Calibration and Validation of Analytical Methods

Analytical Chemistry — BSc in Biotechnology, 2nd year
University of Teramo · A.Y. 2026/2027
Prof. Federico Fanti

LECTURE 7 | ICH · EMA · FDA



Prerequisites and Lecture Objectives

Already known from the statistics lecture

- Systematic and random errors (VIM)
- Trueness, precision, accuracy (ISO 5725)
- Ordinary least squares linear regression
- Residual analysis

Today's objectives

- Classify standards and understand metrological traceability
- Build and evaluate a calibration curve
- Choose the most appropriate calibration method
- Apply validation parameters according to ICH M10, Q2(R2), and Q14



BLOCK A

Standards and Calibration

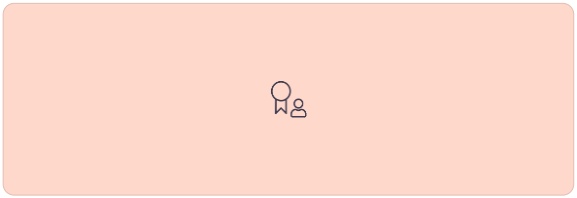
From the preparation of standards to the construction of the working curve: metrological fundamentals and operating procedures.

Hierarchy of Analytical Standards



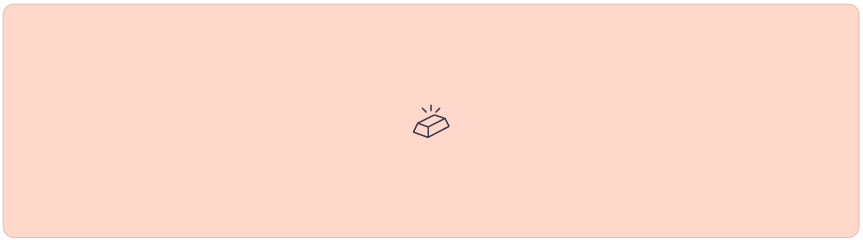
Secondary Standards

Substances that do not meet all PS requirements, calibrated by comparison with PS or CRM.



RM / CRM

Reference Materials (RM) and Certified Reference Materials (CRM): certified values with uncertainty, produced by NIST, JRC, LGC, BAM, accredited to ISO 17034.



Primary Standards (PS)

Highest metrological quality: purity ≥99.9%, stability, non-hygroscopicity, known stoichiometry. Absolute reference without comparison.



Requirements for Primary Standards

Purity

≥ 99.9% with documented analytical certification.

Stability

Stable over time and under standard storage conditions.

Non-hygroscopicity

Does not absorb moisture, ensuring accurate weighed mass.

Known Stoichiometry

Defined molecular formula, exact and verifiable molecular weight.

Certified Reference Materials (CRM)

Definition

CRMs are materials in which one or more property values are certified by a valid procedure, accompanied by a certificate that qualifies them. They reproduce the matrix of real samples.

Metrological Traceability

The traceability chain links every measurement to the SI through documented comparisons with declared uncertainty at each level.

Main Producing Bodies

- **NIST** — National Institute of Standards and Technology (USA)
- **JRC** — Joint Research Centre (EU)
- **LGC** — Laboratory of the Government Chemist (UK)
- **BAM** — Bundesanstalt für Materialforschung (DE)

Accreditation according to **ISO 17034** for reference material producers.

Preparation of Standards: From PS to Working Standards



Each step requires calibrated pipettes and volumetric flasks. Error propagates through serial dilutions: pipetting precision is as critical as the initial weighing.

Absolute vs. Relative Methods

Absolute methods

The relationship between the measured quantity and the amount of analyte is set by physical laws or stoichiometry (gravimetry, titrations). No calibration curve is needed, but in titrations the titrant must be standardized against a primary standard.

Relative methods

They require the construction of a calibration curve describing the signal–concentration relationship. The **majority of instrumental methods** belong to this category: spectroscopy, chromatography, mass spectrometry.

The Analytical Blank: Types and Function

Solvent Blank

Contains only the solvent in which the sample is dissolved. Corrects for the instrumental background of the solvent.

Reagent Blank

Solvent + all reagents used in the preparation. Corrects for additive interferences from the reagents.

Matrix Blank / Procedural Blank

Analyte-free matrix, processed through the same procedure as the sample. Best approximation of the true blank.

Blank subtraction corrects for **additive interferences** and instrumental background, but **not** for the multiplicative matrix effect, which requires other approaches (internal standard, standard addition).

Signal Selection: Peak Area vs. Peak Height

Peak Area

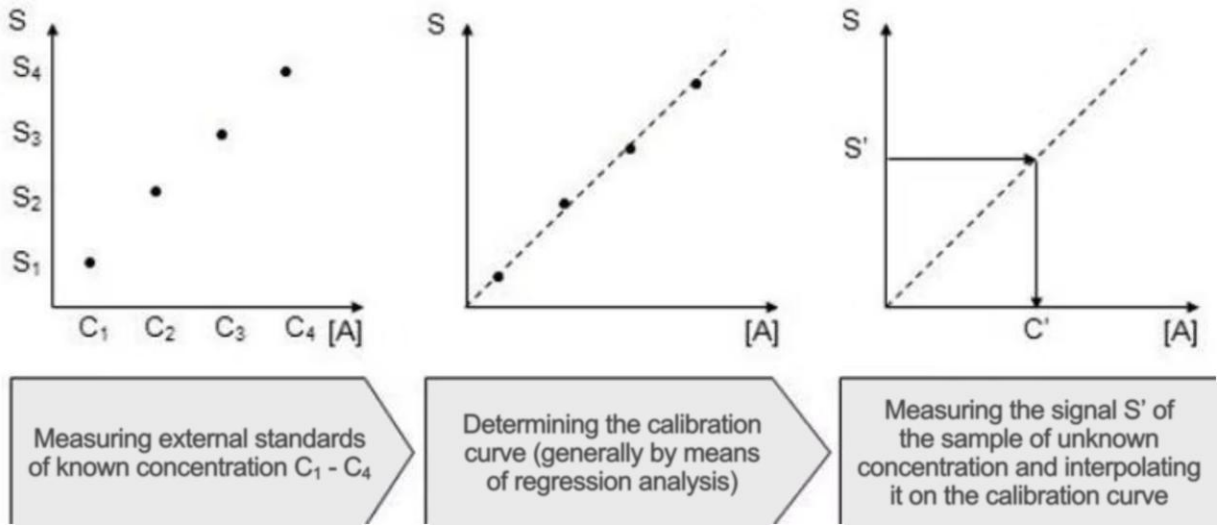
Preferred in current practice. Less sensitive to variations in peak shape (broadening, asymmetry). Captures the entire response of the analyte.

Peak Height

Simpler to measure manually. Subject to errors due to variability in peak shape (width, tailing). Acceptable only if the shape is constant and reproducible.

In chromatographic analysis and LC-MS/MS, **peak area** is the de facto standard for quantification, unless otherwise specified by the method.

Calibration with External Standard (ES)



Procedure

Standards at known concentrations prepared separately; samples treated in the same way; unknown concentration determined by interpolation.

Advantages

Simple and fast; a single curve for all analyses; reduced number of standards.

Critical Assumption

The analyte response must be **identical** in the standard and in the sample: same chemical form, absence of matrix interferences.

Calibration Curve Construction: Operational Requirements

1 At least 6 concentration levels

Distributed from LLOQ to ULOQ, plus blank and zero, covering the entire analytical range.

2 Least squares regression

Calculation of slope, intercept, and coefficient of determination r^2. The intercept must not be forced through zero.

3 Evaluation via residuals and back-calculation

Back-calculated concentrations within ±15% (±20% at LLOQ); at least 75% of calibrators must pass. r^2 alone is not sufficient.

4 New curve for each analytical run

Monitor slope, blank signal, and QC to verify efficiency over time.

Why r^2 Is Not Enough: Residual Analysis

The problem

An r^2 value close to 1 can mask systematic deviations from linearity, especially at the extremes of the range. Two different models can have the same r^2 but very different residuals.

The solution

Analyze the **residual plot**: residuals must be randomly distributed around zero, with no systematic patterns. Verify the **back-calculated concentrations** for each calibrator.

Acceptance criteria (ICH M10)

- Back-calculation within **±15%** of the nominal value
- Within **±20%** at the LLOQ
- At least **75%** of calibrators compliant
- At least **6 calibrators** out of 8 (if 8 levels are used)

Calibration with Internal Standard (IS)

When to use

When the matrix cannot be reproduced in the standards, when injection reproducibility is critical (microsyringe in GC), or when the signal source is intrinsically unstable (flame, ICP plasma).

Principle

A known amount of a reference species (IS) is added **as early as possible in the process** to standards, QCs, and samples at the same concentration (not to blanks). The response signal is the **ratio** $S_{analyte}/S_{IS}$.

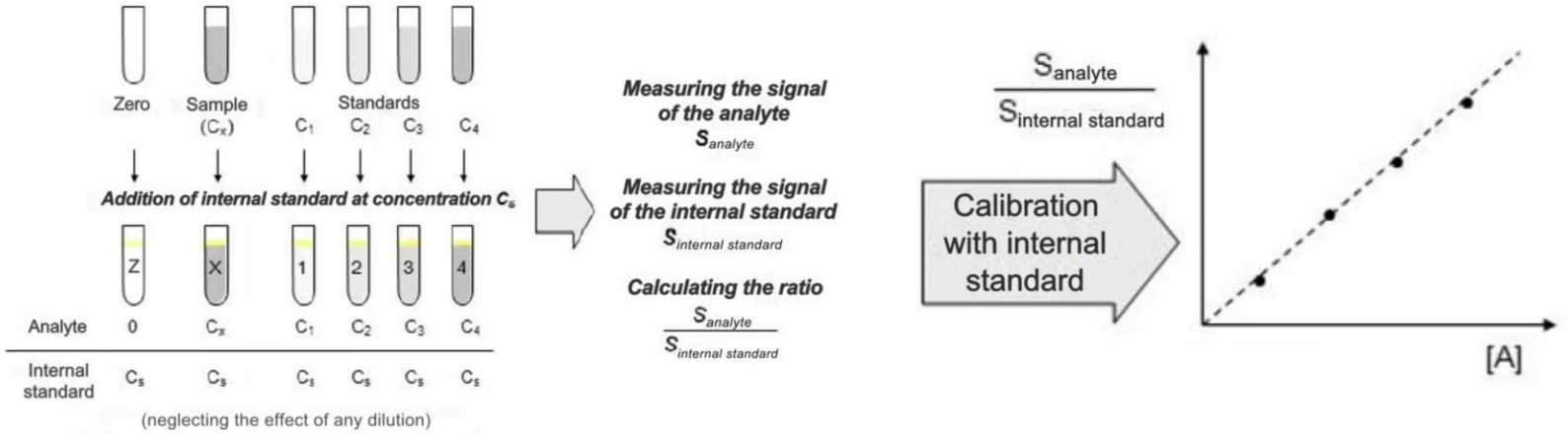
IS Requirements

- Absent in the real sample
- Non-reactive with the analyte or matrix
- Signal clearly distinguishable from all others
- Physicochemical properties similar to the analyte

Optimal Choice in LC-MS/MS

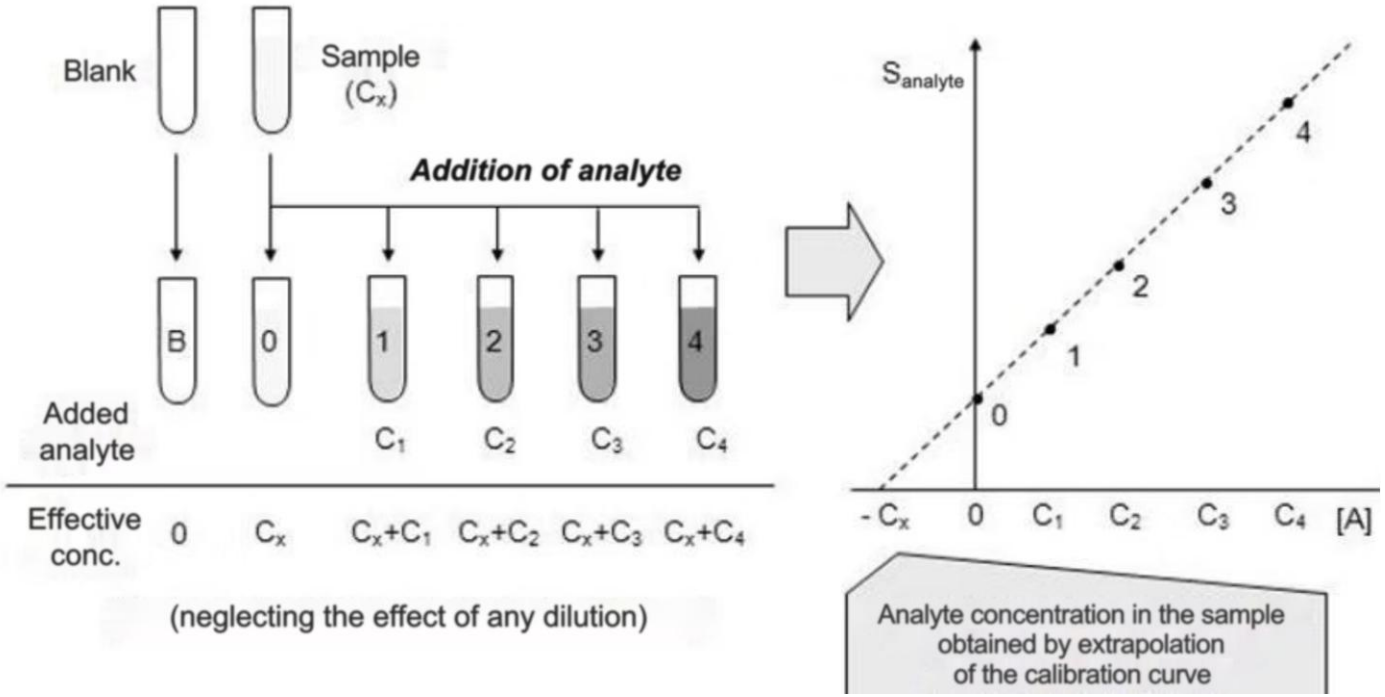
Isotopically labeled internal standard (**SIL-IS**): distinguishable by m/z, chromatographic behavior identical to the analyte.

Operational Scheme: Calibration with Internal Standard



The IS is added at the same concentration C_s to all solutions except the blank. Any instrumental instabilities and matrix interferences affect both signals equally, keeping their ratio constant and producing a calibration line of the signal ratio versus concentration.

Standard Addition Method



Procedure

Aliquots of the sample with unknown concentration C_x are spiked with increasing amounts of pure analyte. The curve is extrapolated to the x-axis: $C_x = b_0/b_1$.

Requirements

Linearity within the addition range; blank correction before extrapolation.

Limitations

Does not correct for constant additive interferences; requires a separate curve for each sample: slow and costly method.

BLOCK B

Validation of Analytical Methods

Parameters, acceptance criteria, and regulatory framework: ICH M10, Q2(R2), Q14, Eurachem, SANTE, and Reg. (EU) 2021/808.



Why Validate an Analytical Method?

Definition

Validation is the process that demonstrates, through documented experimental evidence, that a method is suitable for its intended purpose (*fit for purpose*).

Regulatory framework

- **ICH M10** (2022) — Bioanalysis; adopted by EMA and FDA
- **ICH Q2(R2)** and **Q14** (2023) — Pharmaceutical analytical methods
- **Eurachem Guide** — General chemical laboratories
- **SANTE/11312/2021** — Pesticide residues in food
- **Reg. (EU) 2021/808** — Veterinary drug residues

Overview of Validation Parameters

- **Selectivity / Specificity**
- **Calibration Curve & Range**
- **LOD / LOQ**
- **Trueness**
- **Precision**
- **Recovery**
- **Matrix Effect**
- **Stability**

Additional parameters include: **carry-over**, **dilution integrity**, **robustness**, and **ruggedness**.

Selectivity and Specificity

Definition

Selectivity is the ability of the method to measure the analyte in the presence of other matrix components (endogenous substances, metabolites, co-administered drugs) without significant interference.

The term **specificity** refers to absolute selectivity (response to the analyte only); in practice, the two terms are often used interchangeably.

Experimental Evaluation

Analysis of matrix blanks from **at least 6 lots** of biological matrix (e.g., human plasma from different donors). The signal in the blank at the analyte's retention time must be:

- < 20% of the LLOQ response for the analyte
- < 5% of the internal standard response

Sensitivity: Calibration vs. Analytical

Calibration sensitivity

It is the **slope** of the calibration curve:

$$\text{sensitivity} = \frac{\Delta S}{\Delta [A]}$$

Expresses the change in signal per unit change in concentration. Constant for linear curves.

Analytical sensitivity

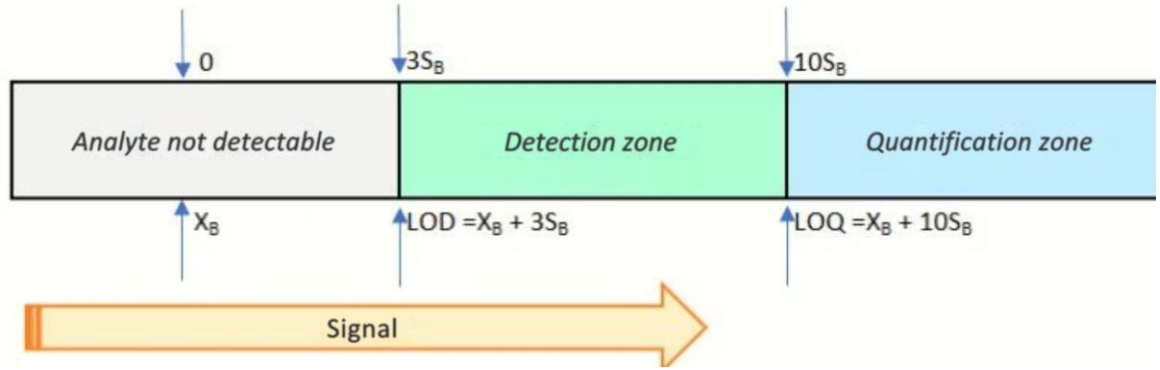
Ratio between calibration sensitivity and the standard deviation of the analytical signal:

$$\gamma = \frac{b_1}{s_S}$$

Depends on concentration even for linear curves, since s_S varies with the signal.

⚠ Note: the term "sensitivity" is often used improperly to indicate the LOD. These are distinct quantities.

LOD and LOQ: Definitions and Formulas



LOD — Limit of Detection

$$\mathrm{LOD} = \frac{3 \cdot s_B}{b_1}$$

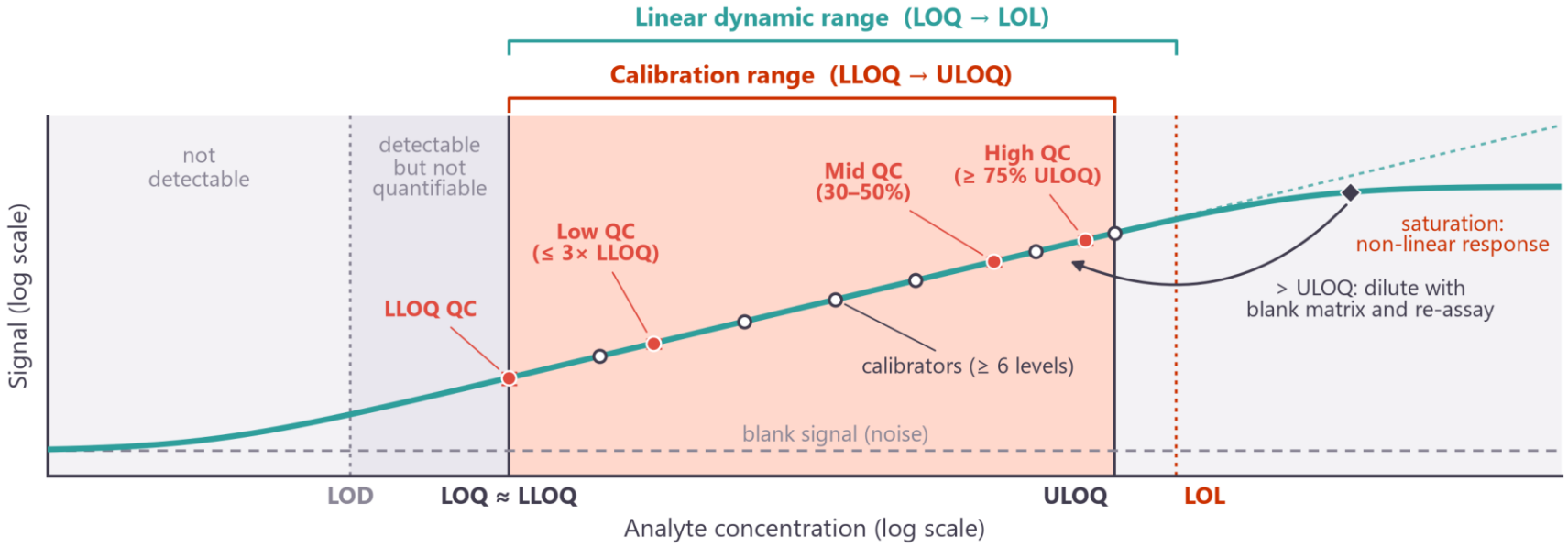
Minimum detectable concentration with a defined confidence level (k=3). Qualitative use: presence/absence of the analyte.

LOQ — Limit of Quantification

$$\mathrm{LOQ} = \frac{10 \cdot s_B}{b_1}$$

Minimum concentration determinable with acceptable precision and accuracy (S/N ≥ 10). In bioanalysis (ICH M10): trueness ±20% and CV ≤ 20%.

Linear Dynamic Range and Calibration Range



Linear Dynamic Range

Range over which the signal is proportional to concentration: from the **LOQ** to the **LOL** (limit of linearity). Above the LOL the detector saturates and the response levels off. It depends on analyte and detector; linearity allows least-squares regression with few standards.

Calibration Range (ICH M10)

From the **LLOQ** (lowest standard, never below the LOQ) to the **ULOQ** (highest standard); it must lie within the linear range. It is built with at least 6 levels plus blank and zero.

QCs cover the whole range:
LLOQ · low (≤ 3× LLOQ)
mid (30–50%) · high (≥ 75% ULOQ)

Out-of-Range Samples

Above the ULOQ, the sample is diluted with blank matrix and re-assayed; the dilution must be validated (dilution integrity). **Below the LLOQ**, the result is reported as "< LLOQ": never extrapolate beyond the calibration curve.

Trueness and Accuracy

Trueness — Bias

$$E = x_i - \mu$$

$$E_{rel}\% = \frac{x_i - \mu}{\mu} \times 100$$

Agreement of the result with the reference value μ. Also expressed as % of the nominal value.

Accuracy = Trueness + Precision

According to ISO 5725 and VIM, **accuracy** is the umbrella term encompassing both trueness (absence of systematic bias) and precision (absence of random error). A result can be precise but not true, or vice versa.

ICH M10 Criteria

Bias within **±15%** of the nominal value; within **±20%** at the LLOQ.

Precision: Repeatability, Intermediate, and Reproducibility

Repeatability (intra-day)

Same procedure, same operator, same instrument, same day. CV% ≤ 15% (≤ 20% at LLOQ).

Intermediate Precision (inter-day)

Variations in day, operator, and instrument within the same laboratory. Reflects the method's variability in routine use.

Reproducibility (inter-laboratory)

Comparison across different laboratories. Basis for standardization and regulatory recognition of the method.

$$\text{RSD\%} = \frac{s}{\bar{x}} \times 100$$

The Horwitz Curve: Expected Precision vs. Concentration

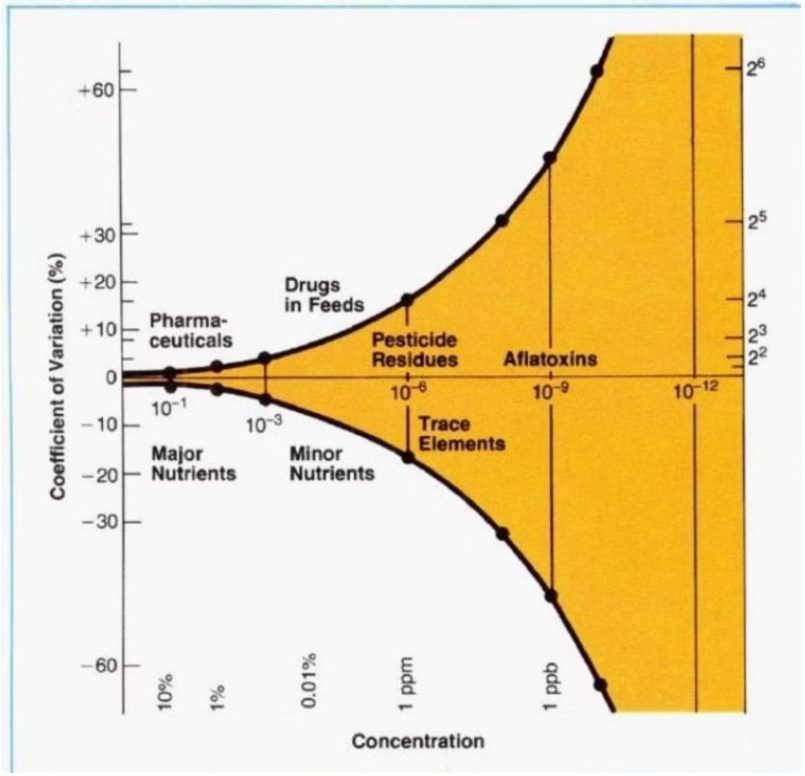
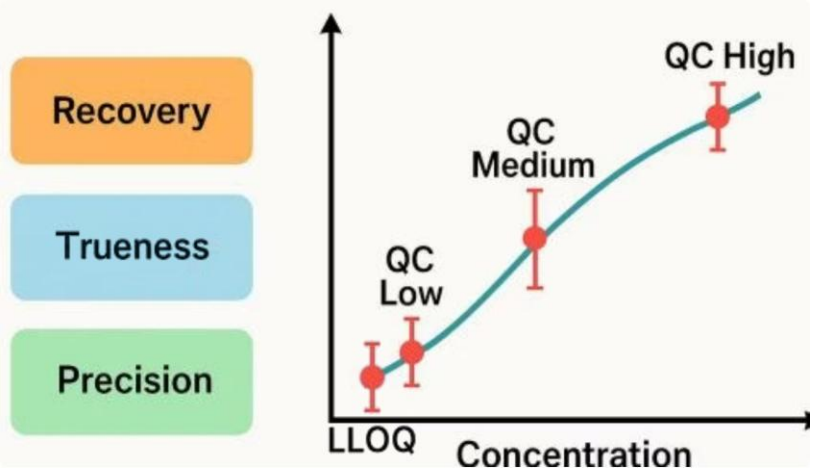


Figure 1. The Horwitz curve shows the relationship between interlaboratory coefficient of variation and concentration on a logarithmic scale. As dilution increases, the expected precision deteriorates in a predictable manner according to the law: $\mathbf{CV\% \approx 2^{(1-0.5 \log C)}}$. This justifies the more permissive criteria (±20%, CV ≤ 20%) at the LLOQ.

Quality Control (QC): Levels and ICH M10 Criteria



LLOQ QC

At the lower limit of quantification. Criteria: ±20%, CV ≤ 20%.

QC Low (LQC)

≤ 3× LLOQ. Criteria: ±15%, CV ≤ 15%.

QC Medium (MQC)

30–50% of the range. Criteria: ±15%, CV ≤ 15%.

QC High (HQC)

≥ 75% of the ULOQ. Criteria: ±15%, CV ≤ 15%.

Causes of Poorer Performance at Low and High Levels

Low levels (near the LLOQ)

- High instrumental noise relative to signal
- Baseline drift
- More pronounced matrix effect
- Co-eluting interferences
- Analyte instability at low concentrations

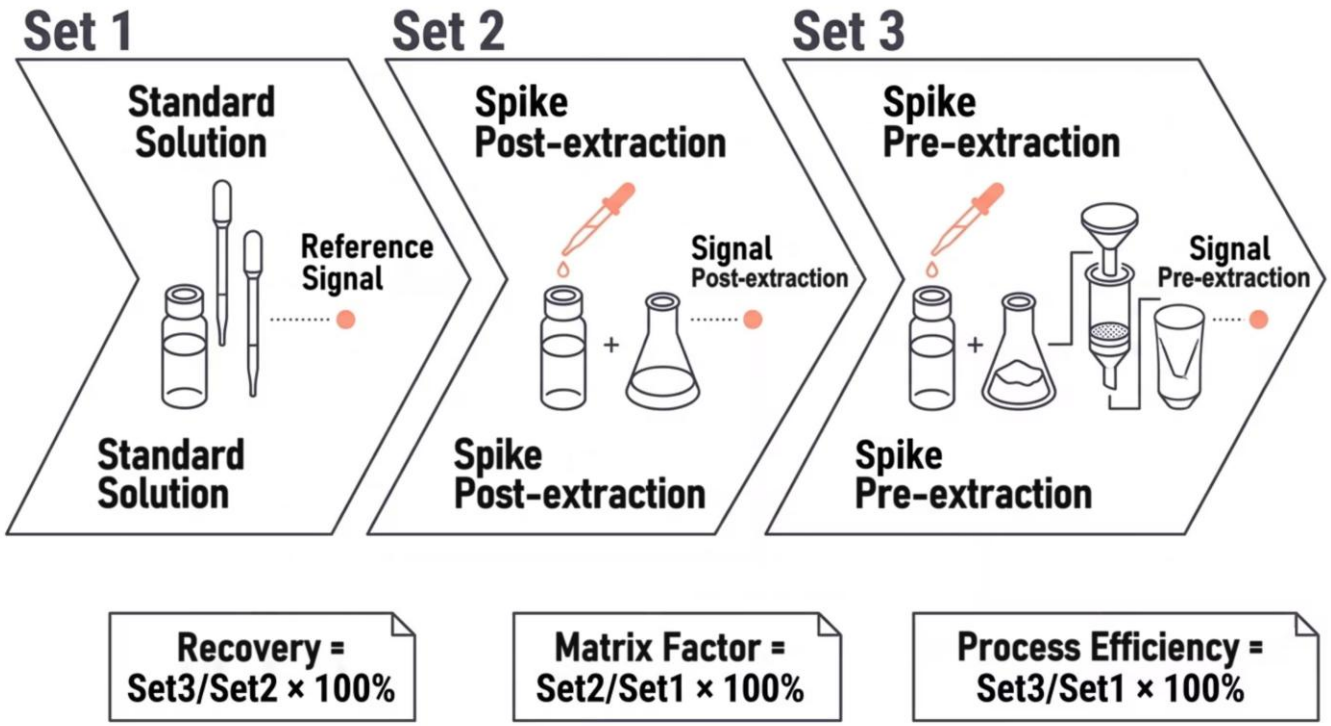
High levels (near the ULOQ)

- Detector saturation
- Deviations from linearity
- Imprecise dilutions
- Co-extraction of interferents
- **Carry-over**: residues from high-concentration samples contaminate subsequent low-concentration samples

Carry-over from high-concentration samples is one of the most insidious sources of error at low levels: it must be assessed by injecting a blank after the most concentrated sample.

Recovery, Matrix Effect and Process Efficiency

Matuszewski scheme: three experimental sets to distinguish different sources of signal loss and variation.



Formulas: Recovery, Matrix Effect, Process Efficiency

Recovery (RE)

$$RE\% = \frac{\text{Set 3}}{\text{Set 2}} \times 100$$

Percentage of analyte recovered after the entire extraction process. It must be constant and reproducible at all QC levels, even if below 100%.

Matrix Factor (MF)

$$MF\% = \frac{\text{Set 2}}{\text{Set 1}} \times 100$$

MF < 100%: ion suppression. MF > 100%: ion enhancement. The **IS-normalized MF** comes from EMA 2011; ICH M10 uses QCs in ≥ 6 lots (±15%, CV ≤ 15%).

Process Efficiency (PE)

$$PE\% = \frac{\text{Set 3}}{\text{Set 1}} \times 100$$

Matrix Effect: Ion Suppression and Ion Enhancement

Mechanism in LC–MS/MS

Matrix components co-eluting with the analyte compete for ionization at the ESI or APCI source. The result is a signal variation independent of the actual analyte concentration.

- **Ion suppression**: MF < 100%, underestimation
- **Ion enhancement**: MF > 100%, overestimation

Evaluation and Mitigation

- MF normalized to the IS (preferably SIL-IS)
- Low and high QCs in ≥ 6 lots: ±15%, CV ≤ 15% (ICH M10)
- **Post-column infusion** technique: constant post-column infusion of the analyte to map suppression zones along the chromatogram
- Optimization of sample cleanup (SPE, PPT, LLE)

Recovery at Different Concentrations

Objective

Verify that the extraction process works equivalently at all QC levels (LQC, MQC, HQC).

Causes of Variability at Low Levels

- Adsorption to container walls
- SPE inefficiency at low concentrations
- Volatilization during evaporation
- Analyte instability

Causes of Variability at High Levels

- Saturation of the SPE stationary phase
- Co-extraction of interferents
- Non-linear effects

High recovery only at high levels is unacceptable. **Recovery must be consistent and reproducible across the entire range**, even if below 100%.

Trueness at Different Concentrations

At Low Levels

Trueness may worsen due to: instrumental noise, baseline instability, more pronounced matrix effect, co-eluting interferences. Bias tends to increase as the LLOQ is approached.

At High Levels

Trueness may worsen due to: detector saturation, deviations from linearity, carry-over, imprecise dilutions. Bias can be positive or negative.

Acceptance Criteria

Trueness must remain within **±15%** of the nominal value (±20% at the LLOQ) at all QC levels. A method that is accurate only "in the middle of the curve" is not valid.

Precision at Different Concentrations

Assessment

Precision is always evaluated both as **intra-day repeatability** (same day, same operator) and as **inter-day intermediate precision** (different days, different operators). It is expressed as CV%.

Criterion

CV% ≤ **15%** at all levels (≤ **20%** at the LLOQ).

Causes of Deterioration at Low Levels

- Instrumental noise and drift
- Injection variability
- Analyte instability

Causes of Deterioration at High Levels

- Detector saturation
- Imprecise dilutions
- Mobile phase instability

Consistency Across the Full Range: The Fundamental Principle

Stable

The parameters do not change significantly from one concentration level to another.

Reliable at the extremes

Performance remains acceptable even at the extremes of the range (LLOQ and ULOQ).

Consistent over time

Results agree across days and operators (intermediate precision).

Accurate across the full curve

A "perfect" curve with QC failing at low levels → method NOT valid.



Stability: Overview of Conditions to Evaluate

Stock solutions

Stability of stock solutions at room temperature and at −20°C/−80°C over extended periods.

Bench-top (short-term)

Stability of the prepared sample on the workbench for the time required for analysis (typically 4–24 h).

Freeze-thaw cycles

At least 3 freeze/thaw cycles to assess analyte degradation.

Long-term

Stability under the intended storage conditions for the duration of the study (weeks/months).

Robustness: Stability Under Small Operational Variations

Definition

Robustness measures the ability of the method to remain reliable in the presence of **small variations** in operating conditions, typical of routine laboratory work.

Key question: *Does the method remain reliable even if something changes slightly?*

Examples of tested variations

- Column temperature ±2–5 °C
- pH variation ±0.1
- Mobile phase composition (e.g., 98/2 → 97/3)
- Flow rate ±0.1 mL/min
- Slightly different extraction volume or time
- Slightly different MS source intensity or UV lamp
- Mixing or centrifugation time

A robust method maintains accuracy, precision, and retention times within acceptable limits despite these small changes. **It must work in real routine conditions, not only under ideal conditions.**

Ruggedness: Stability Across Changes in Context

Definition

Ruggedness evaluates the reproducibility of the method when **broader contextual factors** change: laboratory, instrumentation, operator, consumables.

Key question: *Does the method give similar results regardless of who uses it, where, or how?*

Examples of tested variations

- Different but equivalent instrumentation
- Different laboratories (inter-laboratory studies)
- Columns from different lots
- Operators with different levels of experience
- Reagents and consumables from different brands or lots
- Days far apart in time
- Different environmental conditions (humidity, temperature)

⚠ If simply changing the operator or column is enough to cause results to collapse, the method **is not rugged** and cannot be transferred to other laboratories.

Carry-over and Dilution Integrity

Carry-over

Residual analyte from a previous sample that contaminates the next sample. It is assessed by injecting a matrix blank after the highest-concentration sample (ULOQ). The signal in the blank must be < 20% of the response at the LLOQ.

Dilution Integrity

Verifies that samples with concentrations above the ULOQ can be diluted with blank matrix without loss of accuracy or precision. Tested with samples diluted 2×, 5×, 10× relative to the ULOQ. Acceptance criteria: ±15%, CV ≤ 15%.

Reference Bodies for Bioanalytical Guidelines



Key Roles and Documents

- **FDA** — Bioanalytical Method Validation (2018); superseded by ICH M10 for methods within its scope
- **EMA** — Guideline on Bioanalytical Method Validation (2011), superseded by ICH M10 since 2023
- **ICH M10** (2022) — Harmonised guideline on bioanalytical method validation, adopted by FDA and EMA
- **ICH Q2(R2) / Q14** (2023) — Pharmaceutical analytical methods and analytical development

ICH M10 (2022): Structure and Scope

1

Scope

Validation of bioanalytical methods for the quantification of analytes in biological matrices (plasma, serum, urine, tissues) in the context of pharmacokinetic and toxicokinetic studies.

2

Required Parameters

Selectivity, calibration curve, LLOQ, trueness, precision, recovery, matrix effect, stability, carry-over, dilution integrity.

3

New Features Compared to Previous Guidelines

FDA/EMA harmonization; SIL-IS recommended whenever possible; matrix effect assessed with QCs in ≥ 6 lots; criteria for ligand-binding (LBA) and chromatographic (LC-MS) methods.

ICH Q2(R2) and Q14 (2023): Pharmaceutical Methods

ICH Q2(R2)

Revision of the long-standing guideline on analytical method validation for pharmaceutical products. Updates criteria for specificity, linearity, range, accuracy, precision, LOD, LOQ, and robustness. Linked to the **Analytical Target Profile (ATP)**, introduced by ICH Q14.

ICH Q14

New guideline on **analytical development**: an approach based on method understanding (Analytical Quality by Design, AQbD). Complementary to Q2(R2): while Q2(R2) defines what to validate, Q14 defines how to develop the method in a rational and documented manner.

Guidelines for the Food and Veterinary Sector

SANTE/11312/2021

Guidance document for the validation of analytical methods for pesticide residues in food and feed. Defines criteria for recovery (70–120%), CV% (≤20%), LOQ, and selectivity for multi-residue methods.

Reg. (EU) 2021/808

Regulation on analytical methods for official controls of veterinary drug residues in food of animal origin. Replaces Decision 2002/657/EC. Updates the criteria for CCα and CCβ already defined in that decision.

Eurachem Guide

"The Fitness for Purpose of Analytical Methods": a general guidance document for chemical laboratories, applicable to any sector. Covers validation; for uncertainty see the Eurachem/CITAC QUAM guide.

Comparison of Calibration Methods

Characteristic	External Standard	Internal Standard	Standard Addition
Simplicity	High	Medium	Low
Matrix effect correction	No	Partial (SIL-IS: optimal)	Yes (multiplicative)
Injection variability correction	No	Yes	No
Number of standards per sample	Low	Medium	High (1 curve/sample)
Typical application	Simple matrices	LC-MS/MS, GC-MS	Complex irreproducible matrices



Instrumental Methods: Advantages Over Traditional Techniques

Higher Sensitivity

Quantitation limits far lower than those of classical gravimetric and volumetric techniques.

Automation

Ability to run serial analyses with automated systems, reducing human error and increasing productivity.

Data Management

Acquisition, processing, and storage of analytical data through interfacing with PCs and dedicated software.

Main Instrumental Techniques: Overview

Spectroscopy

Molecular and atomic, absorption and emission: UV-Vis, IR, AAS, ICP-OES, fluorescence.

Electrochemical Methods

Potentiometry, conductimetry, electrolysis, voltammetry. Based on the measurement of potential, current, or conductance.

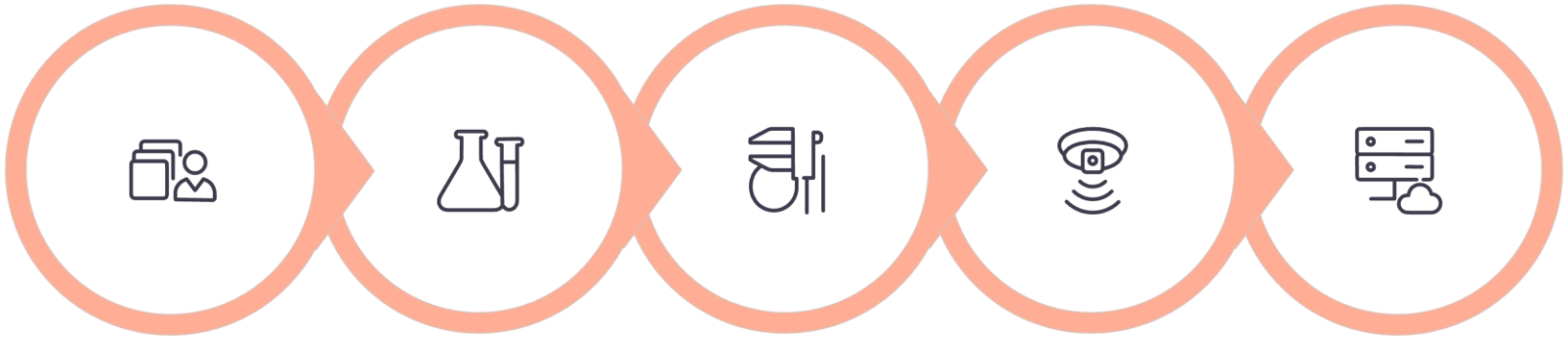
Chromatography

GC, HPLC, ion chromatography. Separation followed by spectroscopic or electrochemical detection.

Mass Spectrometry

MS and hyphenated techniques (LC-MS/MS, GC-MS): separation by mass-to-charge ratio, high specificity and sensitivity.

The Analytical Process: From Sample Preparation to Analysis



Sampling

Standards and blank

Calibration

Analysis

Data processing

Each phase introduces potential sources of error. Method validation must cover the entire process, not just the instrumental step.

Error Propagation in Serial Dilutions

Principle

In a series of dilutions, errors propagate and accumulate. The uncertainty of the final working solution depends on the uncertainty of each individual dilution step.

$$\frac{u_c(c)}{c} = \sqrt{\left(\frac{u_1}{x_1}\right)^2 + \left(\frac{u_2}{x_2}\right)^2 + \ldots + \left(\frac{u_n}{x_n}\right)^2}$$

Practical Implications

- Minimize the number of serial dilutions
- Use calibrated pipettes with known uncertainty
- Prefer Class A volumetric flasks
- Document each step with its corresponding uncertainty
- The stock solution should be prepared by direct weighing with minimum error

Monitoring the Calibration Curve Over Time

1

New Sequence

Build a new calibration curve for each analytical sequence.

2

Verification with 1–2 Standards

With new reagents, verify the existing curve with one or two additional standards before building a new one.

3

Slope Monitoring

The slope (sensitivity) must remain constant over time. Variations indicate instrumental or reagent issues.

4

QC Monitoring

QC samples at different levels confirm the efficiency of the procedure, the instrument, and the operators.

Calibration Line Intercept: Interpretation

Meaning of the Intercept

The intercept represents the blank signal (instrumental background + additive interferences). An intercept significantly different from zero may indicate:

- Blank contamination
- Uncorrected additive interferences
- Uncompensated matrix effect
- Instrumental problem (baseline drift)

Operational Rule

If the intercept is clearly different from zero, it is necessary to evaluate whether this is physically justifiable. If not, review the analytical procedure.

⚠ Never force the line through the origin (0,0): this introduces an artificial systematic error that compromises quantification.

Outliers in the Calibration Curve

1 Identification

After fitting the regression, examine the standardized residuals to spot outliers; Grubbs and Dixon tests apply to replicates of the same level.

2 Justified Exclusion

An outlier may be excluded only if there is documented justification (preparation error, instrumental issue). Arbitrary exclusion is unacceptable.

3 75% Criterion Verification

After exclusion, at least 75% of the calibrators must meet the back-calculation criteria (±15%, ±20% at the LLOQ).

Stable Isotope-Labeled Internal Standard (SIL-IS) in LC-MS/MS

Why It Is the Optimal Choice

The SIL-IS (e.g., deuterated analyte, ^{13}C- or ^{15}N-labeled) has identical chromatographic behavior to the analyte but is distinguishable by m/z. It compensates for:

- Losses during extraction
- Injection variability
- Matrix effect (ion suppression/enhancement)
- MS source instability

Practical Requirements

- Added as early as possible in the process (ideally before extraction)
- Constant concentration in all samples and standards
- Absence of cross-talk between the analyte channel and the SIL-IS channel
- Certified isotopic purity (≥ 98%)

Limitation

High cost; not always available for all analytes.

Summary: Validation Parameters and ICH M10 Criteria

Parameter	General Criterion	Criterion at LLOQ
Trueness (bias%)	±15%	±20%
Precision (CV%)	≤15%	≤20%
Conforming calibrators	≥75% of total	—
Selectivity (blank)	<20% LLOQ response	—
Matrix effect (low/high QC)	±15%, CV ≤15% in ≥6 lots	—
Carry-over (blank post-ULOQ)	<20% LLOQ response	—

Full, Partial and Cross-Validation

Full validation

Required for a new or significantly modified method. Includes all parameters: selectivity, calibration curve, LLOQ, trueness, precision, recovery, matrix effect, stability, carry-over, dilution integrity, robustness.

Partial validation

For minor changes to an already validated method (e.g., change of matrix, new range, new equivalent instrument). Only parameters potentially affected by the change are evaluated.

Cross-validation

Comparison of two validated methods on the same samples (e.g., transfer between laboratories). ICH M10 sets no criteria: bias is assessed statistically (e.g., Bland-Altman, Deming).

Documentation and Traceability in Validation

Validation Plan (VP)

Document drafted before the experiments that describes: objectives, parameters to evaluate, acceptance criteria, experimental design, responsibilities, and timelines.

Validation Report (VR)

Final document reporting all experimental data, statistical calculations, comparison with acceptance criteria, and the conclusion on the validity of the method. Must be signed and archived in accordance with GLP/GMP regulations.

In a regulatory context (GLP studies, registration dossiers), the validation plan and report are inspectable documents. Traceability of every data point is mandatory.

Common Errors in Building the Calibration Curve

Forcing the line through the origin

Introduces a systematic bias. The intercept must be freely estimated by regression.

Using r^2 as the sole criterion

The coefficient of determination does not detect systematic deviations. Always analyze residuals and back-calculated concentrations.

Excluding points without justification

Excluding compliant calibrators without reason invalidates the curve. Every exclusion must be documented.

Reusing the curve with new reagents without verification

With new reagent lots, always verify the curve with at least 1–2 additional standards.

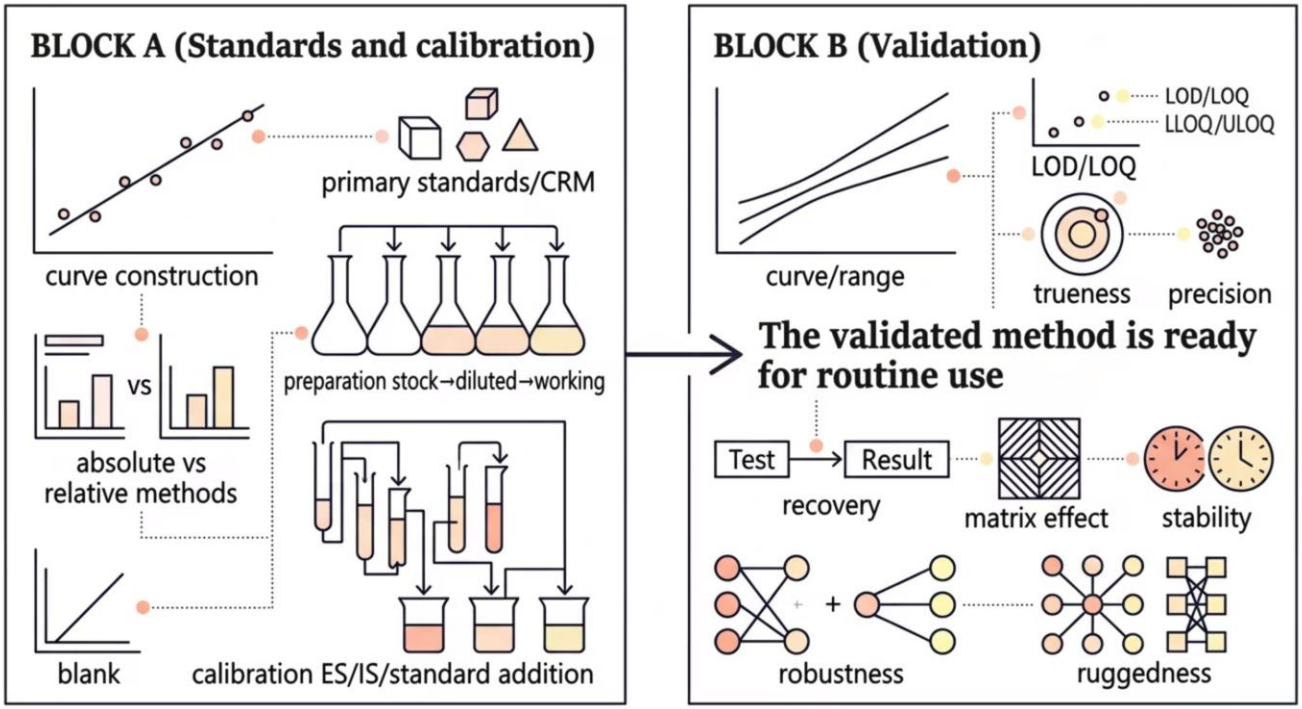
Practical Example: An LC-MS/MS Analytical Sequence



Matrix blank **Zero standard** **Calibrators** **QC triplicates** **Samples**

The injection order is standardized to minimize carry-over and ensure the stability of the curve throughout the entire sequence. End-of-sequence QCs verify instrumental stability over time.

Summary: From Calibration to Validation



Key Take-Home Messages

Metrological traceability is fundamental

Every standard must be linked to the SI through a documented chain of comparisons with stated uncertainty.

r^2 is not sufficient

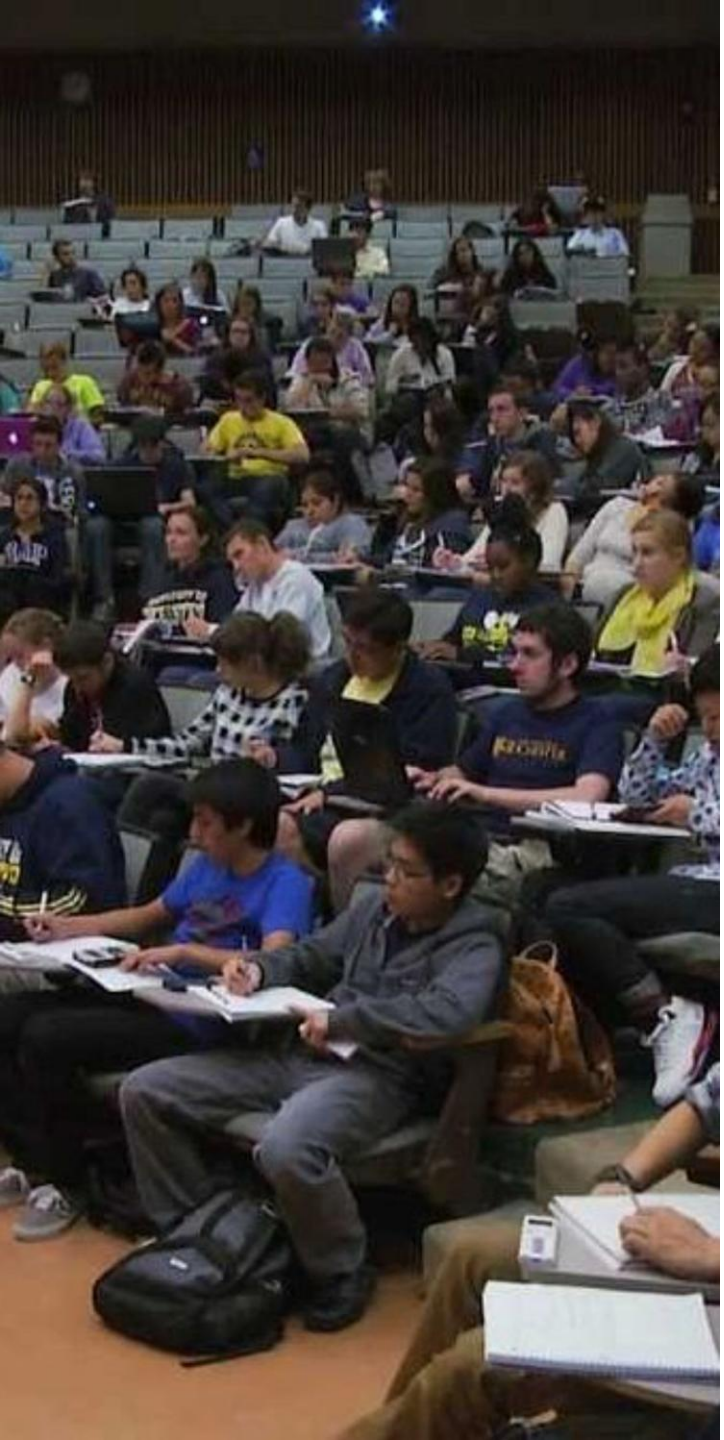
The calibration curve is evaluated using residuals and back-calculated concentrations, not just the coefficient of determination.

Consistency across the entire range is mandatory

A valid method must meet trueness, precision, and recovery criteria from the LLOQ to the ULOQ, not just at the midpoint of the curve.

SIL-IS is the optimal choice in LC-MS/MS

It simultaneously compensates for extraction losses, injection variability, and matrix effect.



Regulatory References and Recommended Readings

Guidelines

- ICH M10 — Bioanalytical Method Validation (2022)
- ICH Q2(R2) / Q14 — Analytical Procedures (2023)
- Eurachem — The Fitness for Purpose of Analytical Methods (3rd ed., 2025)
- SANTE/11312/2021 v2 (2024) — Pesticide residues
- Reg. (EU) 2021/808 — Veterinary drug residues

Reference Texts

- Skoog, Holler, Crouch — *Principles of Instrumental Analysis*, 7th ed.
- Harvey — *Modern Analytical Chemistry*
- Matuszewski et al. — *Anal. Chem.* 2003, 75, 3019–3030
- ISO 5725 — Accuracy of measurement methods