Sample Preparation in Metabolomics

Quenching Metabolism

- Rapid cooling or organic solvents
- Stop enzymatic reactions
- Preserve metabolite levels

Extraction Methods

- Methanol/chloroform extraction
- Solid-phase extraction (SPE)
- Method depends on metabolite class

Matrix Effects

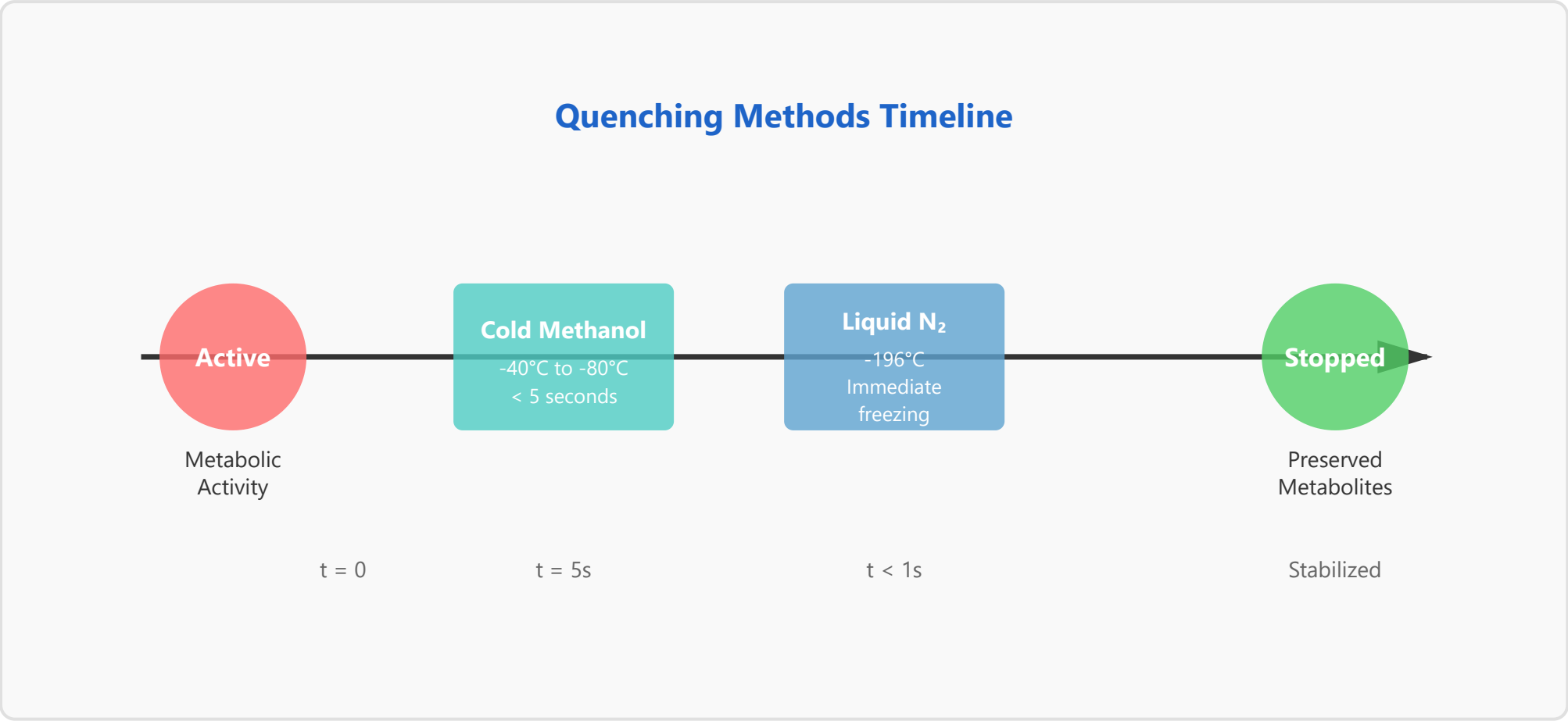
- Ion suppression/enhancement
- Sample cleanup required
- Calibration curve considerations

Internal Standards

- Isotope-labeled compounds
- Normalize for extraction/ionization
- Quality control

1 Quenching Metabolism

Quenching is the critical first step in metabolomics sample preparation that rapidly halts all metabolic activity to capture a true snapshot of the cellular metabolic state. Without proper quenching, metabolite concentrations can change dramatically within seconds due to ongoing enzymatic reactions.



Common Quenching Methods

1. Cold Organic Solvent Method: Typically uses methanol or methanol/water mixtures at -40°C to -80°C. This method simultaneously quenches metabolism and begins extraction. The organic solvent denatures proteins and stops enzymatic activity while extracting intracellular metabolites.

2. Liquid Nitrogen Snap-Freezing: Provides the fastest quenching by instantly freezing samples at -196°C. This method is ideal for tissue samples and can preserve samples for extended periods before extraction.

3. Acid Quenching: Uses strong acids (e.g., perchloric acid) to rapidly denature proteins and stop enzymatic reactions. However, this method may cause degradation of labile metabolites.

Critical Considerations

- **Speed is essential:** Metabolic turnover can occur in seconds, especially for high-energy phosphates like ATP
- **Temperature control:** Cold quenching methods prevent metabolite degradation during the initial handling
- **Sample type matters:** Cell cultures, tissues, and biofluids require different quenching approaches
- **Avoid metabolite leakage:** Some methods can cause cell lysis and metabolite loss into the medium

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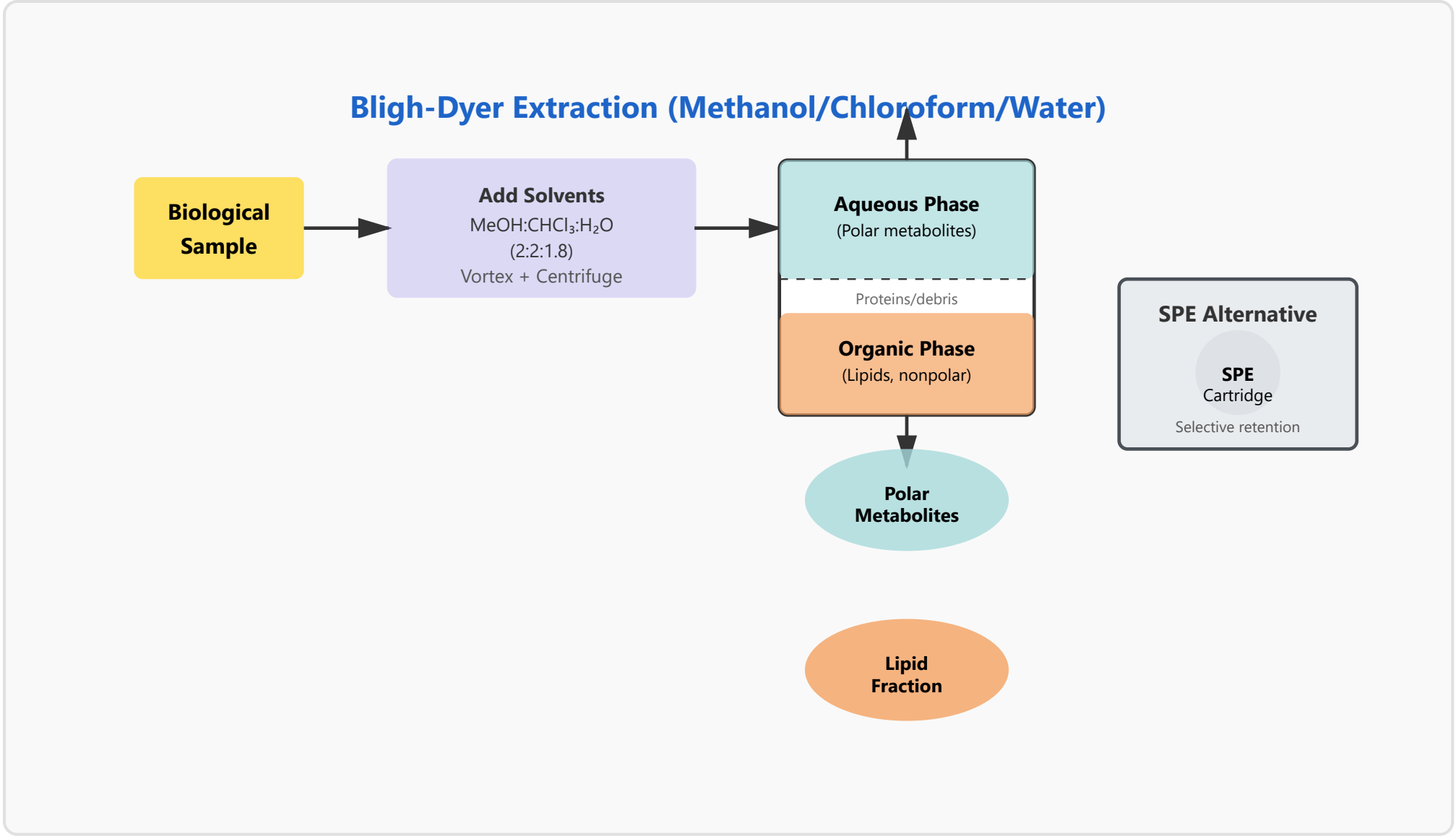
2 Extraction Methods

Metabolite extraction separates compounds of interest from the biological matrix. The choice of extraction method depends on the chemical properties of target metabolites, including polarity, stability, and molecular weight. Different metabolite classes require tailored extraction strategies.



Methanol/Chloroform Extraction (Bligh-Dyer Method)

This biphasic extraction system separates metabolites based on polarity. The method creates two distinct phases: an upper aqueous phase containing polar metabolites (amino acids, organic acids, sugars) and a lower organic phase containing nonpolar metabolites (lipids, steroids). The protein precipitate remains at the interface.

Advantages: Comprehensive extraction, separates metabolite classes, compatible with MS analysis, well-established protocol.

Solid-Phase Extraction (SPE)

SPE uses a solid adsorbent material packed in a cartridge to selectively retain target metabolites while washing away interfering compounds. Different stationary phases (C18, mixed-mode, ion-exchange) provide selectivity for various metabolite classes.

Process: Sample loading → Wash (remove interferences) → Elution (collect target metabolites)

Other Extraction Methods

- **Simple protein precipitation:** Acetonitrile or methanol addition for quick plasma/serum processing
- **Liquid-liquid extraction (LLE):** Separates compounds between two immiscible liquid phases
- **Pressurized liquid extraction:** Uses elevated temperature and pressure for difficult matrices
- **Enzymatic extraction:** Uses enzymes to break down specific cellular components

Method Selection Guidelines

- **Polar metabolites:** Aqueous methanol (80%) or methanol/water mixtures
- **Lipids and nonpolar compounds:** Chloroform/methanol or MTBE-based extractions
- **Comprehensive coverage:** Bligh-Dyer or Folch methods for simultaneous polar/nonpolar extraction
- **Targeted analysis:** SPE for specific metabolite classes with reduced matrix effects
- **Recovery validation:** Always assess extraction efficiency using standards or spiked samples

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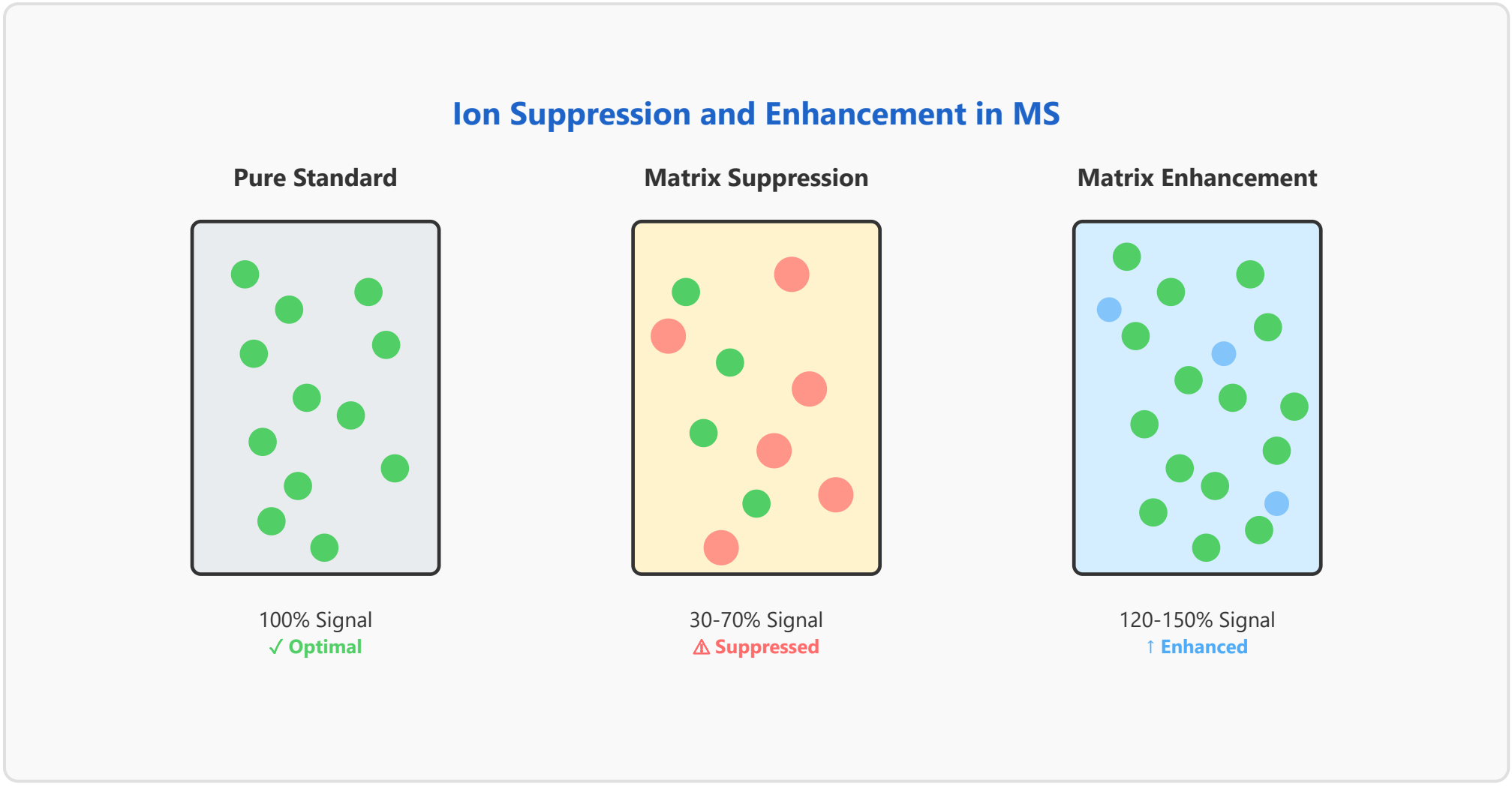
3 Matrix Effects

Matrix effects occur when co-eluting compounds from the biological sample interfere with the ionization of target metabolites in mass spectrometry. These effects can cause significant signal suppression or enhancement, leading to inaccurate quantification even when the metabolite concentration is constant.



Causes of Matrix Effects

- **Ion suppression:** Co-eluting compounds compete for ionization, reducing target analyte signal (most common)
- **Ion enhancement:** Matrix components increase ionization efficiency of target metabolites

- **Source contamination:** Non-volatile salts or lipids accumulate in the ion source
- **pH changes:** Buffer components alter ionization efficiency in the electrospray droplet

Strategies to Minimize Matrix Effects

1. Sample Cleanup: Use SPE, liquid-liquid extraction, or protein precipitation to remove interfering compounds before MS analysis.

2. Chromatographic Separation: Improve LC methods to separate target metabolites from matrix components temporally.

3. Sample Dilution: Reduces matrix concentration but must maintain adequate analyte detectability.

4. Matrix-Matched Calibration: Prepare calibration curves in the same biological matrix as samples to account for constant matrix effects.

5. Standard Addition Method: Add known amounts of analyte to sample aliquots for accurate quantification despite matrix effects.

Assessment and Quality Control

- **Post-column infusion:** Continuously infuse standard while injecting blank matrix to visualize suppression regions
- **Matrix factor calculation:** Compare signal in matrix vs. pure solvent to quantify matrix effects (MF = 1 indicates no effect)
- **Quality control samples:** Analyze QC samples at different concentrations to monitor matrix effect consistency
- **Regular cleaning:** Maintain MS ion source to prevent accumulation-related effects
- **Internal standard correction:** Use isotope-labeled internal standards to normalize for variable matrix effects

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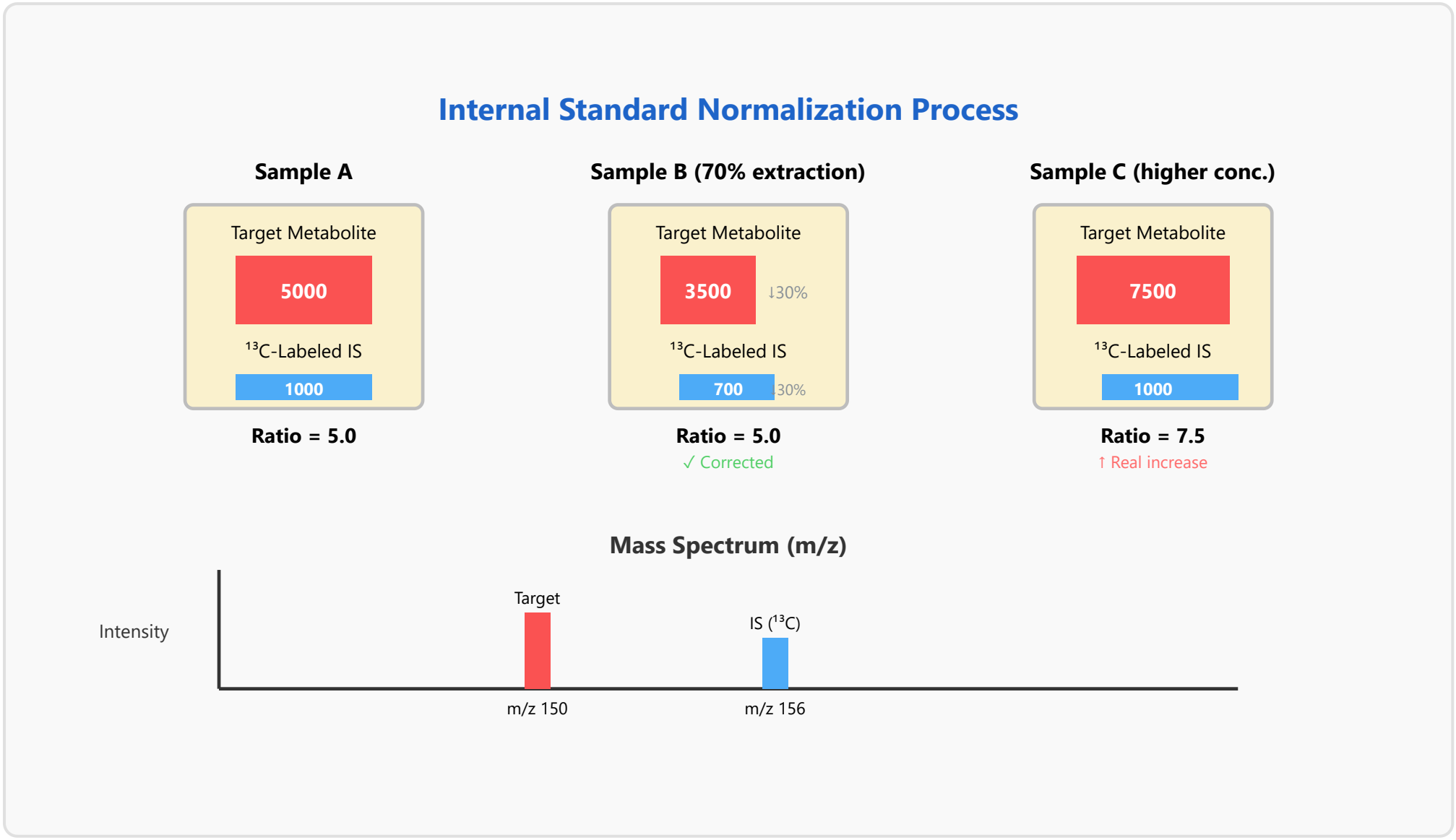
4 Internal Standards

Internal standards (IS) are known compounds added to samples at defined concentrations to correct for variability in sample preparation, extraction efficiency, ionization, and instrument response. Stable isotope-labeled compounds that behave identically to target metabolites are the gold standard for metabolomics quantification.



Types of Internal Standards

1. Stable Isotope-Labeled Standards (SIL-IS): The ideal choice. These are chemically identical to target metabolites but contain heavy isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O). They co-elute perfectly with targets and experience identical matrix effects.

2. Structural Analogs: Compounds with similar structure to targets. Less expensive than SIL-IS but may have different retention times and matrix effects.

3. Universal Standards: Compounds not expected in biological samples, used for general performance monitoring (e.g., caffeine, antipyrine).

Applications and Functions

- **Extraction efficiency correction:** Accounts for losses during sample preparation steps
- **Ionization variability:** Normalizes for fluctuations in MS ionization efficiency
- **Instrument drift:** Corrects for changes in detector response over time
- **Injection volume errors:** Compensates for autosampler imprecision
- **Quality control:** Monitors overall analytical performance across batches
- **Absolute quantification:** Enables accurate concentration determination using calibration curves

Implementation Best Practices

When to add: Add internal standards as early as possible in the workflow, ideally immediately after quenching or at the start of extraction. This ensures they experience all the same processes as endogenous metabolites.

Concentration selection: Choose IS concentrations within the expected range of target metabolites. For quantification, the IS should produce a signal intensity similar to targets.

Multiple standards: Use multiple internal standards to cover different metabolite classes with varying chemical properties (polar, nonpolar, acidic, basic).

Critical Considerations

- **Isotope purity:** High isotopic purity (>98%) is essential to avoid interference with natural abundance peaks
- **No endogenous presence:** Verify that labeled compounds are not naturally present in biological samples
- **Stability:** Ensure internal standards are stable throughout sample storage and preparation
- **Matrix-matched preparation:** Prepare IS stock solutions in the same solvent system as samples
- **Response factor:** Determine relative response factors between IS and targets for accurate quantification
- **Cost vs. coverage:** Balance the cost of SIL-IS with the need for accurate quantification in your application