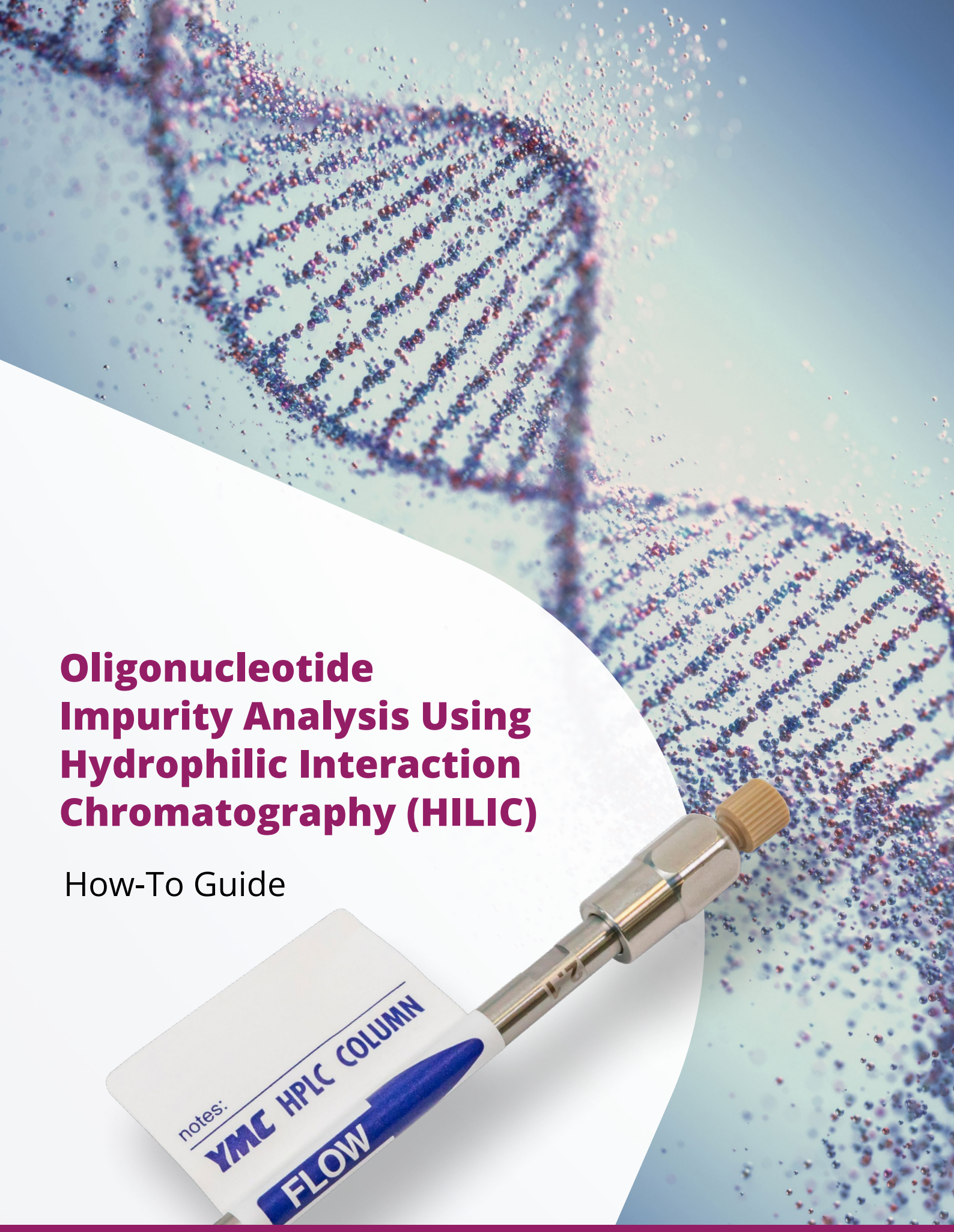
# Oligonucleotide Impurity Analysis Using Hydrophilic Interaction Chromatography (HILIC)

How-To Guide

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**Cover image:** iStock_ktsimage

# Introduction

Oligonucleotides are synthetic DNA/RNA strands used in research and therapeutics, including PCR, gene editing, and antisense therapies. They are synthesized via solid-phase phosphoramidite chemistry, where nucleotides are sequentially added in a 3′ → 5′ direction. However, synthesis errors (e.g., incomplete coupling), side reactions, or degradation generate impurities such as **shortmers** (n–x deletions), **longmers** (n+x additions), and modified full-length products. These impurities can compromise therapeutic efficacy or diagnostic accuracy, necessitating robust analytical methods for characterization. Traditional techniques like ion-pairing reversed phase chromatography (IP-RP) and anion exchange chromatography (AEX) face limitations in sensitivity, selectivity, and MS compatibility. Hydrophilic interaction chromatography (HILIC) has emerged as a powerful orthogonal method, enabling high-resolution separation and mass spectrometry (MS)-compatible impurity profiling.

# Overview of Chromatographic Techniques

| TECHNIQUE | PRINCIPLE | STRENGTHS | LIMITATIONS |
|---|---|---|---|
| **IP-RP** | Separates based on hydrophobic interactions with ion-pairing agents (e.g., triethylamine-HFIP). | High resolution for small impurities. | Ion suppression in MS; corrosive mobile phases. |
| **AEX** | Separates via charge differences under high-pH conditions. | Orthogonal to IP-RP. | Usually incompatible with MS; requires desalting. |
| **HILIC** | Utilises hydrophilic partitioning with polar stationary phases. | MS-compatible; retains polar impurities. | Requires optimization of mobile phase/ column chemistry. |

# Oligonucleotide Characterization Using HILIC

## Step 1: HILIC Column Selection

Key steps and considerations for selecting and using a stationary phase for Hydrophilic Interaction Liquid Chromatography (HILIC) analysis of oligonucleotides.


### 1 Hardware:

- Use **bioinert columns** (e.g., bioinert-coated) to minimize adsorption.
- For non-bioinert columns: Pre-condition with 10–25 sample injections to passivate surfaces.

### 2 Stationary Phase Chemistry:

- **Amide phases:** Well-suited for most ONs (modified/unmodified, siRNA, diastereomers).
- **Diol phases:** Complement amide phases and may also be effective for oligonucleotides, providing partial diastereomer separation.
- **Zwitterionic phases:** Suitable for unmodified ONs but require high salt concentrations.

### 3 Dimensions & Particle Size:

- **Diameter:** 2.1 mm (standard).
- **Length:** 50 mm (fast analysis), 100 mm (high resolution), 150 mm (impurity separation).
- **Particles:** <2 µm for UHPLC; 2.7–5 µm for HPLC.

## 4 Pore Size:

- **Short ONs (<30-mer):** 100–130 Å.
- **Long ONs (>30-mer):** 300 Å or larger.

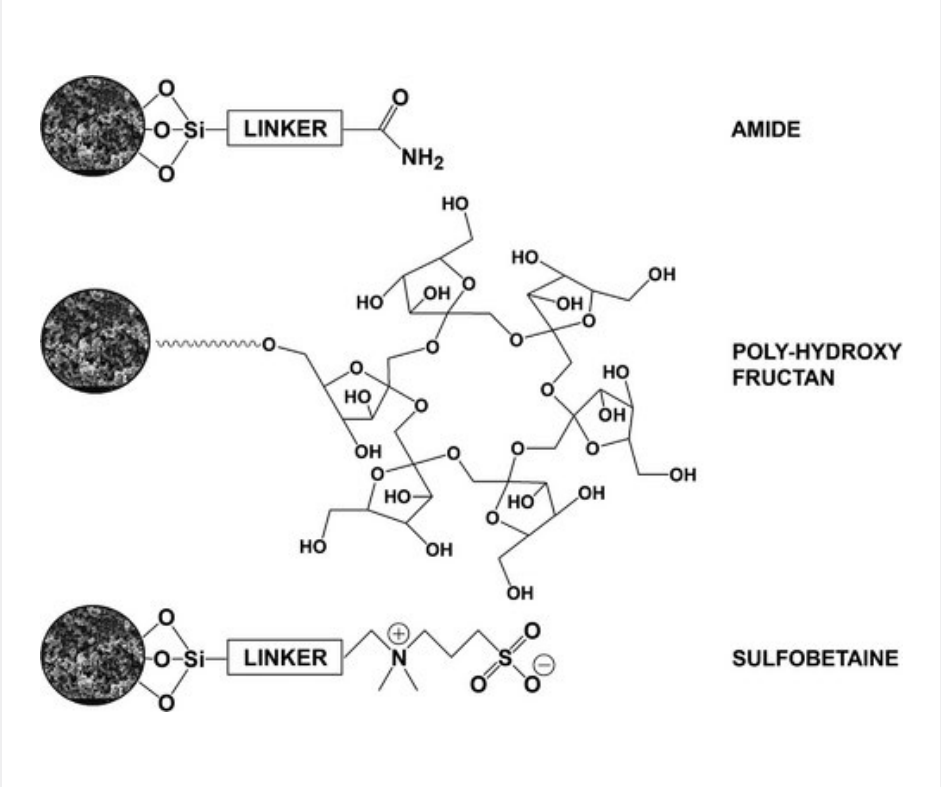


**Figure 1. Schematic structures of stationary phases successfully used for the analysis of oligonucleotides in HILIC.**

**Reference:** Guillarme, D., Fekete, S. and Studzińska, S. (2025), Protocol for Oligonucleotides Characterization Using Hydrophilic Interaction Chromatography. *Journal of Separation Science*. DOI: 10.1002/jssc.70088

# Step 2: Mobile Phase Optimization

## 1 Select Salt Type and Concentration

**Primary choice: 50 mM ammonium acetate** (pH ~6.9).

- Balances chromatographic performance, MS compatibility, and robustness.
- Avoid concentrations <40 mM to prevent retention variability or breakthrough effects.

**Alternatives:**

- Ammonium formate (pH ~6.4) for similar results.
- Ammonium bicarbonate (pH ~7.8) for slightly better resolution of long ONs (e.g., ssDNA ladders).

**Phosphorothioate ONs:** Use ≤50 mM salt to suppress partial diastereomer separation.

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## 2 Adjust Mobile Phase pH

**Optimal range: pH 5–7** to minimize nonspecific adsorption.

- Avoid acidic pH (<6) to prevent electrostatic interactions with metal surfaces.
- For silica columns: Stick to pH 6–7 to avoid column degradation.
- For hybrid silica columns: Higher pH (up to 9) can reduce adsorption but test column stability.

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## 3 Choose Organic Modifier

**Primary solvent: Acetonitrile (ACN).**

- Ensures hydrophilic partitioning and sharp peaks.

**Ternary mixtures:** Add **5–25% methanol** to ACN/water to fine-tune selectivity (e.g., separate ON ladders).

- Note: Methanol may broaden peaks but improves resolution for complex mixtures.

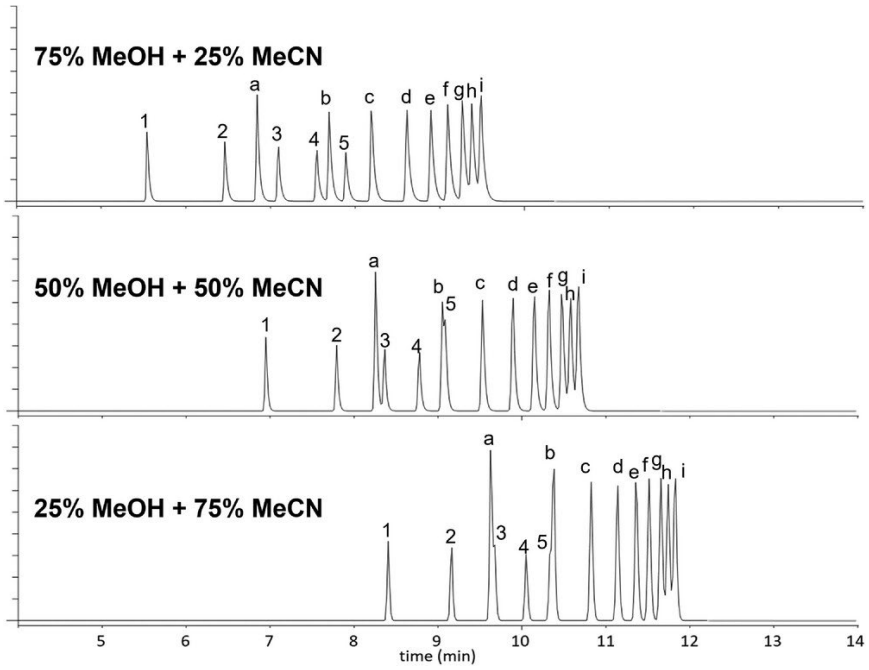


**Figure 2. Comparison of different ternary mobile phase compositions (methanol in acetonitrile).**

**Reference:** Guillarme, D., Fekete, S. and Studzińska, S. (2025), Protocol for Oligonucleotides Characterization Using Hydrophilic Interaction Chromatography. *Journal of Separation Science*. DOI: 10.1002/jssc.70088

## 4 Set Temperature

**Default: 40°C** (balances retention, selectivity, and column stability).

**Adjust based on sample:**

- **Double-stranded ONs (e.g., siRNA):** Set temperature above/below melting point to analyze duplexes or single strands.
- **Diastereomers:** Use <25°C to enhance separation or >60°C to suppress it.
- **High-temperature stability:** Use hybrid silica columns for temperatures up to 90°C.

## 5 Optimize Flow Rate

**Standard: 300–400 µL/min** for 2.1 mm columns.

- Lower flow rates (e.g., 200 µL/min) improve resolution but increase run time.
- Ensure consistent flow/pressure for method robustness (retention decreases with higher pressure in HILIC).

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## 6 Method Validation & Troubleshooting

**Peak broadening:**

- Increase salt concentration (up to 100 mM).
- Reduce methanol content in ternary mixtures.

**Retention variability:**

- Ensure consistent pH (5–7) and salt concentration.
- Use plastic solvent bottles.

**Low resolution:**

- Adjust temperature (see **Step 4**).
- Test ternary mobile phases (ACN/MeOH/water).

### Summary Table: Recommended Mobile Phase Conditions

| PARAMETER | RECOMMENDATION |
|---|---|
| **Salt** | 50 mM ammonium acetate (pH 6.9) |
| **Organic Modifier** | ACN (primary), ≤25% methanol (selectivity tweak) |
| **Temperature** | 40°C (adjust for duplexes/diastereomers) |
| **Flow Rate** | 300–400 µL/min (2.1 mm columns) |
| **Containers** | Plastic (PFA) |

# Step 3: Gradient Optimization

## 1 Start with a Generic Gradient

**Initial conditions:**

- **Mobile Phase A:** Acetonitrile (ACN)
- **Mobile Phase B:** Aqueous buffer (e.g., 50 mM ammonium acetate, pH 6.9)
- **Gradient range:** 70% ACN (initial) to 10% ACN (final).

**Gradient time:**

- **Standard:** 10 minutes for a 50 × 2.1 mm column at 400 µL/min.
- Adjust proportionally for longer columns (e.g., 20 minutes for a 100 mm column).

## 2 Refine Gradient Parameters

**Adjust initial/final ACN %:**

- **Early eluters:** Start with lower ACN (e.g., 60% instead of 70%).
- **Late eluters:** End with lower ACN (e.g., 5% instead of 10%).

**Extend gradient time:**

- For complex mixtures (e.g., ONs with closely related impurities), increase gradient time to improve resolution (e.g., 15–25 minutes).

**Test gradient shape:**

- **Linear gradients:** Simple and effective for most ONs (Figure 5A,B).
- **Concave gradients:** Improve separation of homologs (e.g., ONs and shorter impurities) for uniform peak distribution (Figure 5C).

## 3 Optimize Column Dimensions

- **Short columns (50 mm):** Fast analysis (5–10 minutes) but lower resolution.
- **Long columns (100–150 mm):** Higher resolution for complex samples (e.g., diastereomers, impurities).

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## 4 Consider Premixed vs. Neat Mobile Phases

- **Premixed phases:**
  - Pros: Reduce baseline noise, heat effects, and precipitation risks.
  - Use case: LC-UV methods.
- **Neat phases (ACN + aqueous buffer):**
  - Pros: Minimize leachable impurities for LC-MS compatibility.
  - Cons: Slightly lower robustness due to evaporation effects.

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## 5 Set Re-Equilibration Time

- **Minimum:** 15 column volumes (e.g., ~5 minutes for a 50 × 2.1 mm column at 400 µL/min).
- **Reduce time:**
  - Start gradients with ≥20% water.
  - Increase temperature (e.g., 50°C).

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## 6 Troubleshoot Common Issues

**Poor resolution:**

- Reduce gradient steepness (e.g., 1% ACN/min instead of 6%).
- Switch to a concave gradient.

**Long run times:**

- Shorten column length or increase flow rate (ensure pressure limits are respected).

**Irreproducible retention:**

- Extend re-equilibration time.
- Use plastic solvent containers (PFA) instead of glass.

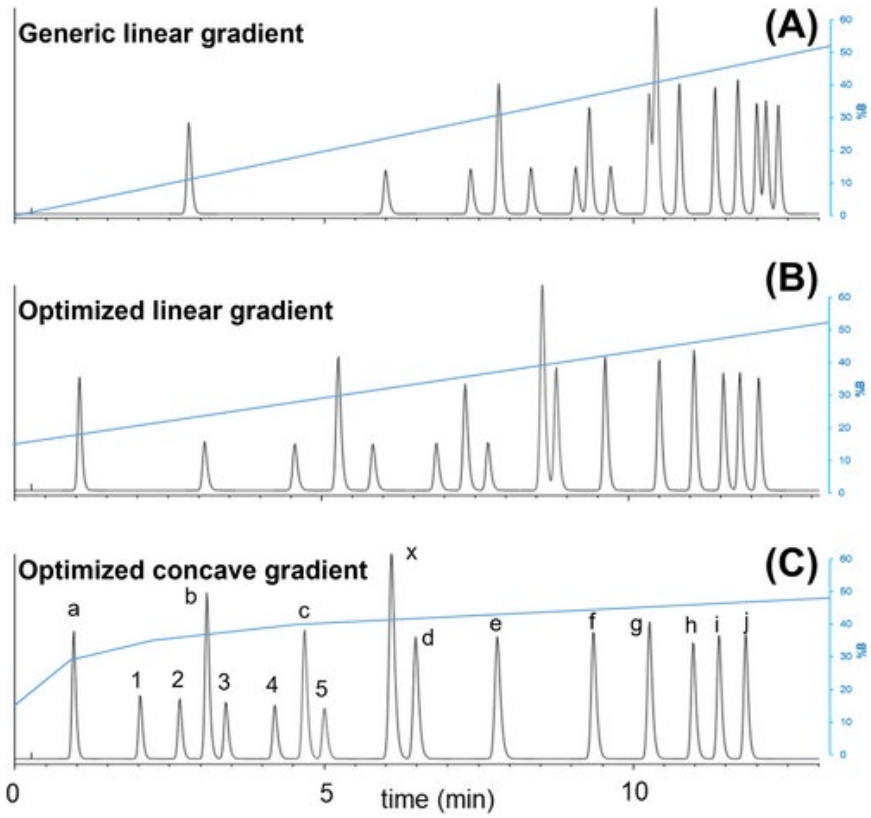


**Figure 3. Comparison of various gradient conditions.**

**Reference:** Guillarme, D., Fekete, S. and Studzińska, S. (2025), Protocol for Oligonucleotides Characterization Using Hydrophilic Interaction Chromatography. *Journal of Separation Science*. DOI: 10.1002/jssc.70088

# Step 4: Coupling with MS

## 1 Ionization Setup: Electrospray Ionization (ESI) in Negative Mode

**Preferred ion source:** Use ESI(−) for ONs due to their polyanionic nature.

**Charge states:**

- Expect **–3 to –5 charges** for 15–25-mer ONs.
- Fewer charge states compared to IP-RPLC, simplifying spectra.

**Advantages over IP-RPLC:**

- No ion-pair reagents (e.g., alkylamines), reducing MS contamination and maintenance.
- Mobile phases are stable for days (vs. hours for IP-RPLC).

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## 2 Mobile Phase Optimization for MS

**Salt Selection:**

- **Primary choice:** 50 mM ammonium acetate (pH 6.8–6.9).
- **Alternatives:**
  - **Ammonium formate:** Similar performance to acetate.
  - **Ammonium bicarbonate:** Potential for higher sensitivity (untested in HILIC).
- **Avoid low salt concentrations** (<10 mM) to prevent peak broadening.

**Organic Modifier:**

- Use **acetonitrile (ACN)** as the primary solvent.
- Avoid methanol-heavy mixtures (causes peak broadening).

**pH Considerations:**

- Maintain **pH 6–7** to balance sensitivity and reduce adducts.
- Slightly alkaline pH improves ionization but risks column stability.

## 3 Addressing Sensitivity Challenges

**Reduce Adducts:**

- **Use plastic containers** (PFA) instead of glass to minimize sodium/potassium leaching.
- **Desalting steps:** Include offline purification if adducts persist.

**Flow Rate Adjustments:**

- **Standard:** 300–400 µL/min for 2.1 mm columns.
- **For nano-ESI:** Lower flow rates (nanoflow) can triple sensitivity but require specialized equipment.

**Salt Concentration Trade-Off:**

- Higher salt (≥50 mM) improves resolution but suppresses signals.
- Lower salt (10–40 mM) boosts sensitivity at the cost of peak shape.

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## 4 MS Parameter Recommendations

**General Settings:**

- **Mass range:** 400–3000 Da (covers most multiply charged ONs).
- **Capillary voltage:** ~4000 V (adjust based on instrument).
- **Gas settings:**
  - Drying gas: 10–12 L/min at 350°C.
  - Nebulizer pressure: 20–30 psi.
- **Fragmentor voltage:** 150–200 V (balance between sensitivity and fragmentation).

**Instrument-Specific Tips:**

- Regularly clean ion sources to offset residual salt buildup.
- Optimize declustering voltages to reduce in-source fragmentation.

# Summary and Outlook

HILIC enhances oligonucleotide impurity analysis by enabling MS-compatible separations, resolving polar impurities, and simplifying 2D-LC workflows. Its synergy with HRAM-MS allows precise identification of low-abundance species (≤0.3%). Future advancements may focus on **automated column-switching systems** and **AI-driven impurity prediction** to reduce method development time. Emerging techniques like supercritical fluid chromatography (SFC) show promise for impurity separation but require further validation. As oligonucleotide therapeutics advance, robust HILIC-MS workflows will remain critical for ensuring product safety and regulatory compliance.

# Further Reading & Resources

1. HILIC analysis of oligonucleotides using bioinert columns
2. When HILIC is a suitable alternative to IP-RP for the analysis of oligonucleotides
3. Determination of optimum method parameters for the analysis of oligonucleotides via HILIC



## Imprint

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