovery), Precision (RSD%), LOD, LOQ, Range, Robustness – as per ICH Q2(R1)

Applications: Official IP/BP/USP method for: Morphine, Digoxin, Atropine, Quinine, Rutin, Sennosides, Vincristine/Vinblastine, Artemisinin, Paclitaxel; Metabolite fingerprinting; Impurity profiling; Bioavailability studies (plasma drug levels)

GAS CHROMATOGRAPHY (GC) AND GC-MS

GAS CHROMATOGRAPHY (GC) & GC-MS

Principle: Volatile analytes (or derivatised to be volatile) vaporised and carried by inert carrier gas (He or N_2) through a long capillary column coated with stationary phase (liquid polymer); components separate based on boiling point and polarity interaction with stationary phase

Carrier Gas: Helium (He): Most common; inert; high sensitivity; Hydrogen (H_2): Faster; better efficiency; Nitrogen (N_2): Cheaper; slower

Column Types: Capillary columns (fused silica, 30–60 m × 0.25–0.32 mm ID); Stationary phase: DB-5 (5% phenyl polysiloxane – general purpose; non-polar), DB-WAX (polyethylene glycol – polar; for alcohols, esters), HP-INNOWAX (polar; menthol, terpenes), DB-1 (most non-polar; for lipids, waxes)

Detectors: FID (Flame Ionisation Detector): Universal for organic compounds; sensitive; most common; MS (Mass Spectrometer): Provides full MS spectrum + Library search; definitive identification; TCD (Thermal Conductivity): Universal; less sensitive; for permanent gases; ECD (Electron Capture): Selective for halogenated compounds, pesticides; very sensitive; NPD (Nitrogen-Phosphorus Detector): Selective for N and P compounds; alkaloids, organophosphorus pesticides

GC-MS: Hyphenated technique: GC separates + MS identifies; EI (70 eV) gives reproducible spectra; NIST library (>300,000 spectra) used for unknown identification; Definitive ID of volatile phytoconstituents

IP Applications: Essential oil analysis: Peppermint oil (menthol NLT 44%); Clove oil (eugenol NLT 85%); Coriander oil (linalool NLT 65%); Fennel oil (anethole NLT 60%); GC-FID official IP method; Residual solvent testing (ICH Q3C); Pesticide residue testing (ICH Q3A)

Derivatisation: Non-volatile compounds made volatile by chemical derivatisation: TMS (trimethylsilyl) ethers for sugars, steroids; Methyl esters for fatty acids; Acetyl esters for alcohols; Oximes for aldehydes/ketones; Extends GC applicability to non-volatile analytes (diosgenin-TMS, artemisinin after derivatisation)

Headspace GC (HS-GC): Volatile analytes analysed from headspace above sample; no extraction needed; used for residual solvents in APIs (USP/BP/EP); also for essential oil vapour profiling

OTHER CHROMATOGRAPHIC TECHNIQUES

ADDITIONAL CHROMATOGRAPHIC METHODS

- **COUNTER-CURRENT CHROMATOGRAPHY (CCC) / CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC):** Uses liquid-liquid partitioning between two immiscible liquid phases; NO solid support → no irreversible adsorption; solute recovery almost 100%; excellent for labile, ionic, or strongly adsorbing compounds; Scale: mg to >100 g in one run; Applications: Isolation of saponins, alkaloids, tannins, peptides; Hylamer-Müller solvent systems
- **DROPLET COUNTER-CURRENT CHROMATOGRAPHY (DCCC):** Older liquid-liquid CC; vertical glass tubes; heavier phase descends as droplets through lighter ascending phase; gentle; for unstable compounds
- **MEDIUM PRESSURE LIQUID CHROMATOGRAPHY (MPLC):** Between gravity CC and HPLC in terms of pressure (3–20 bar); faster than gravity CC; automated; industrially used for Podophyllotoxin, Vincristine purification
- **AFFINITY CHROMATOGRAPHY:** Stationary phase has specific biological ligand (antibody, enzyme, receptor); binds only specific analyte; highly selective; used for protein-drug interaction studies; lectin affinity for glycoproteins/polysaccharides
- **ION-EXCHANGE CHROMATOGRAPHY:** Ionic interactions; anion (DEAE-Sepharose) or cation (CM-Sepharose) exchangers; excellent for alkaloid salts, saponins; used in Quinine isolation from Cinchona
- **SIZE-EXCLUSION CHROMATOGRAPHY (SEC/GFC):** Separation by molecular size; Sephadex G-25/50/100 (aqueous – for peptides, polysaccharides); Sephadex LH-20 (organic solvents – most widely used in phytochemistry for removing tannins, separating flavonoids and alkaloids by hydrophobic mechanism on LH-20)

ELECTROPHORESIS

ELECTROPHORESIS IN PHYTOCHEMICAL ANALYSIS

Electrophoresis is the migration of charged molecules (ions) through a medium under the influence of an applied electric field. Molecules with different charges, sizes, and shapes migrate at different rates and are thus separated. While primarily used for macromolecules (proteins, DNA, RNA), electrophoresis has important applications in phytochemical analysis, particularly for charged phytoconstituents like alkaloid salts, tannins, saponins, and for protein-based authentication of herbal drugs.

BASIC PRINCIPLE: When a potential difference (voltage, V) is applied across a medium, charged molecules move toward the electrode of opposite charge. Migration rate depends on: (1) Net charge (z); (2) Molecular size/shape (larger molecules migrate slower); (3) Field strength (V/cm); (4) Viscosity of medium; (5) Temperature. Rate of migration = zE/f where E = electric field, f = frictional coefficient.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Principle: Proteins/peptides separated in polyacrylamide gel matrix by size and charge; gel formed by polymerisation of acrylamide + bis-acrylamide (cross-linker); pore size controlled by % acrylamide; NATIVE PAGE: Charge + size separation; SDS-PAGE: Size only (SDS denatures protein, gives uniform negative charge)

SDS-PAGE: SDS (Sodium Dodecyl Sulphate): Anionic detergent; binds protein ~1.4 g SDS/g protein → all proteins have same charge density; separated ONLY by size; molecular weight determined by comparison with MW markers (protein ladder)

Gel System: Stacking gel (4% acrylamide, pH 6.8): Concentrates sample into thin band; Resolving gel (8–15% acrylamide, pH 8.8): Separates proteins; Higher %acrylamide → smaller pore → better resolution of smaller proteins

Equipment: Vertical or horizontal gel apparatus; power supply (constant voltage 100–200V or current); staining: Coomassie Brilliant Blue (R-250, sensitivity ~1 µg/band) or Silver staining (sensitivity ~10–50 ng/band) or SYPRO Ruby (fluorescent, very sensitive)

Applications in Phytochemistry: (1) PROTEIN FINGERPRINTING: Authentication of herbal drugs by seed/leaf protein profiles; distinguish closely related species or varieties; detect adulteration (e.g., ginseng species, turmeric vs coleus); (2) Enzyme characterisation: Isolation and characterisation of biosynthetic enzymes (Strictosidine synthase, Taxadiene synthase); (3) Molecular weight determination of plant proteins; (4) Quality control of protein-containing crude drugs (e.g., papain from Carica papaya); (5) Drug-protein interaction studies

Western Blotting: Post-PAGE technique: Proteins transferred to nitrocellulose membrane; probed with specific antibody → detect specific protein; used for enzyme detection, authenticity testing

CAPILLARY ELECTROPHORESIS (CE)

CAPILLARY ELECTROPHORESIS (CE)

Principle: Electrophoresis performed in a narrow-bore fused silica capillary (25–100 µm ID, 20–100 cm long) filled with electrolyte buffer; high voltage (10–30 kV); Electroosmotic Flow (EOF): Buffer flow from anode to cathode induced by ionised silanol groups on capillary wall; extremely efficient (millions of theoretical plates); rapid (5–20 min); small sample (<10 nL injection)

Modes of CE: CZE (Capillary Zone Electrophoresis): Simplest; separation by charge/mass ratio in free solution; for small ions and neutral molecules with EOF; MECC (Micellar Electrokinetic Capillary Chromatography): SDS micelles added as pseudostationary phase; neutral molecules partitioned into/out of micelles; separates neutral phytoconstituents; CEC (Capillary Electrochromatography): Combines CE and HPLC; pressurised flow + electrophoretic drive; packed capillary; CGE (Capillary Gel Electrophoresis): Gel-filled capillary; DNA/RNA sequencing; CIEF (Capillary Isoelectric Focusing): Separates by pI; proteins

Detection: On-column UV detection (most common, 190–280 nm); Fluorescence (laser-induced, LIF – very sensitive, 10^{-15} mol/L); MS (CE-MS: ESI interface); Conductivity detector (ions)

Advantages: Very high efficiency (millions of plates); speed (5–30 min); tiny sample volume (nL); minimal solvent; resolves enantiomers (with chiral additives – β-cyclodextrin); automated; fully quantitative; validated against HPLC

Applications in Phytochemistry: (1) ALKALOID ANALYSIS: Separation of alkaloid enantiomers (atropine vs hyoscyamine using chiral CD-CE); Cinchona alkaloids (quinine, quinidine, cinchonine, cinchonidine); Opium alkaloids; Vinca alkaloids; (2) FLAVONOID PROFILING: Simultaneous CE separation of 10–20 flavonoids in 15 min; (3) PHENOLIC ACIDS: Caffeic, ferulic, chlorogenic acid separation; (4) GLYCOSIDE ANALYSIS: Cardiac glycosides, sennosides; (5) SAPONIN FINGERPRINTING; (6) Herbal authentication; (7) Quality control of herbal products; (8) CHIRAL SEPARATION: Plant metabolite enantiomers

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2D-PAGE)

TWO-DIMENSIONAL PAGE (2D-PAGE)

Principle: Two sequential electrophoretic separations in perpendicular directions: FIRST DIMENSION – IEF (Isoelectric Focusing): Proteins separated by isoelectric point (pI) in a pH gradient gel; each protein migrates to its pI position; SECOND DIMENSION – SDS-PAGE: Gel strip from IEF placed on top of SDS-PAGE gel; proteins separated by molecular weight (MW); Result: 2D map with spots at unique (pI, MW) coordinates

Resolution: Can resolve >2,000 proteins per gel; each spot = one (or few closely migrating) protein(s); revolutionary for proteomics

Gel-to-Gel Variability: Major challenge in 2D-PAGE; DIGE (2D Differential In-Gel Electrophoresis) uses fluorescent dyes (Cy2, Cy3, Cy5) to label two samples before mixing → run on same gel → better comparison

Image Analysis: Software (ImageMaster, PDQuest, Progenesis): Detects, quantifies, and matches spots between gels; identifies differentially expressed proteins

Spot Identification: Spots cut from gel; in-gel digestion with trypsin; peptides analysed by MALDI-TOF or LC-MS/MS; protein identified by database matching (MASCOT, SEQUEST)

Applications in Phytochemistry: (1) HERBAL DRUG PROTEOMICS: Comprehensive protein profiling for authentication; detecting adulteration (substitution of one Panax species for another – distinct 2D-PAGE proteome); (2) PLANT STRESS RESPONSE PROTEOMICS: Changes in protein expression under drought, disease, elicitation; relevant to understanding secondary metabolite production; (3) BIOSYNTHETIC ENZYME DISCOVERY: Identify enzymes induced when secondary metabolites are produced; (4) CULTIVAR DIFFERENTIATION: Distinguish genetic varieties of medicinal plants based on protein profiles; (5) QUALITY VARIATION: Compare protein profiles of herbal drugs from different geographic origins

Modern Alternatives: DIGE (Differential In-Gel Electrophoresis); Label-free quantitative proteomics (LC-MS/MS); iTRAQ, TMT labelling + MS; Protein microarrays – all increasingly replacing classical 2D-PAGE

INTEGRATED APPLICATIONS

APPLICATIONS IN ISOLATION, PURIFICATION & IDENTIFICATION

Strategy for Isolating a New Phytoconstituent

STEP-BY-STEP STRATEGY

- Step 1 – ACTIVITY-GUIDED FRACTIONATION: Biological activity (antimicrobial, anticancer, antioxidant) guides isolation; most active fractions prioritised for further purification
- Step 2 – EXTRACTION: Choose solvent based on activity assay (aqueous = glycosides/alkaloid salts; hexane = terpenoids/fatty acids; methanol = broad spectrum); modern method (UAE, MAE) for speed
- Step 3 – LIQUID-LIQUID PARTITIONING: Sequential fractionation using hexane, $CHCl_3$, EtOAc, n-BuOH; each fraction bioassayed; concentrate on active fraction
- Step 4 – COLUMN CHROMATOGRAPHY: Silica gel (normal phase); VLC first (rapid primary fractionation); then open column CC; then flash CC or MPLC; fractions monitored by TLC
- Step 5 – PREPARATIVE HPLC: Final purification of active fractions to >95% purity; C18 column; UV or ELSD detection; collect pure compound fraction
- Step 6 – STRUCTURE ELUCIDATION: UV → functional groups/chromophores; IR/FTIR → functional groups; ^{1}H-NMR → proton framework; ^{13}C-NMR + DEPT → carbon framework; COSY + HSQC + HMBC → connectivity; NOESY → stereochemistry; MS (HRMS) → molecular formula + fragmentation; X-ray → absolute structure
- • Step 7 – DEREPLICATION (before full isolation): HPLC-PDA + LC-MS/MS → compare with database (SciFinder, DNP, AntiBase) → identify known compounds early → avoid re-isolation

Applications in Crude Drug Identification & Authentication

Technique	Application in Crude Drug Authentication
TLC / HPTLC	Fingerprint chromatographic profiles; WHO recommends HPTLC for herbal authentication; Rf of characteristic markers compared with reference; detect adulteration
UV Spectroscopy	Quick screening; identify class of chromophore; quantify marker compounds in standardised extracts; total flavonoid/phenolic content
FTIR / ATR-FTIR	Identity testing of raw materials (pharmacopoeial); functional group comparison; detect adulteration (foreign oils, synthetic adulterants); NIR for rapid non-destructive analysis

HPLC-PDA	Chromatographic fingerprint + UV spectrum at each peak; gold standard for herbal QC; IP/BP/WHO monographs use HPLC fingerprints
GC-MS	Volatile oil composition profiling; compare with database; detect substitution or adulteration of essential oils; residual solvents; pesticide residues
LC-MS / QTOF	Metabolite profiling; dereplication of known compounds; identify marker compounds; untargeted metabolomics for authentication
NMR Metabolomics	^{1}H-NMR fingerprint of crude extract; chemometric analysis (PCA, PLS-DA); differentiate species, geographical origins, seasonal variation
SDS-PAGE / 2D-PAGE	Protein fingerprint of herbal drugs; authenticate ginseng, saffron, turmeric; detect macro-adulteration (starch proteins from cereal adulterants)
Capillary Electrophoresis	Alkaloid profiling (cinchona, opium); flavonoid fingerprinting; chiral analysis of enantiomeric alkaloids
DNA Barcoding + PCR	Molecular authentication; ITS, matK, rbcL gene sequencing; identifies species even in powdered or processed form

6.3 Hyphenated and Multidimensional Techniques

ADVANCED HYPHENATED TECHNIQUES

- HPLC-PDA-MS (LC-UV-MS): Standard modern phytochemical tool; separates by HPLC → UV spectrum (class identification) → MS (molecular weight + fragments) → simultaneous; one injection gives three levels of information
- HPLC-NMR (LC-NMR): Online or stopped-flow ^{1}H-NMR of HPLC fractions; eliminates isolation step; detect µg amounts; used in dereplication; expensive and complex
- HPLC-NMR-MS (LC-NMR-MS): Triple hyphenation; retention time + UV + NMR + MS for each peak; maximum information without isolation; cutting-edge in natural product research
- GC-MS-MS (GC-MS/MS, Triple Quadrupole GC): MRM-based quantification of trace terpenoids, pesticides; very sensitive (pg/mL levels)
- HPLC-MS/MS (LC-MS/MS): MRM quantification of alkaloids, glycosides, terpenoids in plasma or plant matrices; standard bioanalytical method
- HPLC-CD (Circular Dichroism): Measure optical activity of each HPLC peak; determine absolute configuration of enantiomers without complete isolation
- • ICP-MS (Inductively Coupled Plasma MS): Trace element analysis in herbal drugs; detect heavy metals (Pb, Cd, As, Hg) as per pharmacopoeial limits; extremely sensitive (ppb-ppt levels)

EXAM-ORIENTED MCQs – BASICS OF PHYTOCHEMISTRY & MODERN METHODS

Q1. Phytochemistry is defined as the branch of science dealing with:

a) Animal chemistry b) Chemistry of plant constituents including their isolation, identification, and biological activity c) Only volatile oil analysis d) Synthetic drug chemistry

✓ **Answer: b) Chemistry of plant constituents – their isolation, identification, structural characterisation, biosynthesis, and biological activity**

Q2. The Beer-Lambert Law (A = εcl) is the basis of which spectroscopic quantitative technique?

a) NMR spectroscopy b) Mass spectrometry c) UV-Visible spectroscopy d) IR spectroscopy

✓ **Answer: c) UV-Visible spectroscopy – A = εcl; used for quantification of phytoconstituents like caffeine (272 nm), rutin (354 nm), curcumin (425 nm)**

Q3. In FTIR spectroscopy, a broad absorption around 3200–3550 cm^{-1} indicates:

a) C=O stretch b) O-H stretch (hydrogen-bonded hydroxyl group) c) C-H stretch d) N-H stretch

✓ **Answer: b) O-H stretch (hydrogen-bonded) – alcohols, phenols, carboxylic acids; also indicates tannins, polyphenols in plant extracts**

Q4. The coupling constant (J value, in Hz) in NMR spectroscopy is useful for determining:

a) Number of carbons b) Molecular weight c) Connectivity and stereochemistry of neighbouring nuclei d) UV absorption maximum

✓ **Answer: c) Connectivity and stereochemistry – vicinal ^{3}J coupling shows 3-bond H-H relationships; J values distinguish axial-axial (large J) from axial-equatorial (small J) in ring systems**

Q5. HMBC (Heteronuclear Multiple Bond Correlation) NMR experiment shows correlations between:

a) Adjacent protons (H-H, vicinal) b) Directly bonded H and C c) Protons and carbons 2–4 bonds apart (long-range) d) Carbon-carbon bonds only

✓ **Answer: c) ^{1}H and ^{13}C 2–4 bonds apart – most important 2D NMR for phytoconstituent structure elucidation; connects fragments across heteroatoms and quaternary carbons**

Q6. In Mass Spectrometry, the molecular ion gives which important information?

a) Number of hydrogen atoms only b) Molecular weight of the compound c) Functional groups d) Chemical shift

✓ **Answer: b) Molecular weight – the molecular ion (M^+ in EI; $[M+H]^+$ in ESI) gives the MW of the intact molecule; fundamental for structure elucidation**

Q7. GC-MS uses which type of ionisation that gives reproducible spectra matchable to NIST library?

a) Electrospray Ionisation (ESI) b) MALDI c) Electron Ionisation (EI, 70 eV) d) Chemical Ionisation (CI)

✓ **Answer: c) Electron Ionisation (EI, 70 eV) – gives reproducible fragmentation; >300,000 spectra in NIST database; used for volatile oil component identification (menthol, eugenol, citral)**

Q8. Supercritical CO_2 ($scCO_2$) extraction is classified as a 'green' technology because:

a) The CO_2 produces green colour in extract b) CO_2 is non-toxic, non-flammable, recyclable, and leaves no solvent residues in product c) It is only used for green plant materials d) It requires no energy

✓ **Answer: b) CO_2 is non-toxic, non-flammable, GRAS, recyclable – CO_2 evaporates completely after depressurisation leaving solvent-free extract; preferred for pharmaceutical and food applications**

Q9. Microwave-Assisted Extraction (MAE) is NOT suitable for which type of compounds?

a) Polar phenolics b) Alkaloids c) THERMOLABILE compounds that degrade at elevated temperatures d) Flavonoids

✓ **Answer: c) Thermolabile compounds – MAE generates high temperatures (up to 150°C); thermolabile compounds (e.g., artemisinin endoperoxide, heat-sensitive vitamins) may degrade; UAE (ultrasound) preferred instead**

Q10. In TLC, the Rf value for a compound should ideally be in the range:

a) 0.0–0.1 b) 0.3–0.7 c) 0.8–1.0 d) 1.0–2.0

✓ **Answer: b) 0.3–0.7 – optimal Rf range ensures good separation from impurities above and below; values too low (compounds too polar) or too high (too non-polar) indicate mobile phase needs adjustment**

Q11. Which spray reagent is SPECIFIC for alkaloids in TLC?

a) Anisaldehyde-H_2SO_4 b) $FeCl_3$ c) Dragendorff's reagent ($KBiI_4$) → orange-red precipitate d) Ninhydrin

✓ **Answer: c) Dragendorff's reagent (potassium bismuth iodide) → orange-red spots with alkaloids on TLC; Mayer's (cream) also specific but used in solution**

Q12. HPLC-PDA (Photodiode Array) detector provides which unique advantage over single-wavelength UV?

a) Higher flow rate b) Full UV-Vis spectrum at each HPLC peak → peak purity assessment + compound class identification c) Lower column pressure d) Better retention time reproducibility

✓ **Answer: b) Full UV-Vis spectrum (200–800 nm) captured simultaneously at each peak → peak purity confirmation (spectral homogeneity) + chromophore class identification; essential for phytochemical HPLC**

Q13. Capillary Electrophoresis (CE) is particularly useful for separation of:

a) Very large polymers only b) Non-polar volatile compounds c) Enantiomeric alkaloids (using chiral selectors like cyclodextrins) and ionic phytoconstituents d) Fixed oils and waxes

✓ **Answer: c) Enantiomeric alkaloids – CE with cyclodextrin additives resolves plant alkaloid enantiomers (hyoscyamine/atropine); also excellent for charged compounds (alkaloid salts, phenolic acids, flavonoid glycosides) with high efficiency**

Q14. SDS-PAGE separates proteins primarily based on:

a) Charge only b) Isoelectric point (pI) c) Molecular size/weight (SDS gives uniform charge) d) Polarity

✓ **Answer: c) Molecular size/weight – SDS binds uniformly to proteins (~1.4 g SDS/g protein) giving uniform charge density; migration rate in gel depends only on molecular weight; enables MW determination**

Q15. The purpose of the First Dimension (IEF) in 2D-PAGE is to separate proteins by:

a) Molecular weight b) Hydrophobicity c) Isoelectric point (pI) using pH gradient gel d) Size

✓ **Answer: c) Isoelectric point (pI) – IEF separates proteins in a pH gradient until each reaches its pI (no net charge → stops moving); Second dimension (SDS-PAGE) then separates by MW**

Q16. Which modern extraction technique uses acoustic cavitation at 20–100 kHz?

a) Microwave-Assisted Extraction b) Supercritical Fluid Extraction c) Ultrasound-Assisted Extraction (UAE/Sonication) d) Pressurised Liquid Extraction

✓ **Answer: c) Ultrasound-Assisted Extraction (UAE) – ultrasonic waves cause cavitation (micro-bubble formation and collapse) → intense local pressure → cell wall disruption → enhanced extraction; suitable for thermolabile compounds**

Q17. The NOESY/ROESY NMR experiment is used to determine:

a) Molecular weight b) Chemical shift of carbons c) Spatial proximity (stereochemistry) – through-space H-H correlations within 5 Å d) H-C 2–4 bond correlations

✓ **Answer: c) Through-space H-H correlations within ~5 Å – used for relative/absolute configuration; determine α/β orientation of substituents; distinguish cis/trans isomers; essential for complex terpenoid and glycoside stereochemistry**

Q18. In Electrospray Ionisation (ESI-MS), which type of molecules are best ionised?

a) Non-polar volatile molecules b) Polar, thermolabile, high molecular weight molecules (e.g., glycosides, alkaloid salts) c) Only hydrocarbons d) Inorganic salts only

✓ **Answer: b) Polar, thermolabile, high MW molecules – ESI is a soft ionisation technique; no degradation; suitable for cardiac glycosides, sennosides, saponins, MIA alkaloids that cannot be analysed by GC-MS**

Q19. The Naturstoff reagent (DPBA + PEG 4000) used in HPTLC produces what characteristic colour for flavonoids?

a) Red b) Blue c) Yellow-green fluorescence at UV 366 nm d) Purple

✓ **Answer: c) Yellow-green fluorescence at UV 366 nm – DPBA (2-aminoethyl diphenylborinate) forms fluorescent complexes with flavonoids; intensity and colour vary with subclass: Flavonols → yellow; Flavanones → orange-yellow; Flavones → greenish**

Q20. Which hyphenated technique provides the most comprehensive structural information from a single chromatographic run?

a) HPLC-UV b) GC-FID c) HPLC-PDA-MS (LC-UV-MS) giving retention time + UV spectrum + molecular mass and fragmentation d) TLC-densitometry

✓ **Answer: c) HPLC-PDA-MS (LC-UV-MS) – three levels of information simultaneously: retention time (separation), UV spectrum (chromophore/class), MS fragmentation (MW + structure); standard modern phytochemical dereplication tool**

BEST OF LUCK FOR EXAMS!