

5.1 Thermal analysis: the family and the thermogram

Thermal methods watch a sample while its temperature is driven through a programme, usually a steady heating ramp, and record some physical property against temperature. The record is a **thermogram**, and its steps, peaks and plateaus are the events of the sample's thermal life: melting, crystallisation, desolvation, decomposition, glass transition. Three techniques cover the syllabus: DSC measures heat flow, DTA measures a temperature difference, and TGA measures mass. Between them they answer the pharmaceutical questions of purity, polymorphism, solvate content, stability and compatibility that no spectrum addresses directly.

5.2 Differential scanning calorimetry

In DSC the instrument works to keep the sample and an inert reference following the same temperature programme, and records the heat flow needed to do so. Two designs achieve it. In **heat flux DSC**, sample and reference sit on a common thermoelectric disc inside one furnace, and the temperature difference between them, measured by thermocouples, is converted to heat flow. In **power compensation DSC**, separate small furnaces heat sample and reference, and the instrument varies their relative power to hold both at the same temperature; the power difference is the signal. On the thermogram, an endotherm (melting, desolvation) appears as a peak in one direction and an exotherm (crystallisation, decomposition, curing) as a peak in the other, by the plotting convention of the instrument.

The events read from a drug's DSC curve are standard: the **melting endotherm**, whose onset gives the melting point and whose area gives the enthalpy of fusion; the **glass transition** of an amorphous material, a step change in the baseline (a change in heat capacity, not a peak) at T_g ; crystallisation exotherms when a metastable or amorphous form orders itself on heating; and separate endotherms for different polymorphs or for desolvation before melting. The flagship quantitative application is **purity determination by the van't Hoff method**: a small amount of impurity depresses and broadens the melting endotherm in a mathematically defined way, so the shape of a single melt yields a purity figure, the DSC purity assay used for reference standards. Pharmaceutical applications complete the list: polymorph screening and identification, drug-excipient compatibility screening (a shifted or vanished drug endotherm in a mixture warns of interaction), amorphous content estimation, and lyophilisation cycle design through the glass transition of the frozen concentrate.

5.3 Differential thermal analysis

DTA is the older and simpler instrument: sample and reference are heated in the **same furnace** with a thermocouple in each, and the recorded signal is simply the temperature difference (ΔT) between them. When the sample melts, it lags behind the reference and an endothermic dip appears; when it crystallises or reacts exothermically, it runs ahead and a peak appears. DTA detects the same family of events as DSC, transitions, reactions and decompositions, and historically mapped the phase behaviour of minerals, clays and polymers. Its limitations against DSC are quantitative: ΔT depends on sample packing, thermal conductivity and thermocouple placement, so enthalpies from DTA are approximate, and the baseline is less stable. The honest summary for an exam answer: DTA tells you at what temperature things happen, DSC tells you that and how much heat they involve; pharmaceutical laboratories therefore buy DSC, and

DTA survives in high temperature materials work (its furnaces reach temperatures DSC designs do not) and as the conceptual parent of the family.

5.4 Thermogravimetric analysis

TGA records the mass of a sample continuously on a sensitive microbalance (the thermobalance) while the temperature programme runs, in a controlled atmosphere of nitrogen, air or oxygen chosen to suit the question. The thermogram is a descending staircase: each step is a mass loss event with an onset temperature and a percentage, and the derivative curve (DTG) resolves overlapping steps into peaks. Reading percentages against candidate stoichiometries is the interpretation skill: a first step of about 5 percent near 100 degrees in a hydrate whose formula weight predicts one water of crystallisation confirms the monohydrate; a plateau until 250 degrees followed by a single large step signals a stable anhydrous compound that decomposes without melting events interfering. Pharmaceutical applications: solvate and hydrate quantification (the standard check for how much solvent a crystal lattice holds), moisture and volatile content, decomposition kinetics and shelf life prediction from isothermal and ramped studies, ash and inorganic residue estimation, and oxidative stability comparisons between formulations. Coupling TGA to FTIR or MS of the evolved gases (an echo of Unit 4's hyphenation principle) identifies what each step actually loses.

5.5 Raman spectroscopy

Raman spectroscopy shines monochromatic light, in practice a laser, on the sample and analyses the tiny fraction of light scattered with a change of wavelength. Most scattering is **Rayleigh** scattering at the incident wavelength; about one photon in a million scatters inelastically. If the photon gives energy to a molecular vibration, the scattered light loses that energy and appears at longer wavelength: a **Stokes** line. If it collects energy from an already vibrating molecule, it gains energy: an **anti-Stokes** line, weaker because few molecules are vibrationally excited at room temperature. The Raman shift, the distance of these lines from the Rayleigh line in wavenumbers, equals the vibrational frequency, so the Raman spectrum, like the IR spectrum, is a vibrational fingerprint.

The instrumentation follows from the physics: a laser source (common choices include 532 and 785 nm diode or solid state lasers; 1064 nm excitation in FT-Raman instruments suppresses fluorescence), a filter or monochromator to reject the overwhelming Rayleigh line, and a sensitive CCD detector. The 785 nm laser is the pharmaceutical workhorse because it balances scattering strength against the fluorescence that ruins many 532 nm spectra of coloured drug samples. Applications play to Raman's peculiar strengths: water is a very weak Raman scatterer, so aqueous systems and biological samples are easy; no sample preparation is needed and measurements pass through glass vials and blister packs; and the technique distinguishes polymorphs crisply, which has made handheld Raman analysers standard for raw material identity verification in warehouse receiving, for counterfeit drug screening in the field, and for process monitoring (PAT) of blending and coating.

5.6 Raman versus IR: one vibrational truth, two selections

Aspect	Infrared	Raman
Physical event	Absorption of IR radiation	Inelastic scattering of laser light
Selection rule	Vibration must change the dipole moment	Vibration must change the polarisability
Strong for	Polar bonds: O-H, N-H, C=O	Non-polar bonds: C=C, C-C, S-S, ring modes
Water as solvent	Very strong absorber, hostile	Very weak scatterer, friendly
Sample needs	Thin film, pellet, ATR contact	None; through glass is fine

Aspect	Infrared	Raman
Symmetric stretches	Often weak or inactive	Often strong
Main practical enemy	Water and CO ₂ bands	Sample fluorescence

The two spectra are complementary in the strict sense: for a molecule with a centre of symmetry, vibrations are active in one technique or the other, never both (the mutual exclusion rule), and in ordinary molecules the strong IR bands and strong Raman bands still tend to be different vibrations. An identification argued from both spectra is correspondingly harder to shake, which is why pharmacopoeial identification strategies increasingly pair them.

5.7 Biological standardization and bioassay

Some drug activities, historically those of complex natural products and biologicals, could not be measured by any chemical or physical test: the only assay was the biological response itself. **Biological standardization** therefore compares the preparation against a stable reference standard of the same substance in a defined biological system and expresses potency in units relative to that standard. A **bioassay** is designed as a comparative experiment: graded doses of standard and test are given to groups of animals, cells or tissues; responses are measured; and the potency ratio is computed from the parallel dose response lines, with statistical checks for parallelism and linearity that validate the assay. Designs include quantal (all or none response, such as death or protection) and graded (measured response, such as blood pressure change or blood sugar fall) endpoints, and the classical matched bracket and Latin square layouts that cancel biological variation. The insulin assay by blood sugar lowering in rabbits and the digitalis assay by cardiac effect were the pharmacopoeial mainstays of this approach, and they form the bridge to the immunoassay methods that largely replaced them.

5.8 ELISA

Enzyme linked immunosorbent assay replaces the animal and the radioactive label with an enzyme label and a coloured product. The architecture is general: an antibody (or antigen) is immobilised on the wells of a microplate; the sample's analyte binds; an enzyme conjugated second reagent completes the chosen format; washing separates bound from free; and the enzyme's substrate is converted to a coloured (or fluorescent, or luminescent) product read in a plate reader. Formats matter and should be named correctly. In a **sandwich ELISA** the analyte is caught between an immobilised capture antibody and an enzyme labelled detection antibody, giving high specificity for larger analytes with two epitopes. In a **competitive ELISA** sample analyte competes with a labelled or immobilised reference, so the signal falls as analyte rises, the format used for small molecules with a single epitope. Pharmaceutical and clinical uses surround the student: cytokine and biomarker measurement, anti-drug antibody testing for biologics, residual host cell protein assays in biomanufacturing, vaccine potency support, and screening of hybridomas in antibody production.

5.9 Radioimmunoassay: principle, and the assays of insulin and digitalis

Radioimmunoassay (RIA), developed by Yalow and Berson for plasma insulin, measures minute concentrations by competition. A fixed, limited amount of specific antibody is incubated with a fixed amount of radioactively labelled antigen (classically iodine-125 labelled) and the sample, whose unlabelled antigen competes for the same binding sites. The more analyte in the sample, the less label is bound. Bound and free fractions are separated, the radioactivity of one fraction is counted, and the result is read from a calibration curve; signal therefore falls as concentration rises, the inverse calibration that defines all competitive assays.

The **insulin** assay was the founding application: plasma insulin at picomolar levels, far below chemical detection, became measurable, transforming diabetes research. The **digitalis (digoxin)** assay is the clinical exemplar: digoxin's therapeutic window is narrow and its toxicity dangerous and mimics the disease treated, so serum digoxin monitoring by RIA (and its successors) became routine, with the assay's cross reactivity toward digoxin metabolites and endogenous digoxin like immunoreactive substances taught as its known weakness. RIA's costs are the ones that displaced it: radioisotope handling and licensing, short reagent shelf life set by isotope decay, and waste disposal. Its sensitivity and specificity survive in the ELISA and chemiluminescent assays that inherited its competitive and sandwich logic, and the syllabus retains it because the principle, limited antibody plus competing labelled ligand, remains the template for all binding assays.

5.10 Choosing among the Unit 5 methods

QUESTION	METHOD
At what temperature does it melt, change form or lose its solvent, and with how much heat?	DSC (events and enthalpies), DTA (events only)
How much hydrate, solvent or volatile matter, and at what temperature lost?	TGA (mass steps read as percentages)
Is this raw material the right polymorph, checked through its container?	Raman (through the container, no prep)
Is the hormone, drug or antibody present at trace level in a biological fluid?	Immunoassay: RIA historically, ELISA in modern form

Taken as a whole, Unit 5 teaches a habit rather than a hierarchy: match the physical observable (heat, mass, scattered light, binding) to the property the question actually asks about, exactly as Unit 4 matched separations to detectors.

Frequently Asked Questions

Q: How do the Woodward-Fieser rules distinguish a homoannular from a heteroannular diene, and why does it matter numerically?

A homoannular diene has both double bonds in the same ring and starts from a base value of 253 nm; a heteroannular diene has them in different rings (or is acyclic) and starts from 214 nm. The 39 nm difference is the largest single decision in the calculation, so misclassifying the diene wrecks the prediction before any increment is added. The higher homoannular value reflects its fixed cisoid geometry, which holds the pi systems coplanar and conjugating at maximum effectiveness.

Q: A calculated lambda max comes out 20 nm away from the observed band. What are the likely causes?

Four usual suspects, in order: the diene or enone class was misread (homoannular versus heteroannular, five versus six membered enone ring); a substituent was miscounted, typically ring residues at a ring junction carbon or an exocyclic double bond that counts for two rings; the solvent correction was omitted or applied with the wrong sign; or the real chromophore is twisted out of planarity by steric crowding, which genuinely breaks the rules' assumption of coplanar conjugation. A 20 nm miss is diagnostic information, not arithmetic failure.

Q: Why has ATR largely replaced the KBr pellet in routine IR work?

ATR needs no preparation: the sample is pressed against the crystal and measured as it is, including pastes, films, liquids and aqueous samples that a pellet cannot handle, because the evanescent wave

samples only about a micrometre of depth and water never gets a long path to absorb through. The pellet's grinding, drying and pressing steps, and its moisture artefacts, disappear. The honest trade offs are that ATR sees the surface rather than the bulk, and its relative band intensities differ slightly from transmission spectra, so reference matching should use ATR libraries.

Q: COSY, HSQC and HMBC are all run on one unknown. What does each one prove that the others cannot?

COSY proves which protons are coupled, tracing runs of neighbouring CH groups, but it stops dead at heteroatoms and quaternary carbons. HSQC proves which proton sits on which carbon, assigning every protonated carbon, but says nothing about how the groups connect. HMBC proves the connections across the gaps: its two and three bond correlations leap heteroatoms and quaternary centres and place carbonyls and ring junctions. A structure is solved when the COSY chains, the HSQC assignments and the HMBC bridges are all consistent with one skeleton.

Q: Why is INADEQUATE so rarely run if it gives the carbon skeleton directly?

Sensitivity. The experiment needs two carbon-13 nuclei bonded to each other; at 1.1 percent natural abundance only about one molecule in ten thousand qualifies for any given bond, so the signals are extremely weak and demand concentrated samples and many hours of acquisition. It is kept for structures that defeat indirect methods, novel natural products and disputed skeletons, where one overnight spectrum settles what weeks of inference could not.

Q: How do you tell an alcohol from an amine by mass spectrometry alone?

Both fragment by alpha cleavage, but the charge bearing fragments differ by one mass unit: a primary alcohol gives the oxonium ion $\text{CH}_2=\text{OH}^+$ at m/z 31, while a primary amine gives the iminium ion $\text{CH}_2=\text{NH}_2^+$ at m/z 30, usually its base peak. The molecular ion adds the second test: an amine with one nitrogen has an odd molecular weight by the nitrogen rule, an alcohol's is even. Together, base peak 30 versus 31 and odd versus even M^+ , the distinction is unambiguous.

Q: What does a metastable peak prove, and how is its position calculated?

It proves parentage: that a particular daughter ion was formed from a particular parent ion in a single step in the field free region, rather than in the source among many possible routes. Its apparent mass is $m^* = (m_2^2)/m_1$, daughter mass squared divided by parent mass. The textbook example is toluene: tropylium 91 decomposing to 65 gives a broad metastable peak at 46.4, certifying the loss of acetylene from the tropylium ion.

Q: An unknown's mass spectrum shows M and M+2 in a ratio of about 9 to 6 to 1 including an M+4 peak. What is in the molecule?

Two chlorine atoms. One chlorine gives 3 : 1 across M and M+2 only; two chlorines multiply the pattern out to M+4 with intensities following the binomial expansion of the 3 : 1 ratio, giving approximately 9 : 6 : 1. One bromine would give 1 : 1, and two bromines 1 : 2 : 1, so the M+4 peak with that intensity profile is a chlorine pair's signature. The same pattern check on fragment ions shows which fragments retain both chlorines.

Q: Why is LC-MS, and not GC-MS, the default hyphenated technique in pharmaceutical analysis?

Because most drug molecules and their impurities are polar, thermally fragile or insufficiently volatile, and GC demands that an analyte vaporise intact. LC separates exactly the compounds GC cannot touch, and electrospray ionisation hands them to the mass spectrometer as intact protonated molecules, with MS/MS available for structure and for sensitive bioanalysis. GC-MS remains superior for volatile compounds, where its EI libraries identify peaks outright, but that is the minority of pharmaceutical work.

Q: DSC and DTA both heat a sample beside a reference. What does DSC measure that DTA does not, and why did DSC win in pharmaceutical laboratories?

DTA records only the temperature difference between sample and reference, which marks the temperatures of events but gives unreliable enthalpies because the signal depends on packing and thermal contact. DSC, whether heat flux or power compensation design, measures heat flow quantitatively, so the same run returns transition temperatures and the enthalpies behind them, enabling melting point and enthalpy of fusion from one curve and purity by the van't Hoff treatment of the melt shape. Pharmaceutical questions are quantitative (how pure, how much amorphous, how much hydrate with TGA alongside), so DSC displaced DTA except in high temperature materials work.

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