Best Practices for the full LC MS Workflow

Samples – Preparation – Separation – Mass Spec Analysis – Informatics – Reporting

Best Practices for C18 Separations and Sample Preparation for Mass Spectrometry

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TIPS FOR SUCCESS IN LC-MS

- **Follow published protocols:** Use established protocols for your specific sample type and analysis method to ensure reliability and reproducibility.
- **Validate your methods:** Ensure the chosen methods are appropriate for your specific needs, confirming they meet the required performance criteria.
- **Document your procedures:** Keep detailed records of the sample preparation steps for reproducibility, including any modifications made during the process.
- **Use appropriate controls:** Include blank samples and standards to assess the quality of the data and to identify any potential biases or artifacts in the analysis.

BEST PRACTICES FOR C18 SEPARATIONS AND SAMPLE PREPARATION FOR MASS SPECTROMETRY

OUTLINE AND BASE CONTENT

- Introduction to C18 Separations
- Column Selection and Compatibility
- Mobile Phase Preparation
- Column Equilibration
- Sample Preparation
- During Separation
- Column Maintenance and Storage
- Troubleshooting
- Beyond the Basics
- Sample Preparation for Mass Spectrometry
- Conclusion

Overview

INTRODUCTION TO C18 SEPARATIONS

- C18 columns, also known as reversed-phase columns, are widely used in HPLC (High-Performance Liquid Chromatography) and are a cornerstone of analytical chemistry.
- Optimal performance and longevity of C18 columns depend on adhering to best practices for separations.
- Best practices ensure reliable results, reduce maintenance costs, and prolong the lifespan of the column.

INTRODUCTION TO C18 SEPARATIONS

Best Practices for C18 Separations

C18 columns, also known as reversed-phase columns, are widely used in HPLC (High-Performance Liquid Chromatography) and are a cornerstone of analytical chemistry. To ensure optimal performance and longevity, adhering to best practices for C18 separations is crucial.

Importance of C18 Columns

Widely used in HPLC

Cornerstone of analytical chemistry

Optimal Performance

Adhering to best practices

Ensures longevity of columns

COLUMN SELECTION AND COMPATIBILITY

- Choosing the Correct C18 Column
  - Consider the nature of analytes
  - Evaluate desired separation selectivity
  - Assess required resolution
  - Different C18 columns offer varying selectivity
- Checking Compatibility
  - Verify compatibility with solvents and mobile phases
  - Ensure pH range is within column's operating range
- Considering Column Size
  - Select appropriate column dimensions
  - Base selection on sample complexity
  - Consider desired resolution

CHOOSING THE CORRECT C18 COLUMN

- Nature of Analytes
  - Consider the chemical properties of the analytes
- Desired Separation Selectivity
  - Different C18 columns offer varying selectivities
- Required Resolution
  - Resolution needs may dictate column choice
- Types of C18 Columns
  - C18, C18-AQ, phenyl-hexyl
  - Each type has specific applications
- Verify Compatibility
  - Ensure chosen column is compatible with solvents
  - Check compatibility with mobile phases
- pH Range Verification
  - Ensure pH range of mobile phase is within column's operating range

CONSIDERING COLUMN SIZE

- Column Size Considerations
  - Choose dimensions based on sample complexity
  - Consider desired resolution for analysis

→ Select the appropriate column dimensions (length, inner diameter) based on the sample complexity and the desired resolution.

MOBILE PHASE PREPARATION

- Use high-quality solvents
  - Employ LCMS grade solvents and high-purity water
  - Minimize impurities affecting separation or column lifetime
- Filter mobile phases (LC Solvent Line and Bottle)
  - Use a 0.45 µm or smaller membrane
  - Remove particulate matter to prevent clogging or baseline noise
- Prepare fresh mobile phases
  - Prepare mobile phases within 24 hours
  - Prevent microbial contamination, especially with buffers
- Transition between mobile phases
  - Use a transition phase when switching compositions
- Consider buffer selection

MOBILE PHASE PREPARATION

Use High-Quality Solvents

Employ LC MS grade solvents and high-purity water to minimize impurities that can affect the separation or shorten the column's lifetime.

HPLC grade solvents
High-purity water

Inline Filter Mobile Phases

Filter mobile phases through a 0.45 µm or smaller membrane to remove particulate matter that can clog the column or cause baseline noise.

0.45 µm filters attached to lines solvent bottles.

Prepare Fresh Solutions

Ideally, prepare mobile phases for use within 24 hours to prevent microbial contamination, especially if buffers are included.

Freshly prepared mobile phase

Transition Between Phases

When switching between mobile phases, especially those with different compositions (e.g., water to organic solvent), use a transition phase to minimize column stress and potential damage.

Transition phase solutions
Column stress assessment

SOLVENT SELECTION NOTES

Volatile Buffers

- Use ammonium acetate or ammonium formate
- Minimizes ion suppression

Organic Solvents

- Choose based on sample's solubility
- Ensure compatibility with the instrument

LC-MS EXPERIMENT/WORKFLOW CONSIDERATIONS

OPTIMIZING LC AND MS SYSTEMS

LC System Operation & Optimization - General

- Choose a column that is compatible with the analytes and the mobile phase.
- Optimize the mobile phase composition (organic/aqueous ratio, pH) to achieve good separation and peak shape.
- Optimize the gradient profile to achieve optimal separation and minimize analysis time.
- Choose an appropriate flow rate to balance separation efficiency and analysis time.
- Maintain a constant column temperature to improve reproducibility and peak shape.

Dependent on the molecular system of interest and need.

Select Column Type

C18 Most Common RP
Other Reversed Phase
HILIC
Normal Phase
Size Exclusion

Agilent 1290 Infinity II Stack

Solvents - Buffers

Column Heater

Autosampler

Pumps

LC-MS EXPERIMENT/WORKFLOW CONSIDERATIONS

OPTIMIZING LC AND MS SYSTEMS – C18 COLUMNS

Select Column Type

C18 Most Common RP
Other Reversed Phase
HILIC
Normal Phase
Size Exclusion

Matching Column to solvent system and molecules of interest

C18 columns are a staple in reversed-phase liquid chromatography (HPLC), playing a crucial role in the separation of analytes. They come in various chemistries tailored for specific applications, which influence selectivity, retention, and compatibility with different analytes and mobile phases.

Dependent on the molecular system of interest and need.

The importance of selecting the correct C18 column based on specific analytical needs cannot be overstated. The various chemistries available provide diverse options for optimizing separations in HPLC. By understanding the different properties and characteristics of these columns, you can select the optimal C18 column for your specific analytical needs.

Base Silica C18

- Traditional C18 is the most basic type, where octadecyl (C18) chains are bonded to the silica gel surface. It's a good general-purpose column, offering a good balance of retention and selectivity for various analytes.

Polar-embedded C18

- Includes polar functionalities into the C18 phase. An example is the Acclaim Polar Advantage, enabling better retention and separation of polar analytes.

Hybrid Silica C18

- Utilizes a mixed organic-inorganic silica support, offering enhanced stability and efficiency. Known for wider pH range compatibility and durability, making them suitable for demanding applications.

Other C18 Variations

- Includes Fast C18 for faster separations using larger pore sizes, Wide pore C18 for analyzing larger biomolecules, AQ C18 for high-water-content mobile phases, and C18(2) for higher carbon load, increasing retention for nonpolar analytes.

LC-MS EXPERIMENT/WORKFLOW CONSIDERATIONS

OPTIMIZING LC AND MS SYSTEMS – C18 COLUMNS

Analyte Properties

Consider the polarity, size, and functional groups of the analytes to be separated.

Speed and Efficiency

Consider the need for faster separations or higher efficiency in your analytical processes.

Mobile Phase Compatibility

Select a C18 column compatible with the mobile phase used to ensure optimal performance.

Column Lifetime

Select a column with a durable and stable stationary phase to ensure longevity and consistent results.

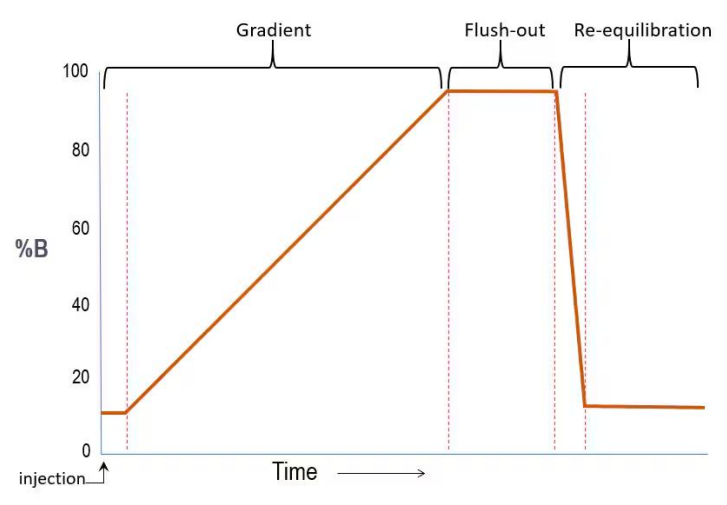
Resolution Requirements

Choose a column with appropriate selectivity to achieve the desired separation between analytes.

Overall Optimization

By understanding these factors, you can select the optimal C18 column for your specific analytical needs.

Example Gradient Delivery – % B is Organic Phase



Column Internal Diameter and Particle Size
Affect Flow Rate with LC and Source Conditions with LC

Column Internal Diameter (mm)	Particle Size (µm)	Typical Flow Rate (mL/min)
4.6	5	1.0
4.6	3	1.5
2.1	5	0.2 - 0.5
2.1	3	0.3 - 0.7
1.0	5	0.05 - 0.1
1.0	3	0.07 - 0.2

See Vendor Specific Websites for more information on pairing samples with methods and columns. There are large numbers of resources and standard-operating-procedures produced and available.

COLUMN EQUILIBRATION PROCESS

Purpose of Equilibration

Equilibration ensures the C18 column is saturated with the mobile phase before sample injection, crucial for consistent and reproducible separations.

Equilibrated column with desired mobile phase

Equilibration Time

Allow sufficient time for equilibration, typically 20 column volumes, to ensure the column is fully saturated with the mobile phase, impacting separation quality.

Time log of equilibration process

Equilibration Procedure

Flow the mobile phase through the column at the desired flow rate. Monitor the baseline signal until it stabilizes, indicating equilibrium.

Stabilized baseline signal

Post-Equilibration Checks

Verify the column's performance through test injections after equilibration. This confirms readiness for sample analysis and provides a reference point.

Test injections data for performance verification

DURING SEPARATIONS

- Monitor Pressure
  - Keep system pressure within acceptable limits
- Optimize Flow Rate
  - Adjust flow rate for desired separation within a reasonable timeframe
- Avoid Pressure Shocks
  - Prevent sudden changes in pressure or flow rate to protect the column
- Maintain Consistent Temperature
  - Avoid large temperature fluctuations to ensure proper separation and column integrity
- Optimize Gradient Conditions
  - Experiment with gradient profiles for optimal separation
- Avoid Overloading the Column
  - Ensure sample volume matches column capacity to prevent peak broadening or tailing

COLUMN MAINTENANCE AND STORAGE

- Clean the column regularly
  - Follow appropriate cleaning procedures
  - Remove contaminants to ensure optimal performance
- Avoid harsh cleaning solutions
  - Use mild cleaning solutions
  - Avoid solvents that can damage the column
- Flush the column regularly
  - Flush with appropriate solvents after each use
  - Prevent contamination and ensure proper storage
- Store the column properly
  - Store in a clean, dry environment
  - Use appropriate storage solutions

    (e.g., acetonitrile or methanol – 50% Organic/50% Aqueous)

TROUBLESHOOTING COMMON ISSUES

Solutions

- Optimize the mobile phase to reduce peak broadening or tailing by adjusting solvent ratios and pH levels.
- Investigate and remove potential sources of contamination to address ghost peaks effectively.
- Ensure the detector is functioning correctly and check for solvent or column issues to resolve baseline noise.

Common Issues

- Peak broadening or tailing can result from incorrect mobile phase composition or flow rate settings.
- Ghost peaks may arise from contamination in the system or improper sample handling.
- Baseline noise often indicates problems with the detector or the mobile phase quality.

BEYOND THE BASICS

- Optimize Column Temperature
  - Impact of temperature on separation
  - Benefits of using a column oven
- Explore Different Column Chemistries
  - Using different C18 chemistries
  - Other stationary phases for enhanced selectivity
  - Improved resolution
- Utilize Automated Column Selection Tools
  - Software tools for optimal column selection
  - Specific application considerations

KEY CONSIDERATIONS

- Purity and Concentration
  - Remove impurities
  - Ensure appropriate analyte concentration for instrument sensitivity
- Solvent Compatibility
  - Choose solvents compatible with ionization method
  - Ensure compatibility with mass spectrometer
- Minimize Interfering Substances
  - Remove salts and detergents
  - Avoid compounds that suppress ionization or cause background noise

TIPS FOR SUCCESS

- **Consult with experts**
  - Seek advice from experienced mass spectrometry users if needed
- **Follow published protocols**
  - Use established protocols for your specific sample type and analysis method
- **Validate your methods**
  - Ensure the chosen methods are appropriate for your specific needs
- **Document your procedures**
  - Keep detailed records of the sample preparation steps for reproducibility
- **Use appropriate controls**
  - Include blank samples and standards to assess the quality of the data

AVOIDING CONTAMINATION

- Avoid Contamination
  - Use clean glassware and reagents
  - Prevent contamination during sample preparation
- Optimize for Specific Analysis
  - Tailor the method to the type of analysis
  - Examples include peptide sequencing, protein quantification, and lipid profiling
- Ensure Data Quality
  - Avoid introducing bias or artifacts
  - Maintain integrity of the final data

Sample Preparation - Basics

KEY CONSIDERATIONS IN SAMPLE PREPARATION

Purity and Concentration	Remove impurities and ensure the analyte is at an appropriate concentration for the instrument's sensitivity.
Solvent Compatibility	Choose solvents compatible with the ionization method and mass spectrometer.
Minimize Interfering Substances	Remove salts, detergents, and other compounds that can suppress ionization or cause background noise.
Sample Preparation Methods	Protein Sample Preparation involves cell lysis, protein extraction, digestion, and purification steps.

SAMPLE PREPARATION FOR MASS SPECTROMETRY

- Purity and Concentration
  - Remove impurities and ensure appropriate analyte concentration
- Solvent Compatibility
  - Choose solvents compatible with ionization method and mass spectrometer
- Minimize Interfering Substances
  - Remove salts, detergents, and other compounds that can suppress ionization
- Sample Preparation Methods
  - Protein: Cell lysis, protein extraction, digestion, and purification
- Solvent Selection
- Avoid Contamination
- Optimize for Specific Analysis
- Data Quality

SPECIFIC EXAMPLES OF SAMPLE PREPARATION - BASICS

Protein Sample Preparation

- Lysate preparation involves breaking open cells to release proteins.
- Protein extraction is followed by digestion to break proteins into peptides.
- Peptide purification is crucial to isolate peptides for analysis.

Lipid Sample Preparation

- Extraction and purification techniques are crucial for lipid analysis.
- Specific methods may include solvent extraction or solid-phase extraction.
- Maintaining sample integrity is essential for accurate lipid profiling.

WEB AND OTHER RESOURCES FOR EXPANDED INFORMATION

https://www.alifesci.com/applications

https://www.agilent.com/en/product/small-molecule-columns/reversed-phase-hplc-columns

https://www.waters.com/waters/en_US/HPLC-Columns/nav.htm?cid=511505&srsltid=AfmBOopgz0XOh2pVCCKUelOzvZcrCBapWaE2cAdsdNN6MciKdw3oBGIv

https://www.phenomenex.com/techniques/hplc-reversed-phase

https://phenomenex.blog/2017/10/05/stationary-phase/

https://www.thermofisher.com/us/en/home/industrial/chromatography/chromatography-learning-center/liquid-chromatography-information/hplc-system-components/how-hplc-columns-work.html?erpType=Global_E1

https://www.chromatographyonline.com/view/hplc-particle-a-look-at-stationary-phase-chemistry-synthesis