Synthetic Oligonucleotide Impurity Analysis: Enhancing the Conventional Single Quad Method Using UPLC-ToF-MS

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Waters Corporation

USP Workshop
04-09-2024
Overview of the Talk

- Benefit of MaxPeak™ Surface Technology
- Benefit of HRMS
- Informatics Tool (Three Apps)
  - Characterization (Intact Mass + CONFIRM Sequence)
  - Impurity Profiling using HRMS + Customized data processing (UNIFI)
Tools for Oligonucleotide Analysis – Waters Corporation

**IPRP-UPLC-UV-MS**

Xevo G3 QTOF MS

BioAccord (TOF MS)

OST Premier 2.1 x 50 mm, 130 Å, 300 Å column

**Software Tools**

- Intact Mass (Classic Workflow)
- Peptide Mapping
- Released Glycans
- Accurate Mass Screening

- UNIFI App: Impurity Screening
- INTACT Mass App: Accurate Mass Measurement using MS 1
- CONFIRM Sequence App: Oligo fragmentation data annotation

✓ Integrated compliance-ready data acquisition, processing and reporting
✓ A shared ecosystem that enables data traceability and transferability

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Some LC Separations Are More Challenging Than Others

Biomolecular analytes prone to metal interactions (NSA):  
Acidic & Phosphorylated Peptides  
Nucleotides  
**Oligonucleotides**  
Sialylated glycans  
Glycoproteins, et.al.

![Phosphodiester Backbone](Photo Credit: Sponk (talk)/Wikimedia Commons)
Too Many Impurities

- Deletion/Extension
- Truncation
- Modifications on FLP
Benefit of the MaxPeak™ Surface Technology

High Performance Surfaces that mitigate unwanted surface interactions and adsorption

On conventional LC systems, metal sensitive analytes are adsorbed on to metal surfaces

Waters™ MaxPeak High Performance Surface is designed to minimize metal-analyte interactions

HPS is hybrid organic-inorganic silica

Anal. Chem. 2021, 93, 14, 5773–5781
**Sample:** MassPREP™ Oligonucleotide Standard

**Conventional BEH™ C18 Column**

<table>
<thead>
<tr>
<th>Min</th>
<th>AU 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.000</td>
</tr>
<tr>
<td>6.0</td>
<td>0.012</td>
</tr>
<tr>
<td>12.0</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Inj. 1

*conditioned*

<table>
<thead>
<tr>
<th>Min</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
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</thead>
<tbody>
<tr>
<td>AU 260 nm</td>
<td>0.000</td>
<td>0.006</td>
<td>0.012</td>
<td>0.006</td>
<td>0.000</td>
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</tbody>
</table>

Inj. 2

**ACQUITY PREMIER BEH C18 Column**

<table>
<thead>
<tr>
<th>Min</th>
<th>AU 260 nm</th>
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<tbody>
<tr>
<td>0.0</td>
<td>0.000</td>
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<tr>
<td>6.0</td>
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</tr>
<tr>
<td>12.0</td>
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<td>0.006</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Inj. 2

*conditioned*

Injection of 2 µL of OST standard, 10 pmol of each oligonucleotide on column, m.p. 25 mM HAA, pH 6

*Conditioning: 500 pmol injection of 35 mer followed by “post conditioning” injection of 10 pmol of standard
Impurities Observed from Regular S.S. Column

- Blank
- FLP + Impurities

Stainless steel OST column, 2.1 x 100 mm

MaxPeak OST Column Recovers More Impurities

MaxPeak OST column, 2.1 x 100 mm

- Blank
- FLP + Impurities

*G*U*A*G*G*U*A*U*U*C*C*A*UTT – 21-mer

11-mer
12-mer
14-mer
15-mer
16-mer
11 Deamination
### Benefit of HRMS

<table>
<thead>
<tr>
<th>Low Resolution MS</th>
<th>High Resolution MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal mass resolution</td>
<td>Isotope resolved</td>
</tr>
<tr>
<td>Difficulty measuring charges ((z))</td>
<td>Can measure multiple charges ((z))</td>
</tr>
<tr>
<td>Good for some targeted analysis</td>
<td>Targeted and untargeted (determines unknowns)</td>
</tr>
<tr>
<td>Requires good chromatographic separation</td>
<td>Fragmentation for structure elucidation</td>
</tr>
</tbody>
</table>
HRMS for Increasing Structural Complexity?

*Nominal mass* MS can not difference them

**Nusinersen FLP:** 2′-O-MOE, PS modified RNA 18-mer


Exact mass = 7122.2763

<table>
<thead>
<tr>
<th>Impurities</th>
<th>Exact Mass (Da)</th>
<th>m/z (-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n - u</td>
<td>6728.2163</td>
<td>1681.0468</td>
</tr>
<tr>
<td>n - c</td>
<td>6729.2003</td>
<td>1681.2928</td>
</tr>
</tbody>
</table>

Ref: In-Depth Impurity Assessment of Synthetic Oligonucleotides Enabled by HRMS (fda.gov)
HRMS Can Measure Impurities with Small Mass Change from FLP


Example: Deamination: + 0.98 Da

5-methyl-cytosine  →  Thymidine

Full length product (FLP)

Deaminated Full length product (FLP) – Peak # 11

[M-3H]^3- charge state
Analytical Strategy for Synthetic Oligonucleotide Impurity Profiling

Informatics Workflow to Support the LC-UV-MS Analytical Strategy

**Oligo Characterizations**

**INTACT Mass App**
- Automated charge deconvolution for accurate mass measurement
- Targeted or untargeted data process
- LC-UV and LC-MS quantitation

**CONFIRM Sequence App**
- Build sequence in scientific library
- Structure elucidation using MS fragment ions
- Sequence coverage viewer
SYNTHETIC Library is Used to Construct Oligo Sequence

INTACT Mass App Data Processing Results


<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Component</th>
<th>Observed mass (Da)</th>
<th>Expected mass (Da)</th>
<th>Mass error (ppm)</th>
<th>Identity result</th>
<th>Observed TIC RT (min)</th>
<th>Observed UV RT (min)</th>
<th>LC area</th>
<th>LC amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D1423 n-OMe[5] &amp; n-OMe5Mec[2] &amp; n-OMe5MMeU</td>
<td>3,593,702</td>
<td>3,593,707</td>
<td>1.5</td>
<td>Pass</td>
<td>4.74</td>
<td>4.69</td>
<td>1,222</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>D1423 n-OMe[4] &amp; n-OMe5Mec[2] &amp; n-OMe2MMeU</td>
<td>3,936,762</td>
<td>3,936,775</td>
<td>-0.4</td>
<td>Pass</td>
<td>7.37</td>
<td>7.34</td>
<td>2,737</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>D1423 n-OMe[2] &amp; n-OMe5Mec[2] &amp; n-OMe5MMeU</td>
<td>4,981,988</td>
<td>4,981,975</td>
<td>2.7</td>
<td>Pass</td>
<td>12.88</td>
<td>12.83</td>
<td>5,276</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>D1423 n-OMe[2] &amp; n-OMe5Mec[2] &amp; n-OMe5MMeU</td>
<td>5,315,046</td>
<td>5,315,048</td>
<td>-0.3</td>
<td>Pass</td>
<td>13.80</td>
<td>13.77</td>
<td>4,704</td>
<td>0.8</td>
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<tr>
<td>6</td>
<td>D1423 n-OMe[2] &amp; n-OMe5Mec &amp; n-OMe5MMeU</td>
<td>5,991,181</td>
<td>5,991,188</td>
<td>-1.2</td>
<td>Pass</td>
<td>17.76</td>
<td>17.72</td>
<td>7,942</td>
<td>1.4</td>
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<tr>
<td>7</td>
<td>D1423 n-OMe[2] &amp; n-OMe5Mec &amp; n-OMe5MMeU</td>
<td>6,684,310</td>
<td>6,684,308</td>
<td>0.3</td>
<td>Pass</td>
<td>19.59</td>
<td>19.55</td>
<td>13,473</td>
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<tr>
<td>8</td>
<td>D1423 n-OMe5MMeU</td>
<td>6,693,332</td>
<td>6,693,320</td>
<td>1.9</td>
<td>Pass</td>
<td>20.13</td>
<td>20.08</td>
<td>13,283</td>
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<tr>
<td>9</td>
<td>D1423 n-OMe5MMeC</td>
<td>6,694,315</td>
<td>6,694,304</td>
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<td>20.39</td>
<td>20.34</td>
<td>26,001</td>
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<tr>
<td>10</td>
<td>D1423 n-OMe5MMeC</td>
<td>7,008,354</td>
<td>7,008,334</td>
<td>2.9</td>
<td>Pass</td>
<td>20.13</td>
<td>20.08</td>
<td>13,283</td>
<td>2.3</td>
</tr>
<tr>
<td>11</td>
<td>D1423 Deamination</td>
<td>7,027,390</td>
<td>7,027,376</td>
<td>2.2</td>
<td>Pass</td>
<td>21.22</td>
<td>21.19</td>
<td>469,430</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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Sequence coverage of the 21-mer oligo analyzed using (DIA) Acquisition

Results: Oligo D423

Sequence coverage: 100.00% (1/1 spectra selected)

<table>
<thead>
<tr>
<th>Spectrum</th>
<th>Retention time window (min)</th>
<th>Acquisition type</th>
<th>Precursor observed mass (Da)</th>
<th>Charge state</th>
<th>% Precursor</th>
<th>% Coverage</th>
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<tbody>
<tr>
<td>21-mer oligo MSK optimization April 14th 2022 041422 YEA615 CD D1423digo M5E CE ramp 40 to 60V 3 TOF M5e 600-5000 40-60V CE</td>
<td>28.75 - 29.25</td>
<td>M5e</td>
<td>7.027,3574</td>
<td>2+ 3+ 4+ 5+</td>
<td>81.74</td>
<td>100.00</td>
</tr>
</tbody>
</table>

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Sequence coverage for an 11-mer impurity (m/z=1197.0, [M-3H]⁻³ precursor)
Customized Workflow for Impurity Profiling - Method Transfer from Single Q to TOF MS

**Targeted Workflow**

- Detects and quantifies named impurities using extracted ion chromatogram (XIC) of mass data
- Quantifies unknown impurities chromatographically resolved from the FLP using integrated area of UV data
- Uses Quantify Assay ToF 2D Chromatographic Workflow

**Untargeted Workflow**

- Identifies unknown impurities coeluting with the FLP peak using m/z information
- Quantifies unknown impurities using extracted ion chromatogram (XIC) of mass data
- Uses Accurate Mass Screening Workflow
Targeted Workflow Automated Data Processing Steps

Steps for analysis of known impurities and impurities chromatographically resolved from FLP

1. Manual Adjustment
2. SST and Calibration Curves
3. Integration of UV data
4. Integration of XICs

Application Note: 720008206
Untargeted Workflow Automated Data Processing Steps

Steps for analysis of unknown impurities coeluting with FLP

1. Identity Test
2. Sodium Adduct Test
3. Harsh vs Soft
4. XIC of Unknown Impurities
Report Generation

*Built-In Report Templates Enable Rapid Interrogation of Data*
Nusinersen is an 18-mer antisense oligonucleotide used to treat spinal muscular atrophy.

The exact mass is 7122.2763 Dalton.

Sequence:

U-*C-A-*C-*U-*U-*U-*C-A-*U-A-A-*U-G-*C-*U-G-G

(methylation on “C” and “U”)

Data Package Available: Nusinersen

Build your own method based on this data package.
Summary

MaxPeak HPS Technology
Greater consistency and repeatability, enhanced sensitivity and dynamic range, less passivation/conditioning time and cost

HRMS and Informatics Solutions
Improved sensitivity and mass resolution to address challenges analyzing increasingly complex oligonucleotides

Oligonucleotide Impurity Analysis Workflow Package
Semi-automated data analysis workflow package to streamline data analysis, reducing time, required training, and risk of error