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B.PHARMA 8TH SEMESTER

BP811ET — ADVANCED INSTRUMENTATION TECHNIQUES

UNIT V — COMPLETE PREMIUM NOTES

HYPHENATED TECHNIQUES

LC-MS/MS · GC-MS/MS · HPTLC-MS

★ PREMIUM PAID NOTES ★ PCI / AKTU Aligned ★

Unit V Contents at a Glance
SECTION 1 — Introduction to Hyphenated Techniques: Definition | Why Hyphenation | Advantages | History
SECTION 2 — Mass Spectrometry Fundamentals: Ion Sources (ESI, APCI, APPI, EI) | Mass Analyzers (Triple Quad QqQ, TOF, Orbitrap, Ion Trap) | Tandem MS (MS/MS) — Product Ion, Precursor Ion, Neutral Loss, MRM
SECTION 3 — LC-MS/MS: Principle | LC-ESI/APCI Interface | Triple Quadrupole | MRM Quantification | Ionisation Suppression | Validation | Pharmaceutical Applications
SECTION 4 — GC-MS/MS: Principle | GC-EI/CI Interface | Triple Quadrupole GC-MS/MS | Scan vs SIM vs MRM | Applications (Residual Solvents, Volatiles, Forensics, Environmental)
SECTION 5 — HPTLC-MS: HPTLC Principle | Coupling Strategies (Direct Elution, DESI, SALDI) | Instrumentation | Applications (Herbal, Forensic, Food)

INTRODUCTION TO HYPHENATED TECHNIQUES

What are Hyphenated Techniques?

Hyphenated techniques (also called **coupled** or **hybrid analytical methods**) are analytical systems created by **online coupling of a separation technique with a detection/identification technique** through an appropriate interface. The hyphen (−) in the name represents the interface that connects the two instruments.

The fundamental concept: **no single analytical technique can simultaneously provide efficient separation, structural identification, and quantification of complex mixtures** — hyphenation combines the best features of each:

Hyphenated Technique	Separation Part	Interface	Detection Part	Primary Strength
LC-MS/MS	HPLC or UHPLC	ESI or APCI source	Triple Quadrupole MS	Quantification of drugs/metabolites in biological matrices
GC-MS/MS	Gas Chromatograph	EI or CI source	Triple Quadrupole or QqQ MS	Volatiles, residual solvents, environmental contaminants
HPTLC-MS	HPTLC plate	Elution, DESI, or SALDI	Any MS analyzer	Herbal authentication, forensic screening
LC-NMR	HPLC	Flow cell	NMR spectrometer	Structure elucidation of unknowns
GC-FTIR	Gas Chromatograph	Light pipe	FTIR spectrometer	Structural ID of GC-separated volatile compounds
ICP-MS	ICP (Inductively Coupled Plasma)	Direct	Mass Spectrometer	Trace element analysis at ppt level

Why Hyphenation? — The Need

Single analytical techniques have critical limitations when analysing real-world pharmaceutical and biological samples:

Limitation of Single Technique	Problem	Solution by Hyphenation
HPLC alone (UV detection)	Cannot distinguish co-eluting compounds that absorb at same λ; cannot identify unknowns	LC-MS adds molecular mass + fragmentation — unambiguous ID even with co-elution
GC alone (FID detection)	No structural information; co-eluting compounds give single FID peak	GC-MS provides mass spectrum — library searchable identity for each peak
MS alone (direct infusion)	Cannot handle complex mixtures — all analytes ionise simultaneously → ion suppression → chimeric spectra	Upstream separation (LC/GC) ensures single analyte entering MS at any time
TLC/HPTLC alone (UV/vis)	No structural ID; limited sensitivity; cannot distinguish compounds with similar Rf	HPTLC-MS adds molecular mass and structure for every spot on plate

Advantages of Hyphenated Techniques

- **Selectivity:** Dual selectivity — separation separates by physical/chemical property; MS detects by mass → two independent dimensions of selectivity
- **Sensitivity:** MS detection achieves femtomolar (10^{-15} mol) sensitivity — far superior to UV or FID
- **Structural information:** MS fragmentation patterns provide unambiguous structural identity — not just retention time
- **Speed:** Modern UHPLC-MS/MS analyses complete in 1–3 minutes; high-throughput 96-sample batches routine
- **Simultaneous multi-analyte:** MRM scan mode measures 50–100 different compounds in a single LC-MS/MS run
- **Quantification accuracy:** Stable isotope-labelled internal standards (SIL-IS) correct for extraction variability and ionisation suppression
- **Regulatory acceptance:** LC-MS/MS is the gold standard for bioanalytical method validation (EMA, USFDA guidances)

Historical Development

Year	Development	Significance
1968	GC-MS coupling established (Gohlke & McLafferty)	First successful online hyphenation
1970s	LC-MS interface attempts (moving belt, thermospray)	Early LC-MS — limited by liquid evaporation problem
1984	Electrospray Ionisation (ESI) demonstrated by Fenn	Nobel Prize 2002 — enabled LC-MS of large biomolecules

Year	Development	Significance
1988	ESI of proteins by Fenn and Yamashita	First multiple-charge states on proteins by ESI-MS
1991	Atmospheric Pressure Chemical Ionisation (APCI)	Enabled small molecule LC-MS with non-polar analytes
1990s	Triple quadrupole LC-MS/MS with MRM	Revolutionised pharmaceutical bioanalysis and pharmacokinetics
2000s	UHPLC-MS/MS; Orbitrap mass analyser	Ultra-high speed; ultra-high resolution (100,000+ resolving power)
2010s	HPTLC-MS coupling (DESI, SALDI)	Direct MS from TLC plates; herbal authentication
2020s	Ambient MS; ion mobility MS (IMS-MS); FAIMS	Emerging techniques for rapid screening without chromatography

MASS SPECTROMETRY FUNDAMENTALS

Understanding LC-MS/MS and GC-MS/MS requires a solid foundation in mass spectrometry — the detection system shared by all hyphenated MS techniques. This section covers the ionisation methods and mass analysers relevant to pharmaceutical hyphenated techniques.

General MS Principle

Mass spectrometry measures the **mass-to-charge ratio (m/z)** of ions. The process: (1) **Ionise** neutral molecules into gas-phase ions; (2) **Separate** ions by m/z in a mass analyser; (3) **Detect** ions and generate a mass spectrum (intensity vs m/z).

m/z = mass of ion (Da or u) / charge number (z)

Ionisation Methods for Hyphenated Techniques

Ionisation is the most critical step — the source must efficiently convert analytes from the LC/GC effluent into gas-phase ions without destroying them. Different sources suit different analyte types and separation methods.

Ionisation Method	Full Name	Principle	Analytes Best For	Used With	Ions Formed
ESI	Electrospray Ionisation	High voltage (3–5 kV) applied to liquid spray; droplets evaporate → multiply charged ions via ion evaporation or charge residue model	Large biomolecules (proteins, peptides), polar drugs, ionic compounds	**LC-MS/MS** (most common)	$[M+nH]^{n+}$, $[M-nH]^{n-}$, adducts $[M+Na]^+$, $[M+K]^+$

Ionisation Method	Full Name	Principle	Analytes Best For	Used With	Ions Formed
APCI	Atmospheric Pressure Chemical Ionisation	LC effluent vaporised at 300–400°C; corona discharge needle ionises solvent → chemical ionisation of analyte	Small, non-polar to moderately polar drugs; lipids; steroids	**LC-MS/MS** (complementary to ESI)	$[M+H]^+$, $[M-H]^-$ (singly charged)
APPI	Atmospheric Pressure Photo-Ionisation	UV photons (10.0 or 10.6 eV krypton lamp) directly photoionise analyte molecules	Non-polar aromatics, PAH, lipids, drugs without basic/acidic groups	LC-MS (niche)	$M^{+}\bullet$ (radical cation) or $[M+H]^+$
EI	Electron Ionisation (Impact)	70 eV electrons bombard vaporised sample in vacuum → $M^{+}\bullet$ formed	**Volatile, thermally stable compounds**; residual solvents	**GC-MS/MS**	$M^{+}\bullet$ (radical cation); extensive fragmentation; NIST library searchable
CI	Chemical Ionisation	Reagent gas (CH_4, NH_3) ions transfer proton to sample	Volatile compounds needing softer ionisation than EI; confirms MW	GC-MS	$[M+H]^+$ (protonated); minimal fragmentation
DESI	Desorption ESI	Charged ESI spray aimed at sample surface (TLC plate); desorbs and ionises	Directly from solid surfaces, TLC spots, tissues	**HPTLC-MS**; ambient MS	$[M+H]^+$, $[M-H]^-$
MALDI	Matrix-Assisted Laser Desorption/Ionisation	UV laser + matrix → desorption/ionisation of large molecules	Proteins, polymers, HPTLC spots	HPTLC-MS (SALDI variant), proteomics	$[M+H]^+$ (singly charged)

⚡ **Critical Exam Distinction:** ESI is the dominant ionisation for LC-MS/MS (polar, ionic, large molecules; multiply charged). EI at 70 eV is the standard for GC-MS (volatile, thermally stable; extensive fragmentation; NIST library). APCI complements ESI for non-polar small molecules in LC-MS. DESI enables direct MS from HPTLC plates without extraction.

Mass Analysers for Hyphenated Techniques

Analyser	Principle	Resolution	Mass Range	Key Feature	Pharma Use
Triple Quadrupole (QqQ)	3 quadrupoles in series: Q1 (select), q2 (fragment), Q3 (select product)	Unit resolution (1 Da)	Up to 3000 m/z	**MRM quantification** — gold standard for bioanalysis	LC-MS/MS and GC-MS/MS pharmacokinetics, TDM, doping
Quadrupole-TOF (Q-TOF)	Quadrupole (Q1) + collision cell + Time-of-Flight	High (>10,000); exact mass	Unlimited	Accurate mass (HRMS); structure elucidation	Metabolite ID; drug impurity characterisation
Orbitrap	Ions orbit electrode; frequency of oscillation → m/z	Ultra-high (>100,000 FWHM)	Up to 6000 m/z	Highest resolution commercial MS; FT-MS	Proteomics; drug metabolism; environmental HRMS
Ion Trap (IT)	RF field traps ions in 3D or 2D space; sequential MS^n possible	Unit to medium	Up to 2000+ m/z	Multi-stage fragmentation (MS^n — MS^3, MS^4...)	Structural elucidation; fragmentation pathway
Magnetic Sector	Magnetic field deflects ions; double-focusing (E+B)	Very high	Wide	Accurate mass (classical HRMS); stable isotope	Isotope ratio; legacy pharma use declining

Tandem Mass Spectrometry (MS/MS) — The Core of Hyphenated MS

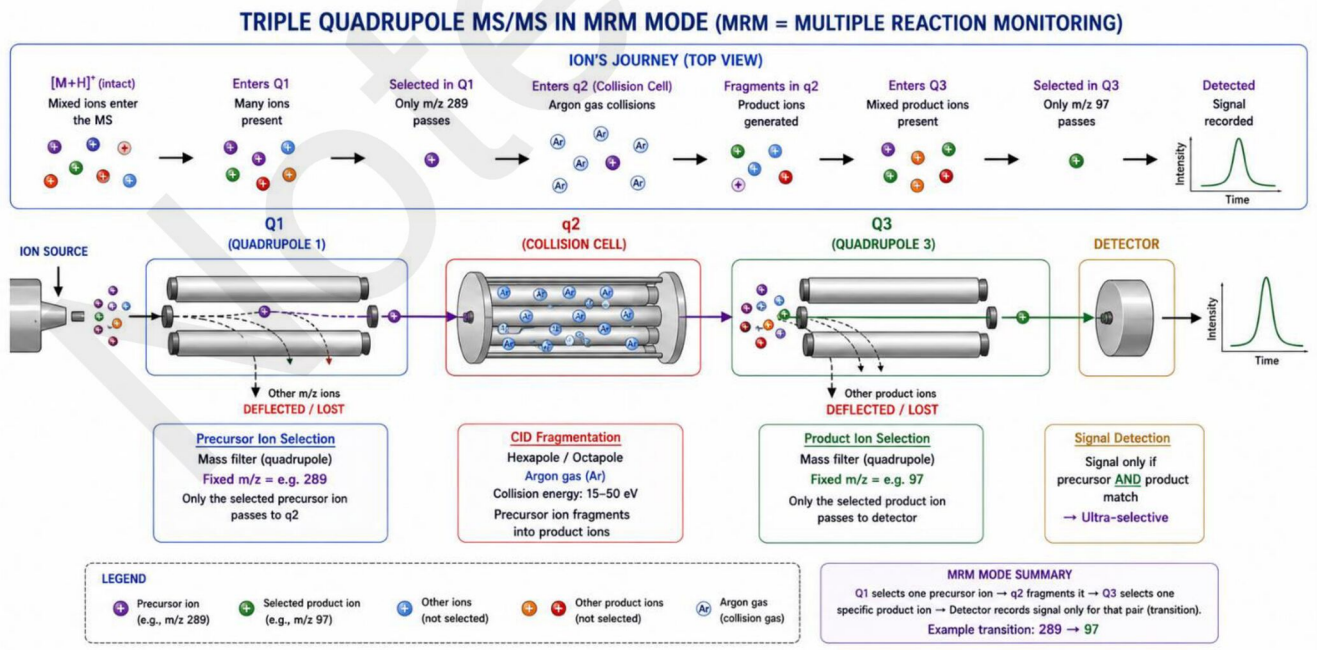
Tandem MS (MS/MS) involves two stages of mass analysis separated by a fragmentation (collision) step. The analyte ion (**precursor ion**) selected in the first MS stage is fragmented in a collision cell by collision with inert gas (Ar, N_2), and the resulting **product ions** (fragment ions) are analysed by the second MS stage.

The four fundamental MS/MS scan modes — all CRITICAL for the exam:

MS/MS Scan Mode	Q1 (First MS)	q2 Collision Cell	Q3 (Second MS)	Data Obtained	Pharmaceutical Application
Product Ion Scan	Fixed at one precursor m/z	Fragments precursor	Scans full mass range	All product ions from one precursor — structural information	Structure elucidation of metabolites; fragmentation library building
Precursor Ion Scan	Scans full mass range	Fragments all ions	Fixed at one product ion	All precursors that give a specific product — identifies compound class	Detect all compounds sharing common structural motif (e.g., all

MS/MS Scan Mode	Q1 (First MS)	q2 Collision Cell	Q3 (Second MS)	Data Obtained	Pharmaceutical Application
					phospholipids by m/z 184)
Neutral Loss Scan	Scans full mass range	Fragments all ions	Scans in sync with Q1 at constant offset (fixed mass difference)	All precursors that lose a specific neutral fragment — identifies compound class	Detect glucuronides (loss of 176 Da); sulfates (loss of 80 Da) in metabolite profiling
MRM (Multiple Reaction Monitoring)	Fixed at precursor m/z	Fragments	Fixed at one specific product m/z	Highly specific signal for ONE compound; ultra-high sensitivity; simultaneous multi-analyte	**Gold standard for quantitative bioanalysis** — drug concentration in plasma/urine for PK, TDM, doping

⚡ **MRM — The Most Important Concept in This Unit:** **MRM (Multiple Reaction Monitoring)** = both Q1 and Q3 FIXED at specific m/z values. Q1 selects the precursor ion (intact drug molecule). q2 fragments it. Q3 selects ONE specific product ion. Only the compound that produces this EXACT precursor → product ion pair gives a signal. This makes MRM the most SPECIFIC and SENSITIVE quantitative MS mode. A single MRM transition is called a 'transition pair': precursor m/z → product m/z. E.g., Testosterone: 289→97 (m/z). Learn this — it appears in every exam.



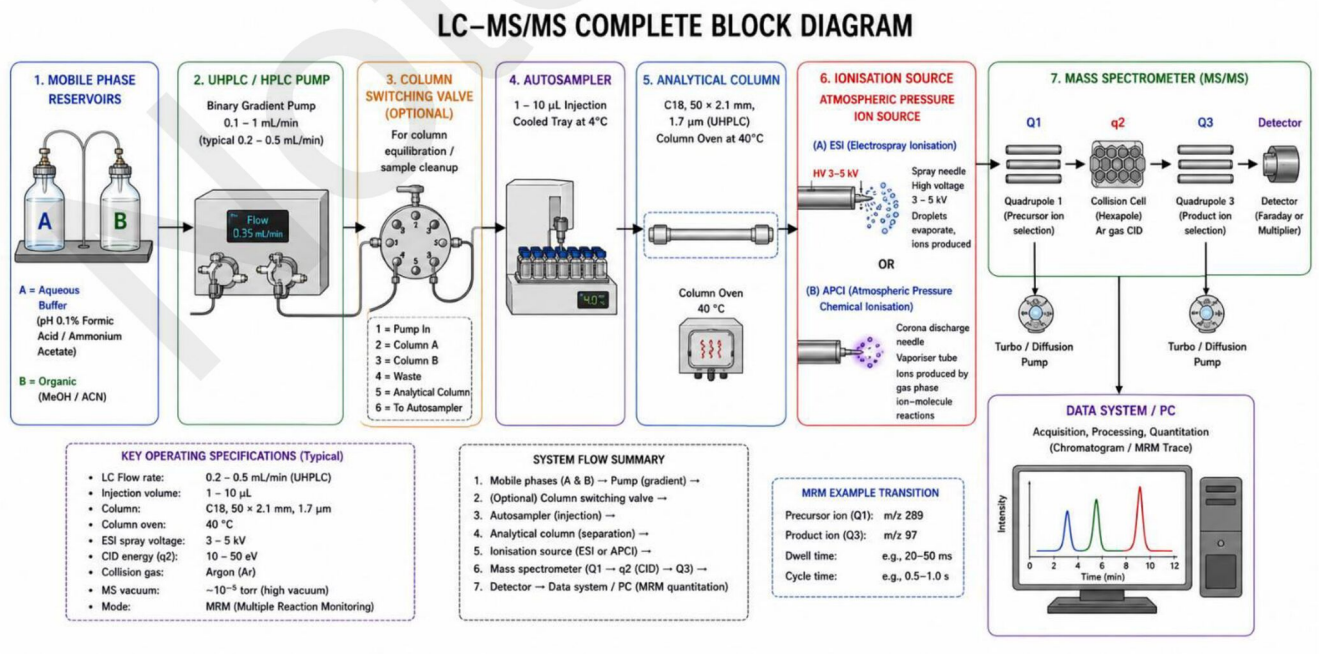
LC-MS/MS (LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY)

Introduction and Importance

LC-MS/MS (Liquid Chromatography coupled to Tandem Mass Spectrometry) is the **most powerful and widely used analytical technique in modern pharmaceutical science**. It combines the separation power of HPLC/UHPLC with the ultra-sensitive, selective detection and structural capability of tandem mass spectrometry.

Capability	Performance	Comparison to HPLC-UV
Sensitivity (LLOQ)	1–100 pg/mL in plasma	100–1000× more sensitive than UV
Selectivity	Dual — retention time + MRM transition	UV: single wavelength — one dimension only
Speed (UHPLC-MS/MS)	1–3 min per sample	5–15 min for HPLC-UV
Simultaneous analytes	50–200 compounds in one run (multi-MRM)	UV: typically 1–3 analytes per run
Matrix tolerance	High — MS selects by m/z even with co-eluters	UV: co-eluting matrix compounds directly interfere
Structural ID	Yes — product ion spectra, exact mass (HRMS)	No — only retention time and UV spectrum

LC-MS/MS System Components and Block Diagram



Component	Function	Key Specification
HPLC/UHPLC Pump	Delivers mobile phase at precise flow rate with gradient capability	Flow: 0.1–1.0 mL/min (UHPLC 0.2–0.5 mL); Pressure: up to 15,000 psi (UHPLC); Gradient accuracy ±0.5%
Column	Separates analytes from matrix and from each other before MS detection	UHPLC: 50×2.1 mm, 1.7 µm BEH C18 (Waters); HPLC: 50–150×4.6 mm, 3–5 µm C18. Short columns favoured for speed.
Mobile Phase	Carries analytes through column; must be MS-compatible (volatile, no involatile salts)	Aqueous: 0.1% formic acid, 5–10 mM ammonium formate/acetate buffer. Organic: methanol or acetonitrile. **NEVER use phosphate buffer — non-volatile → blocks source**
Autosampler	Injects precise sample volume; cooled sample storage	1–10 µL injection; sample tray at 4°C; carryover <0.1%; UHPLC: typically 2 µL
ESI Source	Converts LC effluent to gas-phase ions at atmospheric pressure	Spray voltage: 3–5 kV (pos mode); 2–4 kV (neg mode); source temperature: 100–150°C; Desolvation temperature: 350–600°C; Desolvation gas: N_2 at 800–1000 L/hr
APCI Source	Alternative ionisation for non-polar analytes; vaporises LC effluent before corona discharge ionisation	Vaporiser temperature: 300–450°C; Corona pin current: 2–5 µA; Probe temperature: 400–500°C
Q1 (First Quadrupole)	Selects the precursor ion — isolates one m/z from all ions entering the MS	Unit mass resolution (1 Da); FWHM ~0.7 Da; operates in mass filter or scan mode
q2 (Collision Cell)	Fragments selected precursor ion by Collision Induced Dissociation (CID)	Hexapole or octapole; Ar or N_2 collision gas at 10^{-3} torr; Collision energy: 10–50 eV (adjustable per compound)
Q3 (Third Quadrupole)	Selects the product ion — further filters for specificity	Same as Q1: unit resolution; fixed in MRM or scanned in product ion mode
Detector	Converts ion current to electrical signal	Electron multiplier (dynode) or Faraday cup; Dynamic range 10^5–10^6
Data System	Acquires MRM traces; integrates peaks; generates calibration curves and reports	Waters MassLynx, Sciex Analyst, Agilent MassHunter, Thermo Xcalibur software

ESI Ionisation Mechanism

Electrospray Ionisation (ESI) is the universal interface between HPLC and MS for pharmaceutical analysis. Understanding ESI is critical for method development.

Step-by-Step ESI Process:

1. **Spray formation:** LC effluent passes through a stainless steel capillary needle held at high voltage (3–5 kV). Electric field disperses liquid into a fine spray of highly charged droplets.
2. **Droplet evaporation:** Nitrogen desolvation gas (heated, 350–600°C) evaporates solvent from droplets. Droplets shrink, concentration of charge increases.
3. **Coulomb fission:** When droplet charge density exceeds the Rayleigh limit (surface tension force equals Coulombic repulsion), the droplet undergoes fission — breaks into smaller droplets.
4. **Ion formation (two mechanisms):** (a) **Ion Evaporation Model (IEM):** Small ions directly desorb from highly charged droplet surface when charge density is sufficient. (b) **Charge Residue Model (CRM):** Droplets evaporate completely → fully desolvated ions remain (dominant for large proteins).
5. **Ion transmission:** Ions pass through sampling cone/capillary, skimmer lens, and ion transfer optics into the vacuum region of the MS under a differential pumping system.

ESI Characteristics:

- **Soft ionisation** — minimal fragmentation; molecular ion $[M+H]^+$ or $[M-H]^-$ observed
- **Multiple charging:** Large molecules (proteins, antibodies) form multiply charged ions $[M+nH]^{n+}$ — enables measurement of very large molecules on instruments with limited m/z range
- **Positive mode (ESI+):** Basic analytes (amines, drugs with N) → $[M+H]^+$ or $[M+Na]^+$ — MOST USED in pharma
- **Negative mode (ESI−):** Acidic analytes (carboxylic acids, nucleotides, phospholipids) → $[M-H]^-$
- **Flow rate:** Optimal ESI at 0.1–1 mL/min; microspray/nanospray at 100 nL/min for highest sensitivity

LC Method Development for LC-MS/MS

Mobile Phase Requirements — MS-Compatibility

Mobile phase in LC-MS/MS MUST be **volatile** — components must completely evaporate in the ESI/APCI source without leaving non-volatile residues that block the source or interfere with ionisation.

Mobile Phase Component	MS-Compatible?	Why / Alternatives
0.1% Formic acid in water	✓ COMPATIBLE	Volatile; improves peak shape for basic drugs; promotes positive ESI
0.1% Acetic acid in water	✓ COMPATIBLE	Volatile; milder than formic; common in positive ESI
10 mM Ammonium formate	✓ COMPATIBLE	Volatile ammonium salt; good for basic/acidic drug separation; neutral pH

Mobile Phase Component	MS-Compatible?	Why / Alternatives
10 mM Ammonium acetate	✓ COMPATIBLE	Volatile; pH ~5.5; widely used; compatible with positive and negative mode
10 mM Ammonium bicarbonate	✓ COMPATIBLE	Volatile; pH ~8; good for anionic analytes in negative mode
0.1 M Sodium phosphate buffer	✗ NON-COMPATIBLE — DO NOT USE	Non-volatile sodium salt → deposits in source → blocks source → ion suppression → instrument downtime. Replace with ammonium formate/acetate
Triethylamine (TEA), TFA	⚠ USE WITH CAUTION	TEA: high concentration suppresses ionisation. TFA: strong ion pairing → severe ESI signal suppression. Limit to ≤0.1%

⚡ **Number 1 LC-MS Rule:** NEVER use phosphate buffers, TRIS buffers, or other non-volatile inorganic salts in LC-MS/MS mobile phases. They crystallise in the ESI source, causing blocked source, ion suppression, and instrument failure. Replace with ammonium formate or ammonium acetate — MS-compatible volatile buffers with equivalent buffering capacity.

Column Selection for LC-MS/MS

- **C18 BEH (Bridged Ethyl Hybrid) columns:** Most common; wide pH stability (1–12); UHPLC compatible
- **Short columns (50–100 mm):** Preferred for speed; adequate separation with UHPLC particles (1.7 µm)
- **Narrow bore (2.1 mm ID):** Low flow rate (0.2–0.4 mL/min) → better ESI ionisation efficiency → higher sensitivity vs 4.6 mm ID
- **C8, phenyl, HILIC:** Alternative chemistries for specific analyte classes (lipids, polar metabolites, glucuronides)

Quantitative LC-MS/MS — MRM Method

MRM (Multiple Reaction Monitoring) is the quantitative mode of LC-MS/MS and the **regulatory gold standard** for pharmaceutical bioanalysis (EMA Bioanalytical Method Validation Guideline; USFDA Bioanalytical Method Validation Guidance 2018).

MRM Method Development Steps:

1. **Select precursor ion:** Infuse pure analyte standard → identify $[M+H]^+$ (positive mode) or $[M-H]^-$ (negative mode). Optimise: spray voltage, cone voltage, source temperature.
2. **Select product ions:** Apply CID to precursor; scan Q3 → product ion spectrum. Select 2 product ions: (a) **Quantifier ion** — most abundant, best signal (used for quantification); (b) **Qualifier ion** — second most abundant, unique (used for identity confirmation, ratio check).
3. **Optimise collision energy (CE):** Vary CE (10–50 eV) to maximise product ion yield for each transition.
4. **Validate selectivity:** Inject blank matrix — no signal at MRM retention time (selectivity confirmed).

5. **Prepare calibration curve:** Spiked plasma standards (LLOQ to ULOQ); plot peak area ratio (analyte/IS) vs concentration. Regression: weighted $1/x^2$ least squares.

Internal Standard (IS) in LC-MS/MS

An internal standard is added at a fixed known concentration to EVERY sample, standard, and QC. It compensates for:

- **Extraction variability** — different recovery between samples
- **Injection volume variability** — autosampler imprecision
- **Ion suppression variability** — matrix-dependent ESI signal variation

IS Type	Example	Advantage	Disadvantage
Stable Isotope Labelled (SIL-IS) — BEST	Deuterium-labelled (d_3-drug) or ^{13}C-labelled analogue	Identical chemical behaviour to analyte; co-elutes; corrects FULLY for extraction AND ionisation suppression	Expensive; not available for all drugs; must verify no isotope cross-talk
Structural Analogue	Similar compound of different class (e.g., warfarin as IS for acenocoumarol)	Cheaper; widely available	Different extraction recovery and ionisation efficiency vs analyte; corrects partially
Radiolabelled compound	^{14}C or ^{3}H labelled drug	Used in metabolic fate studies	Cannot be used routinely in MS bioanalysis

Matrix Effects — Ion Suppression and Enhancement

Matrix effects — specifically **ion suppression** — is the most critical challenge in LC-MS/MS bioanalysis. Co-eluting endogenous matrix components (phospholipids, bile salts, polymers) compete with the analyte for ionisation in the ESI source, reducing the signal.

Matrix Component	Suppression Level	Source in Sample	Prevention Strategy
Phospholipids (PC, PE, SM)	Severe — can suppress signal 50–90%	Plasma membrane components; major cause of matrix effects in plasma analysis	Use SPE (HLB or phospholipid depletion plate), protein precipitation + dilution; specific phospholipid removal SPE
Bile acids / salts	Moderate–severe	Bile-derived; present in plasma and urine	Reversed phase SPE; dilute-and-shoot with dilution factor

Matrix Component	Suppression Level	Source in Sample	Prevention Strategy
Polymers (plasticiser, PTFE)	Moderate	Lab consumables (tubes, pipette tips) leaching	Use polymer-free glass tubes; certified low-binding tubes
Ion pairing agents (TFA)	Severe	Added to mobile phase for peak shape	Limit TFA to <0.1%; replace with ammonium formate + low FA
Proteins (albumin)	Moderate	Plasma proteins	Protein precipitation, SPE, or dilution

Matrix Effect Assessment Methods:

- **Post-column infusion method (Bonfiglio):** Infuse analyte continuously via syringe pump post-column; inject blank plasma extracted with same method; dip in LC trace at drug retention time = matrix effect zone
- **Post-extraction spike method (FDA/EMA standard):** Compare signal of analyte in extracted blank matrix (A) vs signal in pure solvent (B). Matrix factor (MF) = A/B. Acceptable: IS-normalised MF = 0.85–1.15 (±15%)

Matrix Factor (MF) = Peak Area (post-extraction spike) / Peak Area (neat standard)

- **Acceptance criteria (EMA/FDA):** IS-normalised MF %CV ≤ 15% across 6 lots of matrix

LC-MS/MS Pharmaceutical Applications

Application	Analytes	Matrix	Key Advantage of LC-MS/MS
Pharmacokinetic (PK) studies	Parent drug + active metabolites in plasma	Plasma, serum, blood	pg/mL sensitivity; all analytes measured simultaneously; definitive structure confirmation
Therapeutic Drug Monitoring (TDM)	Cyclosporine, tacrolimus, sirolimus, methotrexate, imatinib	Whole blood, plasma	Immunosuppressants measured at trough levels (ng/mL) — critical for transplant patients
Metabolite identification	Drug metabolites, phase I (oxidation), phase II (conjugation)	Plasma, urine, bile, microsomes	Q-TOF exact mass → identify unknown metabolites; predict biotransformation pathways
Impurity profiling	Related substances, process impurities, degradation products	API, drug product solution	Identifies impurity structure; detects below ICH Q3A threshold (0.1%)

Application	Analytes	Matrix	Key Advantage of LC-MS/MS
Doping control / sports antidoping	Anabolic steroids, peptide hormones, beta-agonists, EPO	Urine, blood	Ultra-low detection of prohibited substances (WADA laboratories)
Bioequivalence studies	Generic vs innovator drug plasma concentration-time profile	Plasma	Regulatory requirement — USFDA/EMA require validated LC-MS/MS method for BE studies
Tissue distribution / drug-receptor	Drug in brain, heart, tumour tissue	Tissue homogenate	Quantify drug at target site; support PK/PD modelling
Newborn screening	Amino acids, acylcarnitines (inherited metabolic disorders)	Dried blood spot (DBS)	Single 3 mm DBS punch; measures 40+ metabolites by LC-MS/MS — nationwide screening programs
Therapeutic protein quantification	Monoclonal antibodies (mAbs), peptide drugs	Plasma	Signature peptide LC-MS/MS after tryptic digest — regulatory bioanalysis of biologics
Lipidomics / metabolomics	Phospholipids, sphingolipids, fatty acids; endogenous metabolites	Plasma, urine, tissue	Comprehensive profiling of hundreds of lipids or metabolites — biomarker discovery

GC-MS/MS (GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY)

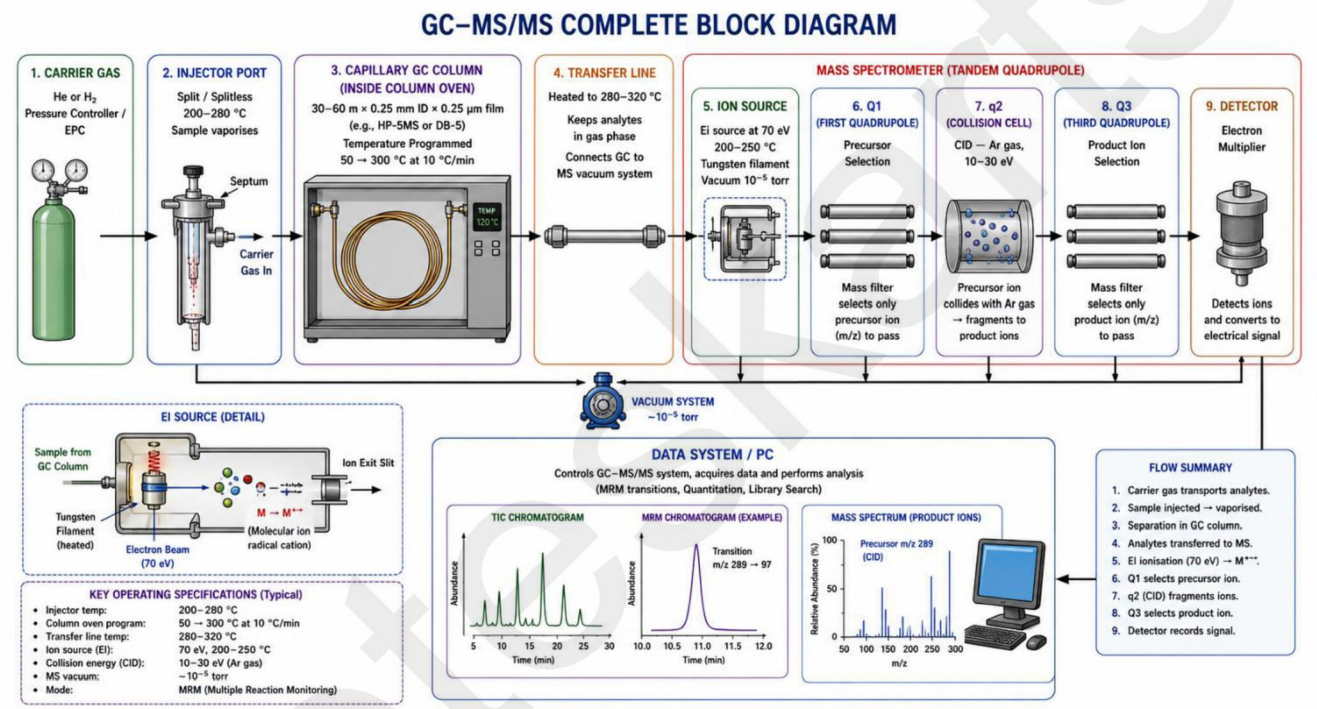
Introduction to GC-MS/MS

GC-MS/MS couples gas chromatographic separation with tandem mass spectrometric detection. It is the **method of choice for volatile and semi-volatile compound analysis** where derivatisation can make polar compounds GC-amenable. The combination is particularly powerful when selectivity and sensitivity both need to be maximised simultaneously in complex matrices.

Feature	GC-MS (Single Stage)	GC-MS/MS (Tandem)
Mode	Full scan EI or SIM	MRM (Q1 fixed + CID + Q3 fixed)
Selectivity	High (RT + EI spectrum)	ULTRA-HIGH (RT + precursor m/z + product m/z)
Sensitivity	Moderate (scan); good (SIM)	Excellent — 5–50× better than GC-MS SIM

Feature	GC-MS (Single Stage)	GC-MS/MS (Tandem)
Matrix tolerance	Moderate — matrix ions can interfere in SIM	Excellent — MRM eliminates most matrix background
Quantification mode	SIM (Selected Ion Monitoring)	MRM (Multiple Reaction Monitoring)
Application	Routine GC-MS; environmental, forensic	Ultra-trace analysis; complex matrices; food safety; residual solvents (ICH Q3C)

GC-MS/MS System Components



GC-MS/MS Component	Function	Specification
Carrier Gas (He)	Mobile phase — carries vaporised sample through column	Helium: most common (inert, high diffusivity, best resolution). H_2: faster, cheaper, now FDA-acceptable. N_2: cheap but poor resolution. Flow: 1–2 mL/min (capillary)
Injector — Split/Splitless	Vaporises liquid sample; introduces to column	Splitless: for trace analysis (most of sample enters column). Split 1:50: for concentrated samples. Temperature: 220–280°C. Injection volume: 1–2 µL
Capillary Column	Separates volatile analytes by bp, polarity	30–60 m × 0.25 mm ID × 0.25 µm film. Phase: HP-5MS (5% phenyl methylsiloxane — universal; MS-grade = low bleed at high T). Temperature program: 50→300°C

GC-MS/MS Component	Function	Specification
Transfer Line	Heated connection between GC oven and MS source; maintains analytes in gas phase	Temperature: 280–320°C (above bp of all analytes); fused silica deactivated transfer line
EI Ion Source	Ionises analytes at 70 eV electron impact; operated at 10^{-5} torr	Filament temperature: 2000°C; Source temperature: 200–250°C; 70 eV standard for reproducible spectra (NIST library)
Triple Quadrupole (Q1-q2-Q3)	MS/MS separation; same principle as LC-MS/MS QqQ	Collision gas: Ar or N_2; CE: 5–30 eV for GC analytes (lower than LC due to lower molecular weight); Unit mass resolution
Electron Multiplier Detector	Converts ion current to measurable electrical signal	Gain: 10^5–10^8; Dynamic range: 10^5–10^6; LOD for FID complement: sub-pg on-column for GC-MS/MS MRM

GC-MS and GC-MS/MS Scan Modes

Scan Mode	Q1	q2	Q3	Use Case	LOD (relative)
Full Scan (EI)	Scans all m/z	Off (no fragmentation)	—	Library searching; unknown identification; metabolite discovery	Lowest sensitivity (full scan)
SIM (Selected Ion Monitoring)	Fixed at 1–5 diagnostic ions	Off	—	Targeted quantification — classic GC-MS method	Better than full scan (5–10×)
Product Ion Scan	Fixed at precursor m/z	On (CID)	Scans all products	Fragmentation confirmation; metabolite structure	Research mode
MRM (Multiple Reaction Monitoring)	Fixed at precursor m/z	On (CID)	Fixed at product m/z	Ultra-sensitive targeted quantification; complex matrices	Best sensitivity (10–100× vs SIM)
NL Scan (Neutral Loss)	Scans	On	Synced scan (fixed offset)	Class identification (drug class, metabolite class)	Research mode

GC-MS/MS vs LC-MS/MS — Key Differences

Parameter	GC-MS/MS	LC-MS/MS
Sample type	Volatile / semi-volatile compounds	Non-volatile, thermally labile, polar compounds
Derivatisation	Often required for polar/non-volatile analytes (TMS, PFB derivatives)	Not usually required
Ionisation	EI (70 eV, hard) or CI (soft)	ESI or APCI (both soft) at atmospheric pressure
Spectral library	NIST EI mass spectral library (350,000+ spectra)	No standardised library (ESI spectra instrument-dependent)
Resolving power	Capillary GC: N = 100,000–500,000 plates	UHPLC: N = 10,000–50,000 plates
Mobile phase compatibility	Inert carrier gas only (He, H_2, N_2)	Wide range of aqueous/organic solvents (volatile only for MS)
Column stability	MS-grade low-bleed capillary columns	Silica-based C18; pH 1–12 stability
Best pharmaceutical use	Residual solvents (ICH Q3C); volatiles; environmental; forensic toxicology; essential oils	Drug PK/TDM; metabolite ID; large molecule bioanalysis; polar drugs
Headspace GC-MS/MS	Yes — ideal for volatile impurities without sample preparation	Not applicable

Pharmaceutical Applications of GC-MS/MS

Application	Analytes	Matrix	Why GC-MS/MS?
Residual Solvent Analysis (ICH Q3C)	Class 1 (benzene, CCl_4), Class 2 (MeOH, ACN, DCM), Class 3 solvents	API, drug product	Headspace GC-MS/MS detects volatile solvents at <1 ppm; MRM eliminates matrix interference; USP ⟨467⟩
Drug of Abuse — Confirmatory Testing	THC, cocaine, opiates, amphetamines (GC-EI for confirmation)	Urine, blood, hair	EI spectra match NIST library → definitive confirmation of screen-positive samples (GC-MS is legal gold standard)
Volatile Organic Compound (VOC) Analysis	Residual monomers (acrylamide, ethylene	Drug substance, packaging	Ultra-low detection limits (<0.01 ppm) needed for mutagenic impurities; GC-MS/MS MRM

Application	Analytes	Matrix	Why GC-MS/MS?
	oxide); genotoxic impurities		provides required sensitivity
Pesticide Residues in Herbal Medicines	200+ organochlorine, organophosphate, pyrethroid pesticides	Herbal drug materials	Multi-residue GC-MS/MS MRM method covers all major pesticide classes; WHO/GACP compliance
Essential Oil Characterisation	Monoterpenes, sesquiterpenes, phenylpropanoids (linalool, eugenol, thymol)	Plant extracts, essential oils	GC separation of complex terpenoid mixtures; EI MS identification; quality control of aromatherapy products
Fatty Acid Profiling (FAME Analysis)	Fatty acid methyl esters from omega-3, omega-6 supplements	Fish oil capsules, pharmaceutical lipid excipients	Derivatisation to FAME → GC separation → EI MS identification; pharmacopoeial test
Environmental Pharmaceutical Pollution	Antibiotics, hormones, analgesics in water	Wastewater, environmental water	Ultra-trace (ng/L) detection by GC-MS/MS after SPE concentration
Genotoxic Impurity Testing	Alkyl sulfates, alkyl sulfonates, aldehydes, epoxides	API from fermentation or synthetic routes	ICH M7 guidance: GC-MS/MS for ppm-level genotoxic impurities; acceptance limit often 1.5 µg/day intake

HPTLC-MS (HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY)

Introduction to HPTLC

HPTLC (High Performance Thin Layer Chromatography) is an advanced form of TLC that uses specially manufactured, smaller-particle silica plates with highly uniform particle size distribution (2–5 µm), enabling better separation efficiency, speed, and quantification compared to classical TLC.

Parameter	Classical TLC	HPTLC
Particle size	10–12 µm	2–5 µm (more uniform)
Plate height	Higher	~10× lower → better separation
Development distance	10–15 cm	5–8 cm
Development time	30–60 min	5–15 min
Detection sensitivity	Lower (visual or UV lamp)	Higher — densitometry with CCD camera; fluorescence
Sample application	Manual spotting	Automated TLC Sampler (ATS): 5–1000 nL precision
Quantification	Semi-quantitative (visual)	Quantitative — CAMAG TLC Scanner / VideoScan
MS coupling	Not feasible practically	Yes — HPTLC-MS via direct elution, DESI, SALDI

HPTLC System Components

- **Plate:** HPTLC silica gel 60 F_{254} (20×10 cm); normal phase (silica) or RP-18 (reversed phase)
- **Automated Sample Applicator (ATS 4):** Precise spray-on application of 5–1000 nL per band; programmable track positions
- **Development chamber:** Automatic Developing Chamber (ADC 2): controlled humidity and solvent saturation; Twin-Trough chamber
- **Detection (pre-MS):** UV lamp (254 nm and 366 nm); derivatising reagents (ninhydrin, vanillin-H_2SO_4); CCD documentation
- **Densitometry:** CAMAG TLC Scanner 3: quantitative scanning at 190–900 nm; fluorescence mode

HPTLC-MS Coupling Strategies

The challenge of HPTLC-MS coupling is transferring separated analytes from the solid silica plate into the gas phase for MS analysis. Three main strategies have been developed:

Strategy 1 — Direct Elution (Elution-Based HPTLC-MS)

Aspect	Description
Instrument	CAMAG TLC-MS Interface (commercially available) coupled to any ESI-MS
Principle	A circular elution head is pressed onto the HPTLC spot. Solvent (methanol, ACN, or 0.1% formic acid) flows through the head, dissolves the analyte from the silica, and carries it via a transfer capillary directly into the ESI source of the MS.
Procedure	(1) Run HPTLC as normal. (2) Document plate. (3) Press elution head onto each band/spot sequentially. (4) Pump solvent (0.1–0.3 mL/min). (5) Record MS spectrum (full scan or MRM) for each spot.
Advantages	Commercially available; works with any ESI-MS; full MS spectrum for structural ID; compatible with all HPTLC plates
Disadvantages	Destructive to plate (elution spot removed); sequential (one spot at a time); some analyte left on plate; limited to normal phase silica
Resolution	1 mm elution head diameter — can resolve bands as close as 2–3 mm

Strategy 2 — DESI-MS (Desorption Electrospray Ionisation)

Aspect	Description
Principle	DESI sprays charged ESI droplets (methanol/water, 0.1% formic acid) at the HPTLC plate surface at an angle (~50°). The charged droplets desorb and ionise analyte molecules from each TLC spot. Generated ions enter the MS inlet.
Instrument	DESI source coupled to any mass spectrometer (ion trap, Q-TOF, triple quad, Orbitrap)
HPTLC-DESI-MS procedure	(1) Run HPTLC. (2) Mount plate on motorised stage under DESI source. (3) Scan stage (x-y) → raster across plate. (4) MS continuously records ions — generates 2D MS image (ion map of entire plate)
Advantages	Non-destructive (plate preserved); true IMAGING — generates 2D spatial distribution of compounds across entire plate; fast imaging of full plate in minutes; no sample preparation post-TLC
Disadvantages	DESI source not yet widely available; signal suppression from silica surface; requires specialised DESI-MS hardware; quantification less accurate than direct elution
Application	Alkaloid distribution in plant material; forensic drug identification on TLC; lipid imaging; food authentication imaging

Strategy 3 — SALDI-MS (Surface-Assisted Laser Desorption/Ionisation)

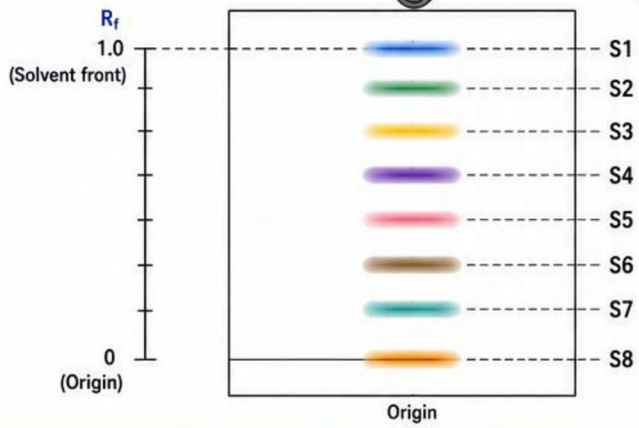
Aspect	Description
Principle	Modification of MALDI using the TLC silica plate itself (or a MALDI matrix applied to the plate) as the desorption/ionisation substrate. UV laser (337 nm or 355 nm) fired at plate → desorbs and ionises analytes from each spot.
Instrument	MALDI mass spectrometer (TOF or Orbitrap) with modified target stage for TLC plates
Procedure	(1) Run HPTLC → air dry. (2) OPTIONAL: spray MALDI matrix (DHB, CHCA) on plate. (3) Mount in MALDI instrument. (4) Fire laser at each spot → collect mass spectra. (5) Can image entire plate.
Advantages	High sensitivity; no solvent extraction step; imaging capability; compatible with MALDI-TOF (fast, sensitive); can analyse entire plate as one imaging experiment
Disadvantages	Requires MALDI instrument (expensive); plate must be compatible with MALDI vacuum; some signal suppression from silica; limited resolution in TOF mode without matrix
Best use	Alkaloids, glycosides, lipids, peptides from plant extracts; requires MALDI matrix optimisation for each compound class

HPTLC–MS SYSTEM DIAGRAM — DIRECT ELUTION METHOD

TOP: HPTLC PLATE AFTER DEVELOPMENT

(Pre-MS Documentation)

UV–VIS / DENSITOMETRIC DOCUMENTATION SYSTEM
(pre-MS documentation)

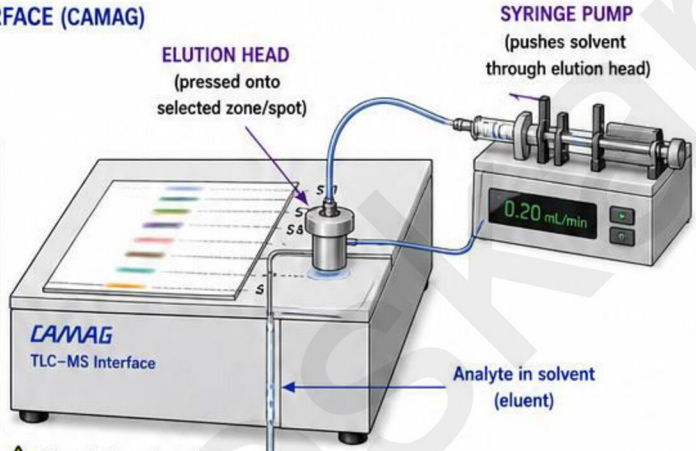


HPTLC PLATE

- Developed in chamber
- Dried
- Documented (UV 254 / 366 nm or visible / derivatized)
- Zones S1–S8 eluted sequentially

MIDDLE: TLC–MS INTERFACE (CAMAG)

DIRECT ELUTION



INTERFACE FEATURES

- Precise elution from defined zone
- Minimal band broadening
- Clean elution with selected solvent
- Compatible with ESI-MS

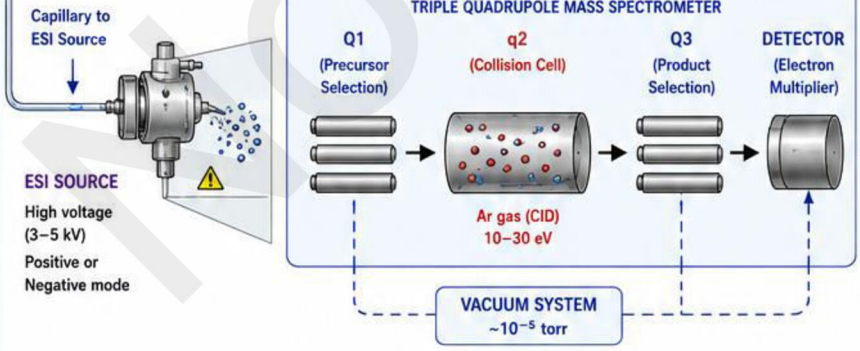
⚠ Keep plate surface clean and dry during analysis

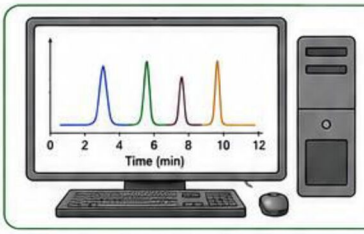
SEQUENCE OF OPERATION

1. Spot/Zone 1 (S1) → Spectrum 1
2. Spot/Zone 2 (S2) → Spectrum 2
3. Spot/Zone 3 (S3) → Spectrum 3
8. Spot/Zone 8 (S8) → Spectrum 8

Sequential elution and MS acquisition for all zones

BOTTOM: ESI MASS SPECTROMETER

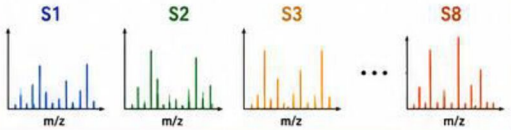




Functions

- Control of TLC–MS interface
- MS data acquisition (MRM or Scan)
- Data processing
- Spectral library search
- Reporting

MS Spectra (Examples)



KEY PARAMETERS	Elution Flow Rate:	Elution Solvents:	ESI Voltage:	CID Energy (q2):	MS Vacuum:
	0.1 – 0.4 mL/min	MeOH / MeOH–H_2O / MeOH–H_2O–Formic acid, etc.	3 – 5 kV	10 – 30 eV	~10^{-5} torr

HPTLC-MS Applications

Application Area	Specific Use	Why HPTLC-MS?
Herbal Drug Authentication	Identify adulterants and authentication markers in Ayurvedic, Unani, homeopathic plants. E.g., distinguish Withania somnifera (Ashwagandha) from Sida (adulterant)	HPTLC gives visual fingerprint; MS confirms identity of specific alkaloids/glycosides by molecular mass — combined = definitive authentication without isolation
Phytochemical Profiling	Profile alkaloids (vincristine, quinine), flavonoids, glycosides, terpenes in plant extracts	Rapid screening: 20 samples in one HPTLC run; MS confirms identity of each spot without HPLC isolation
Forensic Drug Analysis	Identify illicit drugs on seized drug exhibits; distinguish drug from cutting agent	Visual spot + MS identity = legal evidence; DESI-HPTLC-MS can image full plate of seized material
Pharmaceutical QC	Identity test of APIs and herbal extracts; detect substitution/adulteration	Pharmacopoeial HPTLC plates (Ayurvedic Pharmacopoeia) + MS confirmation
Food Authentication	Detect food adulterants — horse meat in beef (species markers), paprika adulteration with Sudan red dyes	HPTLC separates complex food matrix; MS confirms analyte identity without full HPLC method development
Mycotoxin Screening	Aflatoxins B1, B2, G1, G2 in food and pharmaceutical excipients	HPTLC fluorescence + MS confirmation of individual spots — rapid screening without lengthy LC-MS/MS
Lipid Analysis	Phospholipid classes, triglycerides, fatty acids in biological and formulation samples	TLC separates lipid classes by class (PC, PE, TG) → HPTLC-MS identifies individual molecular species
Antibiotic Identification	Tetracyclines, aminoglycosides in bulk drug and formulation	HPTLC visual + MS — confirms presence of correct antibiotic and detects degradation products

MASTER COMPARISON: LC-MS/MS vs GC-MS/MS vs HPTLC-MS

Parameter	LC-MS/MS	GC-MS/MS	HPTLC-MS
Separation technique	HPLC or UHPLC	Gas Chromatography	High Performance TLC
Interface	ESI or APCI (atmospheric pressure)	EI (70 eV) or CI (vacuum)	Direct elution, DESI, or SALDI/MALDI
Ionisation type	Soft (ESI, APCI)	Hard (EI) or Soft (CI)	Soft (ESI in direct elution; laser in SALDI)
Analyte volatility required	No — non-volatile compounds OK	Yes — must be volatile or derivatisable	No — solid plate; no volatility needed
Analyte polarity range	Polar to non-polar (with right column)	Non-polar to moderately polar (with derivatisation)	Wide — normal phase (polar) or RP-18 (non-polar)
Typical m/z range	50–3000+ Da (ESI multiply charged to MDa)	50–1000 Da (GC amenable range)	50–2000 Da (DESI or ESI range)
Throughput	High (96 samples/batch automated)	Moderate (sequential analysis)	Moderate–high (20 samples/plate)
Sensitivity (LLOQ)	pg/mL in plasma (typical: 0.1–1 ng/mL)	pg–ng range in headspace	ng–µg per spot range
Structural information	Yes — product ion spectra; exact mass (Q-TOF, Orbitrap)	Yes — EI spectra; NIST library searchable	Yes — MS spectrum per spot; full scan or MRM
Regulatory acceptance	Gold standard — EMA/FDA bioanalytical guideline	Gold standard — ICH Q3C residual solvents; USP ⟨467⟩; forensic confirmation	Emerging — Ayurvedic Pharmacopoeia; herbal QC
Mobile phase / carrier	Volatile aqueous-organic solvents (no phosphate)	Inert carrier gas (He, H_2, N_2)	Normal phase: hexane-EA-MeOH; RP: MeOH-water
Sample preparation	SPE, protein precipitation, LLE + clean extracts	Headspace GC, LLE, derivatisation	Minimal — direct spotting of extract

Parameter	LC-MS/MS	GC-MS/MS	HPTLC-MS
Pharmaceutical use	PK; TDM; bioequivalence; metabolite ID; impurity profiling	Residual solvents; forensic; environmental; genotoxic impurities	Herbal authentication; forensic screening; QC identity

IMPORTANT QUESTION BANK

A. 2-Mark Questions with Model Answers

Q1. Define hyphenated techniques. Give two examples.

Ans: **Hyphenated techniques** are analytical systems created by online coupling of a separation technique with a detection/identification technique through an interface. The hyphen represents the interface connecting the two systems. Examples: (1) **LC-MS/MS:** HPLC coupled to tandem mass spectrometry via ESI/APCI — for pharmaceutical bioanalysis. (2) **GC-MS/MS:** Gas chromatograph coupled to triple quadrupole MS via EI — for residual solvents and forensic toxicology. Others: HPTLC-MS, LC-NMR, ICP-MS, GC-FTIR.

Q2. What is MRM? Why is it preferred for quantitative bioanalysis?

Ans: **MRM (Multiple Reaction Monitoring):** A tandem MS/MS scan mode in which BOTH Q1 (precursor ion) and Q3 (product ion) are simultaneously fixed at specific m/z values — only ions matching the EXACT precursor-to-product transition generate signal. **Why preferred:** (1) ULTRA-HIGH SELECTIVITY — only the compound generating both the specific precursor AND product ion passes; (2) ULTRA-HIGH SENSITIVITY — signal-to-noise ratio dramatically improved vs full scan; (3) MULTI-ANALYTE — 50–100 MRM transitions monitored simultaneously in one LC run; (4) REGULATORY STANDARD — EMA/USFDA bioanalytical method validation guidelines require MRM-based quantification.

Q3. What is electrospray ionisation (ESI)? State the ions formed.

Ans: **ESI** is a soft atmospheric pressure ionisation method used to interface HPLC with MS. **Principle:** High voltage (3–5 kV) applied to LC effluent creates charged droplets from a nebuliser needle. N_2 desolvation gas (350–600°C) evaporates solvent. Droplets undergo Coulomb fission until individual ions are released into gas phase. **Ions formed:** Positive mode: $[M+H]^+$ (protonated molecule), $[M+Na]^+$, $[M+K]^+$, $[M+nH]^{n+}$ (multiply charged for proteins). Negative mode: $[M-H]^-$ (deprotonated). ESI is suitable for polar, ionic, and large biomolecules.

Q4. State two key differences between EI and ESI ionisation.

Ans: **EI (Electron Ionisation) vs ESI (Electrospray Ionisation):** (1) **Ionisation type:** EI is HARD — 70 eV electrons cause extensive fragmentation ($M^{+\bullet}$ radical cation + many fragment ions). ESI is SOFT — minimal fragmentation; primarily $[M+H]^+$ or $[M-H]^-$. (2) **Analyte type:** EI used only for VOLATILE, thermally stable compounds (GC-MS). ESI used for non-volatile, thermally labile, polar to large biomolecular compounds (LC-MS). (3) **Library:** EI spectra standardised at 70 eV → NIST library searchable. ESI spectra instrument-dependent → no universal library.

Q5. Explain matrix effect (ion suppression) in LC-MS/MS.

Ans: **Matrix effect (ion suppression):** Reduction in analyte signal in ESI-MS caused by co-eluting endogenous matrix components competing with the analyte for ionisation at the ESI droplet surface. **Main causes:** Plasma phospholipids (PC, PE, SM) are the most problematic — severe suppression (50–90%). Bile salts, polymers, TFA in mobile phase. **Assessment:** Matrix Factor (MF) = signal of

post-extraction spike / signal of neat standard. Acceptable: IS-normalised MF %CV ≤ 15%. **Reduction strategies:** Better sample cleanup (phospholipid-specific SPE), UHPLC to improve chromatographic separation, dilute-and-shoot, switch to APCI (less susceptible).

Q6. What is the principle of HPTLC-MS direct elution coupling?

Ans: **HPTLC-MS Direct Elution (TLC-MS Interface):** After HPTLC development and documentation, a circular elution head (CAMAG TLC-MS Interface) is pressed onto each separated spot on the HPTLC plate. **Principle:** Solvent (methanol or 0.1% formic acid, 0.1–0.3 mL/min) pumped through the elution head dissolves the analyte from the silica gel surface and carries it via a capillary transfer tube directly into the ESI source of the mass spectrometer. The MS records the full mass spectrum or MRM signal for each spot sequentially. **Advantages:** Commercial system; compatible with any ESI-MS; provides structural information (MS spectrum) for every HPTLC spot without isolation.

Q7. What are the mobile phase requirements for LC-MS/MS?

Ans: **MS-compatible mobile phases must be VOLATILE** — completely evaporate in the ESI/APCI source without leaving non-volatile deposits. **Compatible:** 0.1% formic acid (most common), 0.1% acetic acid, 5–10 mM ammonium formate, 5–10 mM ammonium acetate — all ammonium salts. Methanol and acetonitrile as organic modifier. **INCOMPATIBLE (never use):** Sodium phosphate, potassium phosphate, TRIS, sodium acetate — non-volatile; crystallise in source → blockage → signal loss → instrument damage → expensive maintenance. **Rule:** Ammonium = always OK. Sodium/potassium = never.

Q8. State three pharmaceutical applications of GC-MS/MS.

Ans: Three pharmaceutical applications of GC-MS/MS: (1) **Residual Solvent Analysis (ICH Q3C):** Headspace GC-MS/MS measures Class 1 (benzene <2 ppm) and Class 2 solvents (methanol, ACN, chloroform) in APIs and drug products. MRM mode gives ultra-low detection limits with minimal matrix interference. USP ⟨467⟩ method. (2) **Drugs of Abuse Confirmatory Testing:** Urine forensic toxicology — GC-EI-MS/MS definitively identifies THC, cocaine metabolites, opiates, amphetamines. EI spectrum matched to NIST library = legal gold standard confirmation. (3) **Genotoxic Impurity Testing (ICH M7):** Alkyl sulfates, aldehydes, epoxides in API — GC-MS/MS MRM detects at ppm levels mandated by ICH M7 (typically ≤1.5 µg/day acceptable intake).

B. 5-Mark Questions

Q1. Explain the principle and instrumentation of LC-MS/MS with a block diagram. (5 marks)

Q2. Describe the four MS/MS scan modes in triple quadrupole mass spectrometry. (5 marks)

Q3. Describe GC-MS/MS with instrumentation and pharmaceutical applications. (5 marks)

Q4. Explain the principle and strategies for HPTLC-MS coupling. (5 marks)

Q5. Discuss matrix effects in LC-MS/MS. How are they assessed and minimised? (5 marks)

C. 10-Mark Question

Q1. Write a comprehensive note on LC-MS/MS — principle, ESI ionisation, instrumentation, MRM quantification, matrix effects, and pharmaceutical applications. (10 marks)

Q2. Compare LC-MS/MS, GC-MS/MS, and HPTLC-MS on principle, instrumentation, ionisation, and pharmaceutical applications. (10 marks)

PREVIOUS YEAR-STYLE QUESTIONS

PYQ 1. What are hyphenated techniques? Describe the principle and instrumentation of LC-MS/MS. [AKTU 7 marks]

PYQ 2. Explain GC-MS/MS with instrumentation and applications. [5 marks]

PYQ 3. Explain HPTLC-MS coupling strategies and applications. [5 marks]

PYQ 4. What is MRM? Explain the four scan modes of triple quadrupole MS. [5 marks]

PYQ 5. Compare LC-MS/MS and GC-MS/MS with respect to principle, ionisation, and pharmaceutical applications. [5 marks]

50 MCQs — HYPHENATED TECHNIQUES (UNIT V)

1. The 'hyphen' in hyphenated techniques represents:

A. A mathematical subtraction operation

B. The interface connecting the separation technique to the detection technique ✓

C. The wavelength range of detection

D. The mobile phase composition

Explanation: The hyphen in LC-MS/MS, GC-MS/MS etc. represents the INTERFACE that connects the separation system (LC or GC) to the detection system (MS). This interface must convert analytes from the separation format into a form compatible with the mass spectrometer (gas-phase ions). Without the interface, the two instruments cannot work together.

2. The most common ionisation method used in LC-MS/MS is:

A. Electron Ionisation (EI) at 70 eV

B. Chemical Ionisation (CI)

C. Electrospray Ionisation (ESI) ✓

D. Matrix-Assisted Laser Desorption Ionisation (MALDI)

Explanation: ESI (Electrospray Ionisation) is the dominant ionisation method for LC-MS/MS. It operates at atmospheric pressure, handles liquid LC effluent, is soft (produces $[M+H]^+$ or $[M-H]^-$ without fragmentation), and is compatible with polar, ionic, and large biomolecular analytes. EI is used in GC-MS (requires vapour phase analytes). MALDI is used for HPTLC-MS.

3. In MRM scan mode, which statement is correct?

A. Q1 scans all m/z; Q3 is fixed

B. Both Q1 and Q3 are fixed at specific m/z values ✓

C. Q3 scans all m/z; Q1 is fixed

D. Both Q1 and Q3 scan simultaneously

Explanation: MRM (Multiple Reaction Monitoring): BOTH Q1 (fixed at precursor m/z) AND Q3 (fixed at product m/z) are simultaneously fixed. Only ions matching the EXACT precursor→product pair generate a signal. This double selection gives the highest specificity and sensitivity of all MS/MS scan modes. It is the gold standard for quantitative pharmaceutical bioanalysis.

4. The standard electron energy used in EI ionisation (GC-MS) is:

A. 3–5 eV

B. 10 eV

C. 70 eV ✓

D. 200 eV

Explanation: 70 eV is the international standard for EI (Electron Ionisation) in GC-MS. This energy gives reproducible fragmentation patterns regardless of instrument (because it's well above the first ionisation potential of most organic compounds). Standardisation at 70 eV enables NIST spectral library searching — spectra collected on one instrument match those on another. Higher or lower eV gives different fragmentation patterns.

5. Which mobile phase additive is INCOMPATIBLE with LC-MS/MS?

A. 0.1% Formic acid

B. 10 mM Ammonium formate

C. 50 mM Sodium phosphate buffer ✓

D. 0.1% Acetic acid

Explanation: Sodium phosphate is NON-VOLATILE — it does not evaporate in the ESI/APCI source and instead crystallises, blocking the source capillary and ion optics. This causes severe ion suppression, baseline instability, and instrument contamination requiring expensive cleaning. ALWAYS use ammonium formate, ammonium acetate, formic acid, or acetic acid — all volatile at LC-MS source temperatures.

6. In GC-MS/MS, the ion source operates at approximately what vacuum?

A. Atmospheric pressure (760 torr)

B. 10 torr

C. 10^{-5} torr ✓

D. No vacuum needed

Explanation: GC-MS ion source requires HIGH VACUUM at approximately 10^{-5} torr (turbomolecular pump). This is necessary because: (1) EI ionisation requires gas-phase analytes in vacuum; (2) Prevents ion-molecule collisions in the flight path; (3) Ensures ions travel undisturbed from source to detector. This is fundamentally different from LC-MS/MS (ESI operates at atmospheric pressure — no vacuum in the source region).

7. HPTLC uses silica gel particles of approximately:

A. 50–100 µm

B. 10–12 µm (classical TLC)

C. 2–5 µm ✓

D. 0.5–1 µm

Explanation: HPTLC (High Performance TLC) uses 2–5 µm silica gel particles with highly uniform size distribution. Compare: classical TLC uses 10–12 µm particles. Smaller, more uniform particles give: lower plate height (HETP), better separation efficiency, faster development, shorter migration distance, and higher sensitivity. These properties make HPTLC suitable for MS coupling.

8. The CAMAG TLC-MS Interface is used for which HPTLC-MS coupling strategy?

A. DESI-MS (spray desorption)

B. Direct elution — solvent pumped through elution head onto TLC spot → ESI-MS ✓

C. SALDI — laser desorption from TLC plate

D. MALDI-TOF from whole plate

Explanation: The CAMAG TLC-MS Interface uses DIRECT ELUTION: an elution head (small circular rubber seal) is pressed onto each HPTLC spot sequentially. A pump flows solvent (MeOH or 0.1% FA) through the head, dissolving the analyte from silica, and transfers it via a capillary directly to the ESI source of the mass spectrometer. It is the only commercially available (CAMAG) HPTLC-MS coupling system.

9. What does the 'q2' in a triple quadrupole (Q1-q2-Q3) refer to?

A. The second quadrupole mass analyser for ion selection

B. The collision cell (hexapole or octapole) where CID fragmentation occurs ✓

C. A second independent mass spectrometer

D. The detector that counts ions

Explanation: q2 (lowercase) = the COLLISION CELL — not a quadrupole mass analyser but a hexapole or octapole rf-only device filled with inert gas (Ar or N_2) at 10^{-3} torr. Ions from Q1 collide with gas atoms → Collision Induced Dissociation (CID) → fragment ions. The 'q' (lowercase) distinguishes it from Q1 and Q3 (quadrupoles that actually select m/z). CID collision energy: 10–50 eV for LC-MS/MS; 5–30 eV for GC-MS/MS.

10. ESI produces multiply charged ions $[M+nH]^{n+}$ for large molecules. What is the advantage?

A. Increases the m/z of the ion making it harder to detect

B. Allows measurement of very large molecules (MW > 100,000 Da) on instruments with limited m/z range ✓

C. Makes the molecule less stable

D. Requires more fragmentation to identify

Explanation: Multiple charging (z = 2, 3, 4... up to 50+ for proteins) DIVIDES the m/z: m/z = MW/z. A 50,000 Da protein with +25 charge appears at m/z 2001 — well within the m/z range of a standard quadrupole. This enables measurement of huge biomolecules (antibodies 150,000 Da) on instruments with m/z < 3000 range. The charge state distribution in the ESI spectrum can be deconvoluted to give the true molecular mass.

11. Product Ion scan in triple quadrupole MS involves:

A. Q1 fixed; Q3 scans all products — gives all fragment ions from one precursor ✓

B. Q1 scans; Q3 fixed at one product

C. Both Q1 and Q3 fixed

D. Q1 scans; Q3 scans with fixed offset

Explanation: Product Ion Scan: Q1 FIXED at one specific precursor m/z; q2 fragments; Q3 SCANS through all m/z values → collects ALL product (fragment) ions from that one precursor. This gives the complete fragmentation spectrum — used for structural characterisation of metabolites and building MRM transition libraries. Compare: MRM = both Q1 AND Q3 fixed. Precursor Ion = Q1 scans, Q3 fixed.

12. The neutral loss scan mode in MS/MS is used to detect:

A. Ions that have gained mass

B. All precursors that lose a specific mass (neutral fragment) upon CID ✓

C. Ions that don't fragment

D. Products from only one specific precursor

Explanation: Neutral Loss Scan: Q1 and Q3 both scan simultaneously, with Q3 always set at a fixed offset LOWER than Q1 (Q3 = Q1 − neutral mass). Only compounds that lose the specific neutral fragment (e.g., 176 Da = glucuronic acid; 80 Da = SO_3) generate a signal. Used in metabolomics to screen all compounds of a specific metabolic class (all glucuronides, all sulfates, all acetyl-conjugates) in one scan.

13. In LC-MS/MS, APCI (Atmospheric Pressure Chemical Ionisation) is preferred over ESI for:

A. Proteins and peptides

B. Non-polar lipids and moderately polar small molecules ✓

C. Multiply charged ions

D. Large biomolecular complexes

Explanation: APCI is preferred for NON-POLAR to MODERATELY POLAR SMALL MOLECULES such as lipids, steroids, vitamins (retinol, tocopherol), and non-basic drugs that ionise poorly by ESI. APCI vaporises the LC effluent thermally (300–450°C) before corona discharge ionisation — gives proton transfer without the charge residue mechanism of ESI. For proteins and multiply charged ions: ESI is the only option.

14. DESI in HPTLC-MS stands for:

A. Direct Extraction from Silica Interface

B. Desorption Electrospray Ionisation ✓

C. Differential Elution System Interface

D. Double Extraction Spray Ionisation

Explanation: DESI = Desorption Electrospray Ionisation. A charged ESI solvent spray is directed at the HPTLC plate surface at an oblique angle. The charged droplets pick up and ionise compounds from the plate surface. Ions fly off the plate and enter the MS inlet. DESI enables 2D MS IMAGING of TLC plates — the entire plate can be imaged to show spatial distribution of every compound, non-destructively.

15. The mass analyser that provides the highest mass resolution (>100,000 FWHM) is:

A. Triple Quadrupole

B. Ion Trap

C. Orbitrap ✓

D. Magnetic Sector

Explanation: Orbitrap provides ULTRA-HIGH MASS RESOLUTION (typically 60,000–500,000 FWHM depending on scan speed). Ions orbit around a central electrode at frequencies proportional to their m/z — detected by Fourier Transform (FT-MS). Orbitrap is used in high-resolution MS (HRMS) for: exact mass metabolite identification, unknown compound characterisation, and pharmaceutical impurity profiling. Triple quadrupole: unit resolution (1 Da). Ion trap: unit to moderate.

16. Headspace GC-MS/MS is specifically used for pharmaceutical:

A. Protein characterisation

B. Residual solvent analysis (ICH Q3C, USP ⟨467⟩) ✓

C. Blood drug concentration (PK studies)

D. Amino acid profiling

Explanation: Headspace GC-MS/MS is the primary method for RESIDUAL SOLVENT ANALYSIS per ICH Q3C and USP ⟨467⟩. Sample heated in sealed vial → volatile solvents partition into headspace → headspace gas injected onto GC column → separated → EI MS/MS detection by MRM. Detects Class 1 (benzene <2 ppm), Class 2 (methanol <3000 ppm), and Class 3 solvents in APIs and drug products without complex sample preparation.

17. The stable isotope labelled internal standard (SIL-IS) in LC-MS/MS corrects for:

A. Column degradation

B. Both extraction variability AND ion suppression variability ✓

C. Only extraction recovery variability

D. Only chromatographic retention time shifts

Explanation: SIL-IS (e.g., d_3-drug, ^{13}C-drug) has IDENTICAL chemical behaviour to the unlabelled analyte: same extraction recovery, same chromatographic retention time, same ESI ionisation efficiency. Therefore it corrects for BOTH: (1) Extraction variability between samples — SIL-IS and analyte both lose/gain equally; (2) Ion suppression variability — SIL-IS and analyte suppressed equally by matrix. The ratio (analyte area / IS area) is stable even when absolute signals vary.

18. In GC-MS, the HP-5MS column coating used is:

A. 100% polysiloxane

B. 5% phenyl, 95% methylsiloxane — 'MS-grade' low-bleed ✓

C. Polyethylene glycol (Carbowax)

D. Cyano-propyl polysiloxane

Explanation: HP-5MS (or equivalent DB-5MS, Rtx-5MS) is the most universal GC-MS column: 5% phenyl, 95% dimethylpolysiloxane. 'MS-grade' means it has been specially deactivated to have LOW BLEED at high temperatures (250–325°C) — column bleed appears as background ions that interfere with MS detection. HP-5MS gives good separation for most GC-amenable compounds and minimal bleed up to 300°C. PEG columns are for polar compounds (alcohols, aldehydes).

19. The Q-TOF mass analyser (Quadrupole-Time-of-Flight) is used in pharmaceutical analysis for:

A. Quantitative MRM bioanalysis — gold standard

B. High-resolution exact mass measurement for metabolite identification and unknown structure determination ✓

C. Residual solvent headspace analysis

D. HPTLC imaging

Explanation: Q-TOF (Quadrupole + Time-of-Flight) provides HIGH RESOLUTION MASS SPECTROMETRY (HRMS) with accurate mass measurement (typically < 5 ppm mass accuracy). Used in pharmaceutical analysis for: (1) Metabolite identification — exact mass gives molecular formula; (2) Impurity characterisation — determine structure of unknown impurities; (3) Drug-drug interaction studies — identify all metabolites; (4) Pharmacovigilance — unexpected biotransformation products. Triple Quadrupole QqQ is used for quantitative MRM bioanalysis.

20. APPI (Atmospheric Pressure Photo-Ionisation) in LC-MS uses:

A. 70 eV electrons

B. UV photons from a krypton lamp (10.0 or 10.6 eV) ✓

C. High voltage electrospray

D. Corona discharge needle

Explanation: APPI uses UV photons (most commonly from a krypton discharge lamp: 10.0 eV or 10.6 eV) to directly photoionise analyte molecules. APPI is complementary to ESI and APCI — it excels for NON-POLAR AROMATIC compounds (polycyclic aromatic hydrocarbons, steroids, retinoids, lipophilic drugs) that have poor ESI or APCI response. Produces $M^{+\bullet}$ (radical cation) or $[M+H]^+$ when dopant (e.g., acetone, toluene) is used.

21. The ion ratio in LC-MS/MS is used for:

A. Calculating the molecular weight of the analyte

B. Confirming compound identity — ratio of qualifier to quantifier MRM transition must match reference ✓

C. Determining the injection volume accuracy

D. Measuring extraction recovery

Explanation: ION RATIO = signal from qualifier MRM transition / signal from quantifier MRM transition (expressed as %). Must be within ±20–30% of the ion ratio in the reference standard at the same concentration. This is an IDENTITY CONFIRMATION criterion — confirms that the peak in the sample is really the target compound and not a co-eluting impurity that happens to give the same quantifier transition. Required by EMA/FDA bioanalytical validation guidelines.

22. Which LC-MS/MS application requires measurement of drugs in dried blood spots (DBS)?

A. Therapeutic drug monitoring of digoxin

B. Newborn screening for inherited metabolic disorders (amino acids, acylcarnitines) ✓

C. Drug of abuse confirmatory testing

D. Residual solvent analysis

Explanation: DBS (Dried Blood Spot) LC-MS/MS is used for NEWBORN SCREENING — a 3 mm punch from a neonatal heel-prick blood spot on filter paper contains <5 µL blood. LC-MS/MS measures 40+ metabolites (amino acids, acylcarnitines, fatty acid oxidation markers) in a single 2-min run to screen for >30 inherited metabolic disorders (PKU, MSUD, MCAD deficiency, etc.). Nationwide newborn screening programs in most countries now use LC-MS/MS.

23. The transfer line temperature in GC-MS/MS should be:

A. Same as column oven temperature (ambient)

B. Higher than the highest GC oven temperature — prevents condensation of analytes ✓

C. Lower than the injection port temperature

D. Room temperature

Explanation: The GC-MS transfer line (connection between GC oven exit and MS ion source) must be HEATED to a temperature AT LEAST as high as the highest temperature of the GC oven program (typically 280–320°C). If transfer line is cooler, analytes condense in the line, causing: loss of signal, peak broadening, peak tailing, contamination buildup, and memory effects. Transfer line temperature = one of the most critical GC-MS parameters.

24. In the context of HPTLC-MS, SALDI stands for:

A. Sequential Analyte Loading and Detection Interface

B. Surface-Assisted Laser Desorption/Ionisation ✓

C. Solid-Analyte Liquid Desorption Interface

D. Silica-Assisted Laser Diffraction Imaging

Explanation: SALDI = Surface-Assisted Laser Desorption/Ionisation. A variant of MALDI where the TLC silica plate surface itself (or with added MALDI matrix like DHB, CHCA) serves as the laser desorption substrate. UV laser pulses desorb and ionise compounds from each TLC spot. Compatible with MALDI-TOF or Orbitrap instruments. Advantages: high sensitivity; imaging capability; no organic solvent extraction needed.

25. The Orbitrap mass analyser works by:

A. Magnetic field deflection of ion trajectories

B. Ions orbiting around a central electrode at frequencies proportional to their m/z; FT-MS detection ✓

C. Time-of-flight through a drift tube

D. 4 parallel rods with DC+RF voltages

Explanation: Orbitrap: ions are TRAPPED in orbital motion around a central spindle electrode. The frequency of axial oscillation is inversely proportional to the square root of m/z. Image current from oscillating ions is detected and converted to mass spectrum by Fourier Transform (FT-MS) — hence ultra-high resolution (>100,000 FWHM). Developed by Alexander Makarov (1999/2005). Used in Thermo Fisher Orbitrap Elite, Exploris, Astral instruments.

26. A bioanalytical method shows matrix factor (MF) of 0.65 for the analyte and 0.68 for the SIL-IS. The IS-normalised MF is:

A. 0.65

B. 0.95 ✓

C. 0.68

D. 0.88

Explanation: IS-normalised MF = MF_analyte / MF_IS = 0.65 / 0.68 = 0.956 ≈ 0.95. Acceptance criterion: IS-normalised MF = 0.85–1.15 (i.e., within ±15% of 1.0). The value 0.95 PASSES — the SIL-IS effectively corrects for the matrix suppression. Even though both analyte and IS are suppressed (both MF < 1.0 = suppressed), the RATIO is close to 1.0, meaning the SIL-IS tracks the analyte suppression perfectly. This is the key advantage of SIL-IS over structural analogues.

27. An LC-MS/MS method for testosterone uses precursor m/z 289 → product m/z 97. What does the '289 → 97' represent?

A. Two different testosterone metabolites

B. The MRM transition — precursor ion (m/z 289) fragmenting to product ion (m/z 97) in q2 ✓

C. The column dead volume time in seconds

D. The UV wavelengths used

Explanation: '289 → 97' is the MRM TRANSITION PAIR for testosterone. m/z 289 = $[M+H]^+$ of testosterone (MW = 288 Da). In q2 collision cell with Ar gas at specific collision energy, testosterone $[M+H]^+$ fragments to give product ion at m/z 97 (loss of steroid A-ring fragment, loss of water + sidechain). This specific 289→97 transition is monitored in Q3 of the triple quadrupole. It is the 'fingerprint' for testosterone — no other compound in plasma gives this exact precursor→product combination at this retention time.

28. Why is helium (He) preferred over nitrogen (N_2) as GC carrier gas for GC-MS/MS?

A. Helium is heavier — better separation

B. Helium provides better separation efficiency (higher diffusivity) and is compatible with MS vacuum ✓

C. Nitrogen is too reactive with the MS detector

D. Helium is cheaper and more available

Explanation: Helium is preferred because: (1) HIGHEST DIFFUSIVITY among carrier gases → faster analyte transfer in column → optimal efficiency at higher flow rates (steeper Van Deemter curve minimum); (2) INERT — no reaction with analytes or column stationary phase; (3) COMPATIBLE with MS vacuum — He is efficiently pumped by turbomolecular pumps; (4) Low molecular mass → fast elution. N_2: cheaper but lower diffusivity → poorer peak shape at optimal flow. H_2: best diffusivity but safety concern (flammable); now gaining acceptance in pharmaceutical labs.

29. In LC-MS/MS, the desolvation gas temperature (typically 350–600°C) is important because:

A. It sterilises the solvent

B. It evaporates solvent from ESI droplets — insufficient temperature leads to incomplete desolvation and poor sensitivity ✓

C. It decomposes matrix interferents

D. It sets the column oven temperature

Explanation: ESI desolvation gas (heated N_2) EVAPORATES the solvent from charged droplets. If temperature is too LOW: solvent molecules not fully evaporated → solvent-analyte clusters form → non-specific adducts in spectrum → decreased sensitivity. If temperature is too HIGH: thermally labile analytes (labile drugs, peptides) may degrade. Optimal desolvation temperature is method-specific (typically 400–500°C for most small molecule drugs) and must be optimised during LC-MS/MS method development.

30. NIST mass spectral library is used for compound identification in:

A. LC-MS/MS — ESI spectra

B. GC-MS/MS — EI (70 eV) spectra ✓

C. HPTLC-MS — DESI spectra

D. All of the above equally

Explanation: NIST mass spectral library (350,000+ spectra) contains exclusively EI (70 eV) mass spectra — the standard for GC-MS compound identification. Because EI at 70 eV is STANDARDISED, spectra generated on one instrument match those in the library. ESI spectra (LC-MS/MS) are NOT standardised — same compound gives different fragmentation on different instruments at different CID energies → no universal LC-MS/MS library. DESI and HPTLC-MS: no spectral library either.

31. Which GC-MS/MS mode gives the best sensitivity for trace analysis of residual solvents?

A. Full scan EI — complete spectrum

B. SIM (Selected Ion Monitoring) — GC-MS only

C. MRM (Multiple Reaction Monitoring) — GC-MS/MS ✓

D. All give equal sensitivity

Explanation: MRM (GC-MS/MS) gives the BEST sensitivity for trace analysis: (1) Background eliminated — only specific precursor→product pairs pass (matrix noise virtually zero); (2) Signal-to-noise ratio dramatically improved vs SIM (10–50× better); (3) Allows detection at <1 ppm for ICH Q3C Class 1 solvents. Full scan: worst S/N (all ions included). SIM: better than full scan but still admits all ions at selected m/z including matrix ions. MRM: two stages of selection → lowest background.

32. The qualifier ion in LC-MS/MS bioanalysis must satisfy:

A. It must be the same m/z as the quantifier ion

B. Its ratio to the quantifier ion must be within ±20–30% of the ratio in the reference standard ✓

C. Its area must be larger than the quantifier ion

D. It must elute at a different retention time

Explanation: Qualifier ion = a SECOND MRM transition monitored simultaneously with the quantifier transition. The ion ratio (qualifier area / quantifier area) must be within ±20–30% of the same ratio in a spiked calibrator at the same concentration level. This identity criterion confirms that the detected peak is truly the target compound — not a co-eluting matrix component that happens to give the same quantifier m/z. Required by EMA/FDA/WADA bioanalytical guidelines.

33. UHPLC columns used in LC-MS/MS typically have particle sizes of:

A. 10–12 µm

B. 5 µm

C. 3 µm

D. 1.7–1.8 µm ✓

Explanation: UHPLC (Ultra High Performance LC) uses sub-2 µm particles — typically 1.7 µm (Waters BEH) or 1.8 µm (Agilent RRHD). Smaller particles → lower theoretical plate height (HETP) → better separation efficiency → 3–5× faster analysis than conventional HPLC (5 µm). Operating pressure up to 15,000 psi (UHPLC) vs 6,000 psi (conventional HPLC). Standard for high-throughput LC-MS/MS in pharmaceutical bioanalysis.

34. In HPTLC-MS via DESI, what additional capability does it provide compared to direct elution?

A. Higher sensitivity per spot

B. 2D spatial MS imaging of the entire TLC plate simultaneously ✓

C. Better structural information per compound

D. Cheaper analysis per sample

Explanation: DESI-HPTLC-MS provides 2D SPATIAL MS IMAGING of the entire plate. A motorised x-y stage rasters the HPTLC plate under the DESI spray → MS continuously records → generates ion maps showing spatial distribution of every compound across the plate. This reveals how compounds are distributed in the plate — very useful for natural product profiling, tissue imaging (DESI-MSI), and visualising compound migration. Direct elution: sequential, spot-by-spot — no imaging.

35. The 'precursor ion' in MS/MS is also called:

A. Product ion or daughter ion

B. Parent ion or mother ion ✓

C. Fragment ion

D. Neutral loss ion

Explanation: Precursor ion = PARENT ION or mother ion — the intact molecular ion isolated by Q1 before fragmentation in q2. After CID in q2, the precursor breaks into PRODUCT IONS (also called daughter ions, fragment ions). Terminology: Precursor ion (parent) → CID → Product ions (daughters). In MRM: 'precursor m/z → product m/z' = parent → daughter transition. The terms 'parent' and 'daughter' are older terminology now replaced by 'precursor' and 'product' per IUPAC recommendation.

36. An LC-MS/MS method for measuring a basic drug (MW 310 Da, amine group) in plasma: The drug appears as $[M+H]^+$ at m/z 311. In product ion scan, major fragments appear at m/z 211, 183, 150. For MRM quantification, which choice is BEST?

A. 311→311 (no fragmentation, SIM equivalent)

B. 311→211 as quantifier; 311→183 as qualifier ✓

C. 311→183 as quantifier; 311→211 as qualifier (less abundant used for quantification)

D. Use full scan mode for best sensitivity

Explanation: MRM selection rules: QUANTIFIER = most abundant, most sensitive product ion. QUALIFIER = second most abundant (different structural information). Here: m/z 211 is the most abundant fragment → 311→211 as QUANTIFIER. m/z 183 is less abundant → 311→183 as QUALIFIER. Option C reverses them (wrong — quantifier should be most abundant for best precision). Option A = SIM equivalent (no MS/MS selectivity — no fragmentation). Option D = full scan gives worst sensitivity in MRM mode.

37. A GC-MS/MS analyst observes that the transfer line temperature is set 10°C BELOW the final GC oven temperature. The likely result is:

A. Better peak resolution

B. Improved sensitivity

C. Late-eluting peaks tailing severely or not reaching detector; analyte condensation in transfer line ✓

D. No effect — transfer line temperature has minimal impact

Explanation: If transfer line temperature < final GC temperature: LATE-ELUTING ANALYTES (highest boiling point compounds) will CONDENSE in the transfer line when the GC oven reaches temperatures above the transfer line temperature. This causes: severe peak tailing, peak broadening, reduced signal intensity, ghost peaks in subsequent runs (condensed analyte re-evaporates slowly), and transfer line contamination. Transfer line must always be AT LEAST as hot as the highest GC oven temperature — typically set 5–15°C HIGHER.

38. In a neutral loss scan for glucuronide metabolites, Q3 is set at 194 when Q1 is at 370. What is the neutral loss being scanned?

A. 194 Da

B. 176 Da ✓

C. 370 Da

D. 564 Da

Explanation: Neutral loss = Q1 − Q3 = 370 − 194 = 176 Da. Loss of 176 Da = loss of glucuronic acid group (glucuronyl moiety, $C_6H_8O_6$, MW = 176.12 Da). The precursor at m/z 370 could be a glucuronide conjugate of a drug with MW = 193 Da (170+H + glucuronide = 170+1+176 = 347... actually MW_drug = Q1 − 176 − 1 + 1 = 194 as $[M+H]^+$ of the parent after neutral loss). Neutral loss scan of 176 Da detects ALL glucuronide metabolites simultaneously — a powerful metabolite profiling tool.

39. A pharmaceutical company must validate an LC-MS/MS method per USFDA guidelines. Which matrix effect criterion must be met?

A. Matrix factor must equal 1.0 exactly

B. IS-normalised matrix factor %CV ≤ 15% across at least 6 independent lots of the same matrix type ✓

C. Matrix factor must be > 1.0 (signal enhancement preferred)

D. No matrix effect assessment required if SIL-IS is used

Explanation: Per USFDA Bioanalytical Method Validation Guidance (2018) and EMA guideline: IS-normalised matrix factor (MF) must be assessed using at least 6 independent sources (lots) of the same matrix type (e.g., 6 different donors of human plasma). The %CV of IS-normalised MF across these 6 lots must be ≤ 15%. This criterion ensures the method gives consistent results regardless of which donor's plasma is used — critical for clinical studies.

40. For GC-MS analysis of a mixture containing both low-boiling (50°C) and high-boiling (280°C) compounds, which column oven program is most appropriate?

A. Isothermal at 280°C throughout

B. Isothermal at 50°C throughout

C. Temperature programme: start at 40–50°C → hold → ramp to 280°C at 10°C/min ✓

D. Start at 280°C → cool to 50°C during run

Explanation: TEMPERATURE PROGRAMMING is essential for mixtures with wide boiling point range. Start at LOW temperature (40–50°C): allows low-boiling components to elute with adequate retention and separation. Then RAMP at 10°C/min to 280°C: progressively elutes higher-boiling compounds. Hold at 280°C: ensures all high-boiling compounds elute. Isothermal at 280°C: low-boiling compounds elute immediately, unresolved (too hot). Isothermal at 50°C: high-boiling compounds never elute (too cold). Temperature programming maximises resolution across the entire compound range.

41. A clinical trial requires quantification of a monoclonal antibody (MW 148,000 Da) in human plasma by LC-MS/MS. Which approach is required?

A. Direct ESI-MS of intact antibody at m/z 148,001

B. Signature peptide approach: tryptic digest of antibody → LC-MS/MS quantification of specific peptide fragment ✓

C. GC-MS/MS after derivatisation to make antibody volatile

D. HPTLC-MS for antibody characterisation

Explanation: Monoclonal antibodies (150 kDa) CANNOT be directly quantified by conventional triple quadrupole LC-MS/MS (limited m/z range ~3000). The SIGNATURE PEPTIDE approach is used: (1) Antibody captured from plasma (optionally); (2) Tryptic digestion produces specific peptide fragments unique to that antibody (MW 800–2500 Da); (3) A signature peptide is selected (specific sequence, good LC retention, good ionisation); (4) LC-MS/MS MRM quantifies the signature peptide. Signature peptide concentration = antibody concentration. This is the regulatory-accepted bioanalytical approach for therapeutic mAbs.

42. HPTLC-MS is particularly valuable for herbal drug authentication because:

A. It is faster than all other methods

B. It provides both a visual fingerprint (TLC pattern) AND molecular mass confirmation (MS) for each spot — definitive authentication without isolation ✓

C. It has the highest sensitivity of all analytical methods

D. It uses no solvents and is completely green

Explanation: HPTLC-MS combines TWO complementary types of information: (1) Visual TLC fingerprint — unique Rf values, colours, and band pattern characteristic of the authentic herb; AND (2) MS spectrum — molecular mass and fragmentation confirming identity of each specific compound (alkaloid, glycoside, terpenoid) in each spot. Together, this provides definitive authentication of herbal drugs and detection of adulterants or substitution. LC-MS/MS alone lacks the visual fingerprint; classical HPTLC alone lacks structural confirmation — HPTLC-MS combines both.

43. In LC-MS/MS bioanalysis, a calibration curve is prepared in extracted blank plasma (matrix-matched calibration) rather than pure solvent because:

A. Blank plasma is cheaper than solvent

B. Plasma matrix amplifies the signal, making calibration more sensitive

C. Matrix-matched calibration accounts for ion suppression and extraction recovery — ensures calibrators have the same matrix effects as study samples ✓

D. Pure solvent calibrations are not allowed by regulatory guidelines

Explanation: Matrix-matched calibration (prepare calibrators in extracted blank matrix): ensures that CALIBRATORS experience the SAME matrix effects (ion suppression, extraction recovery) as actual study samples. If calibrators prepared in pure solvent: no matrix effect on calibrators → calibrators give higher signal than matrix-spiked study samples at same concentration → OVERESTIMATION of sample concentration → incorrect PK parameters → regulatory failure. This is the reason FDA/EMA require calibrators in the same blank matrix as the study samples.

44. GC-MS/MS using MRM provides better selectivity than GC-MS using SIM because:

A. MRM has lower sensitivity (fewer ions detected)

B. MRM uses two stages of mass selection (precursor AND product), eliminating matrix co-eluting ions that might have the same m/z as the precursor ✓

C. SIM monitors only one ion while MRM monitors the complete spectrum

D. MRM requires no sample preparation

Explanation: MRM vs SIM selectivity: SIM monitors one m/z in Q1 → any co-eluting compound with the same precursor m/z contributes to signal → potential false positives. MRM adds Q3 selection of product ion → co-eluting compound must have BOTH the same precursor m/z AND fragment at the same product m/z → astronomical selectivity improvement. In complex matrices (urine, food, environmental), hundreds of co-eluting compounds may share the same precursor m/z; MRM eliminates virtually all of them.

45. An LC-MS/MS analyst uses 0.1% trifluoroacetic acid (TFA) in mobile phase for sharper peaks. What problem might arise?

A. No problem — TFA is perfectly MS-compatible

B. TFA causes severe ion pair suppression in ESI — dramatically reduces signal of basic analytes ✓

C. TFA causes column damage

D. TFA makes the peaks too narrow for integration

Explanation: TFA is an ion-pairing agent — at concentrations >0.01%, TFA forms non-volatile ion pairs with basic analytes ([analyte-TFA]⁺ complex) that dramatically reduce analyte ionisation in the ESI source (ion suppression of 90–99%). Solution: replace TFA with formic acid (0.1% formic acid) — gives comparable peak shape for basic drugs with minimal ESI suppression. If TFA is essential for peak shape: use post-column addition of propionic acid in 2-propanol to quench TFA's ion-pairing effect before ESI.

46. Which pharmaceutical regulatory guideline specifically governs GC analysis of residual solvents in drug substances and products?

A. ICH Q2(R1) — analytical method validation

B. ICH Q3C — Impurities: Guideline for Residual Solvents ✓

C. ICH M7 — Assessment and control of genotoxic impurities

D. ICH Q6A — Specifications for pharmaceutical substances

Explanation: ICH Q3C (Residual Solvents) specifically lists all solvents in 3 classes: Class 1 (carcinogenic — benzene ≤2 ppm, CCl_4, 1,2-dichloroethane); Class 2 (non-genotoxic but potentially toxic — methanol, acetonitrile, chloroform, DCM, etc. with specific PDEs); Class 3 (low toxicity — ethanol, acetone, ethyl acetate — PDE > 50 mg/day). GC headspace method (USP ⟨467⟩) is the pharmacopoeial method for ICH Q3C compliance. ICH M7 covers genotoxic impurities (different compounds).

47. In ESI-MS, the 'charge envelope' of a protein observed as multiple peaks at different m/z represents:

A. Multiple protein conformations

B. Multiple charge states $[M+nH]^{n+}$ — each peak is the same protein with different numbers of protons attached ✓

C. Multiple proteins of different molecular weights

D. Isotope distribution of the protein

Explanation: ESI of large proteins produces a CHARGE ENVELOPE — a series of peaks at different m/z values, each representing the SAME PROTEIN with a different number of protons (different z): $[M+8H]^{8+}$, $[M+9H]^{9+}$, $[M+10H]^{10+}$, etc. Each peak: $m/z = (M + z \times 1.008) / z$. The charge envelope can be DECONVOLUTED mathematically to give the true molecular mass M. This charge state distribution is characteristic of ESI of biomolecules and is fundamentally different from EI or MALDI (which give mainly singly charged ions).

48. For GC-MS analysis of ibuprofen (MW 206, carboxylic acid, non-volatile), which modification would make it GC-amenable?

A. Increase column temperature to 400°C

B. Derivatisation — e.g., methylation with diazomethane to form methyl ester (more volatile, thermally stable) ✓

C. Use larger injection volume

D. Switch to HPLC column

Explanation: Ibuprofen (carboxylic acid) is not sufficiently volatile for direct GC injection. DERIVATISATION converts polar groups to less polar, more volatile forms. For carboxylic acids: METHYLATION (methyl ester, R-COOCH₃) using diazomethane, BF₃/MeOH, or trimethylsilyl-diazomethane. For hydroxyl groups: TMS (trimethylsilyl) or acetyl derivatives. For amines: pentafluoropropionyl (PFP) or acetyl derivatives. After derivatisation, ibuprofen methyl ester (MW 220) is readily GC-amenable and gives NIST-searchable EI spectrum.

49. A forensic laboratory needs to CONFIRM (not just screen) the presence of cocaine metabolite benzoylecgonine (BE) in urine. Which method is the regulatory gold standard for confirmation?

A. LC-MS/MS MRM — because it provides highest sensitivity

B. GC-MS with EI (70 eV) — EI spectrum + NIST library = definitive legal identity confirmation ✓

C. HPTLC-MS — most selective

D. Immunoassay (EMIT) — used for screening and confirmation

Explanation: GC-EI-MS is the FORENSIC GOLD STANDARD for CONFIRMATION of drugs of abuse (SAMHSA guidelines, WADA, legal standard). Reason: EI at 70 eV gives REPRODUCIBLE fragmentation spectrum that matches the NIST library — this provides a scientifically defensible, court-admissible identification. LC-MS/MS MRM is used in some forensic confirmations but lacks a standardised spectral library. Immunoassays are for SCREENING only — not legally accepted for confirmation. GC-MS (with EI) identity confirmation is unambiguous.

50. A researcher wants to identify an unknown compound in a herbal extract without isolating it. The compound appears as a spot on HPTLC at Rf 0.45 and fluoresces at 366 nm. Which hyphenated approach gives the most information?

A. LC-MS/MS after dissolving the whole plate

B. HPTLC-MS via direct elution interface → full scan ESI-MS → molecular mass + fragmentation pattern for identification without isolation ✓

C. GC-MS/MS after extraction

D. UV spectrophotometry of the spot

Explanation: HPTLC-MS via direct elution (CAMAG TLC-MS interface) gives: (1) Molecular mass $[M+H]^+$ or $[M-H]^-$ from full scan ESI-MS; (2) Product ion spectrum (CID fragmentation) for structural characterisation; (3) HPTLC fingerprint (Rf, colour, fluorescence) for preliminary class identification. All this WITHOUT ISOLATING the compound — the spot is directly eluted into the MS. This is the unique advantage of HPTLC-MS for natural product research: rapid screening and tentative identification of hundreds of spots on one plate without the time-consuming isolation and purification steps required for NMR or stand-alone MS.