

Comprehensive impurity profiling of synthetic oligonucleotides by high-resolution mass spectrometry intact mass data processing

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U.S. Food and Drug Administration

ASMS Conference on Mass Spectrometry and Allied Topics

June 7, 2023; Houston, Texas

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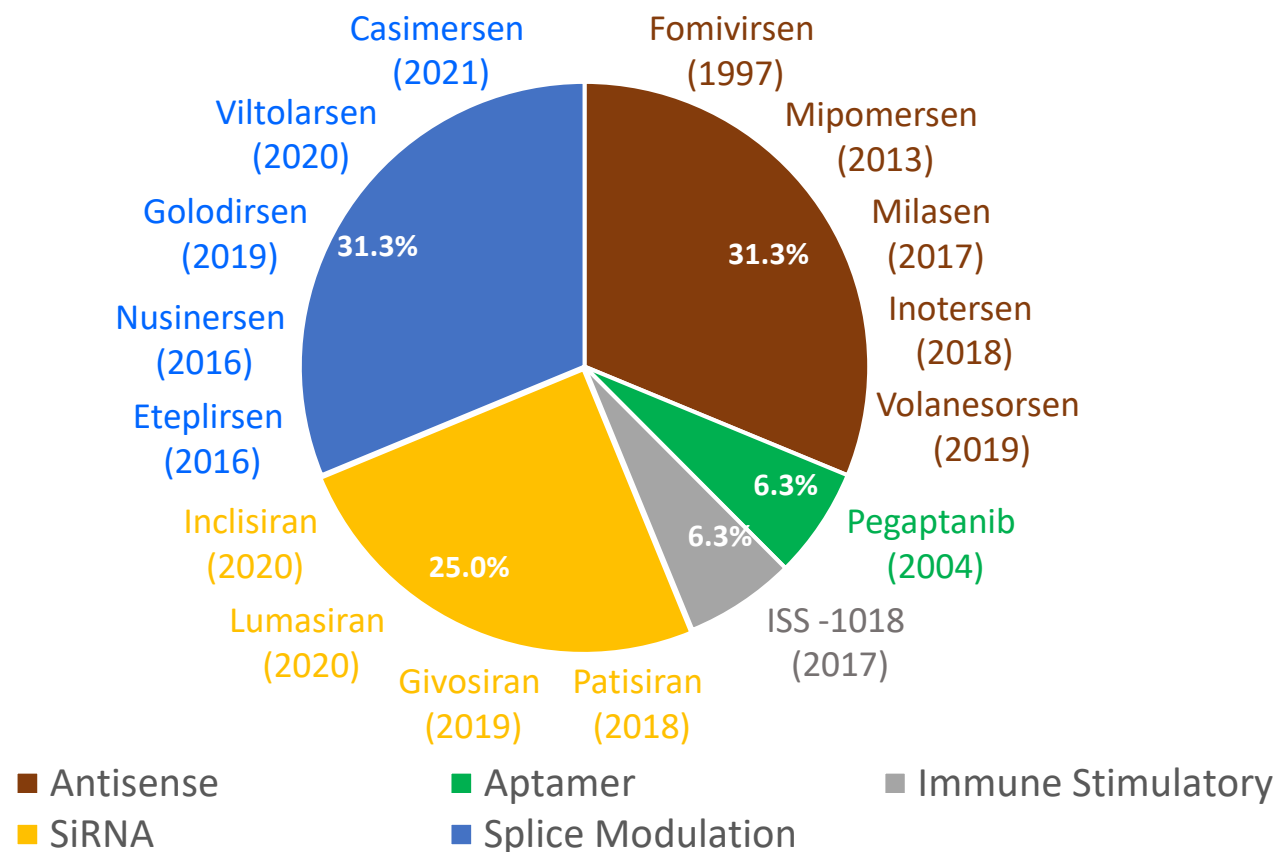
A close-up photograph of a person's hands. The left hand holds an orange plastic pill bottle, tilted to pour three white, oval-shaped capsules into the palm of the right hand. The background is blurred, focusing attention on the medication.

Pharmaceutical quality is
assuring *every* dose is safe and
effective, free of contamination
and defects.

- Introduction
- BioPharma Finder software
 - Intact Mass Analysis workflow
- Intact Mass Analysis Parameter Optimization
 - Sequence Manager
 - Source Spectra
 - Sliding Windows Settings
 - Deconvolution Isotope Table
- Case study
 - Impurity analysis of different batches
- Conclusion

Introduction

○ Synthetic Oligonucleotide Therapeutics (ONTs)

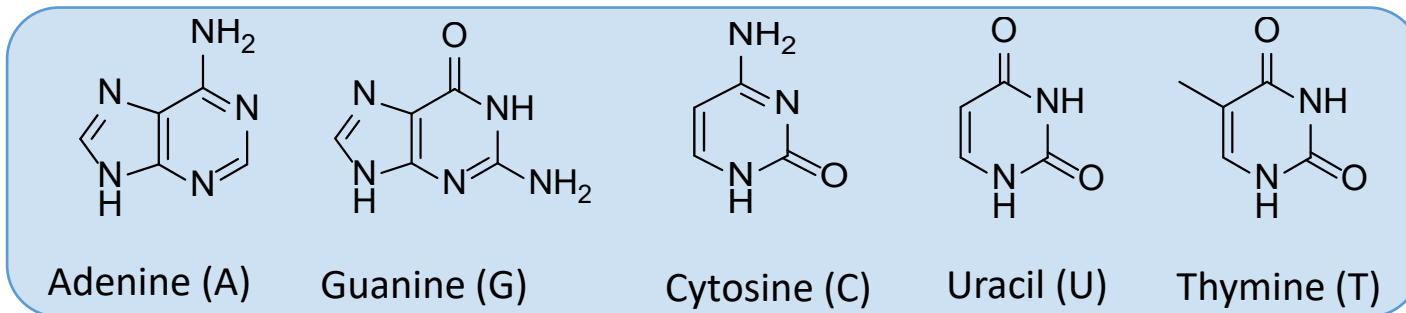
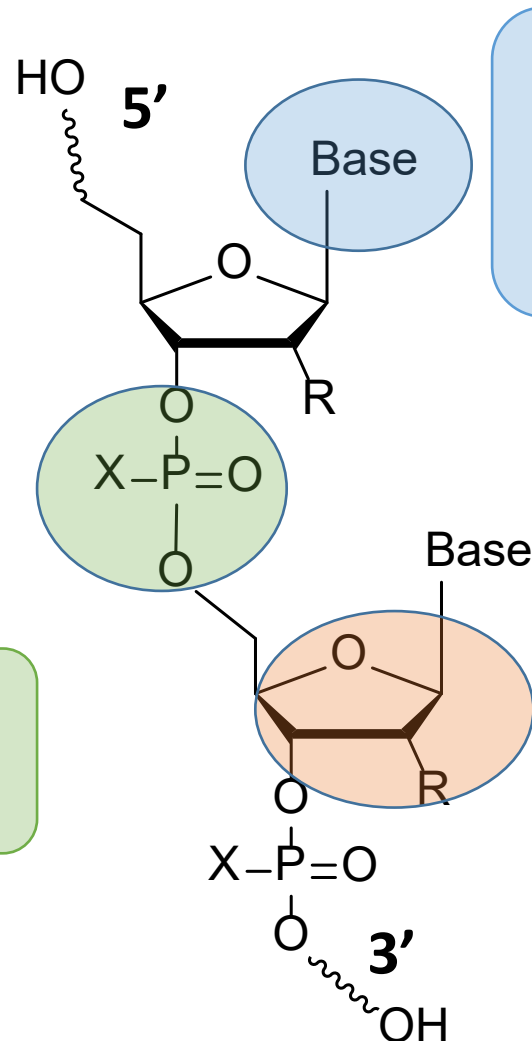


- Potential to treat a broad spectrum of indications from rare/life-threatening to common diseases
- Impurity profiling is critical for ensuring quality, efficacy and safety of complex ONT drugs

T. Rupp and H. Cramer. CMC and Regulatory Aspects of Oligonucleotide Therapeutics. RNA Therapeutics: The Evolving Landscape of RNA Therapeutics, Elsevier, 2022, 263-320

Introduction

○ Synthetic Oligonucleotide Therapeutics (ONTs)



X = O⁻ Phosphodiester
S⁻ Phosphorothioate
CH₃ Methylphosphonate

R = OCH₃ 2'-O-methyl
OCH₂CH₃ 2'-O-ethyl
OCH₂CH₂OCH₃ 2'-O-methoxyethyl (MOE)
OCH₂CH₂OC₂H₅ 2'-O-ethoxyethyl (EOE)
OCH₂CH=CH₂ 2'-O-allyl

- Product-related impurities of ONTs

- Deletion / addition sequences

n-1, n-2, n+1, n+2...

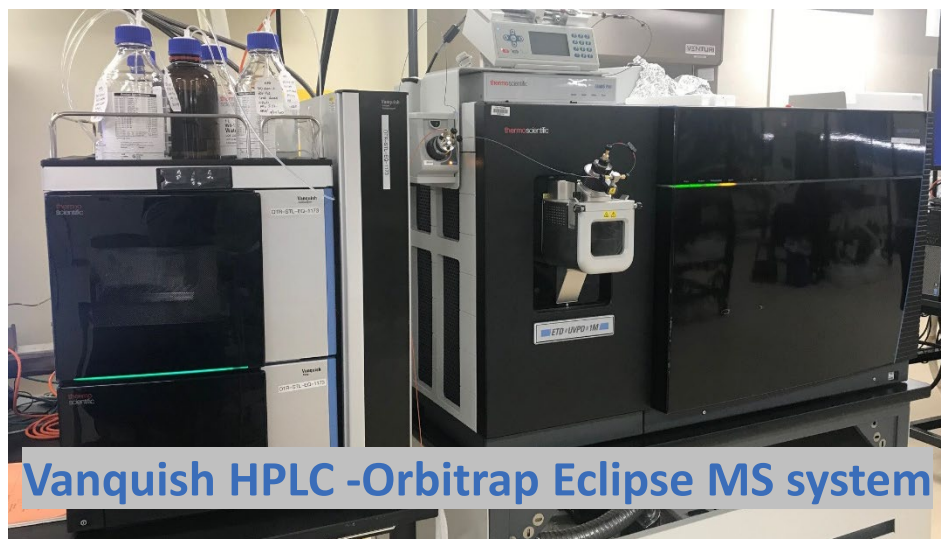


○ n-A ○ n-T ○ n-C ○ n-G

- Truncations (terminal deletion)
- Internucleotide linkage modifications
- Sugar moiety modifications
- Base modifications
- High-molecular-weight impurities

Introduction

○ HILIC-HRMS for oligonucleotide impurity profiling



ASMS poster #313427 (Rabiul Islam)

ASMS ThOH am #313595 (Kui Yang)

Abdullah et al. J Mass Spectrom 2022; e4819

Column: Shodex HILICpak VN-50 2D

MPA: 30% ACN & 70% H₂O, 20 mM NH₄Ac, pH 5.5

MPB: 70% ACN & 30% H₂O, 20 mM NH₄Ac, pH 5.5

Gradient:

0-1 min 10%, 1-10 min 10-25%, and 10-12 min 25% MPA.

Test molecule: 2'-O-MOE, phosphorothioate (PS) modified RNA 18-mer (Nusinersen)

U-C-A-C-U-U-U-C-A-U-A-A-U-G-C-U-G-G (C and U are methylated)

Manufacturer	Manuf. date	Batch
GenScript	2020 July	U670TFG080

Used as is.

○ Intact Mass Analysis workflow

thermo scientific BioPharma Finder RNA_Int_SeqSpe Help

Home Intact Mass Analysis Load Results Queue Parameters Spectra Comparison Intact Workbook

Component Detection Identification Report Save Method

Set the parameters for component detection. [Prev] [Next] [Finish]

Chromatogram Parameters

Use Restricted Time ☐

☒ Time Limits 0.002 to 15.008

☐ Scan Range 1 to 11727

m/z Range 600.0000 to 2,000.0000

Chromatogram Trace Type TIC

Sensitivity High

Rel. Intensity Threshold (%) 1

Source Spectra Method

☒ Sliding Windows

Generate the source spectra by using the sliding windows algorithm.

Sliding Windows Definition

RT Range 1.000 to 12.000

Target Avg Spectrum Width 0.100 minutes

Target Avg Spectrum Offset

☐ Scan Offset 1

☒ % Offset (legacy) 25

Sliding Windows Merging Parameters

Merge Tolerance 10.0 ppm

Merge Scheme Legacy Merge Scheme

Max RT Gap 1.000 minutes

Min. Number of Detected Intervals 3

☐ Auto Peak Detection

Generate the source spectra by using large molecule chromatographic peak detection.

☐ Average Over Selected Retention Time

Generate the source spectra by selecting a single scan (or averaging by dragging across multiple scans) on the chromatogram.

Deconvolution Algorithm

☐ ReSpec™ (Isotopically Unresolved)

☒ Xtract (Isotopically Resolved)

Output Mass Range 600 to 15,000

Output Mass ☒ M ☐ MH+

S/N Threshold 3.00

Rel. Abundance Threshold (%) 0.00

Charge Range 2 to 8

Min. Num Detected Charge 1

Isotope Table Sequence-Specific

☒ Show Advanced Parameters

Calculate XIC ☐

Fit Factor (%) 80

Remainder Threshold (%) 25

Consider Overlaps Resolution at 400 m/z ☒

☐ Raw File Specific

☒ Method Specific 120000.00

Negative Charge ☒

Charge Carrier ☒ H+ (1.00727663)

☐ K+ (38.9631585)

☐ Na+ (22.9892213)

☐ Custom

Minimum Intensity 1

Expected Intensity Error 3

RNA_full_363.raw

Chromatogram Mode ☒ Averaging ☐ Auto Zooming

RNA_full_363 NL: 2.31E8

Relative Intensity

RT (min)

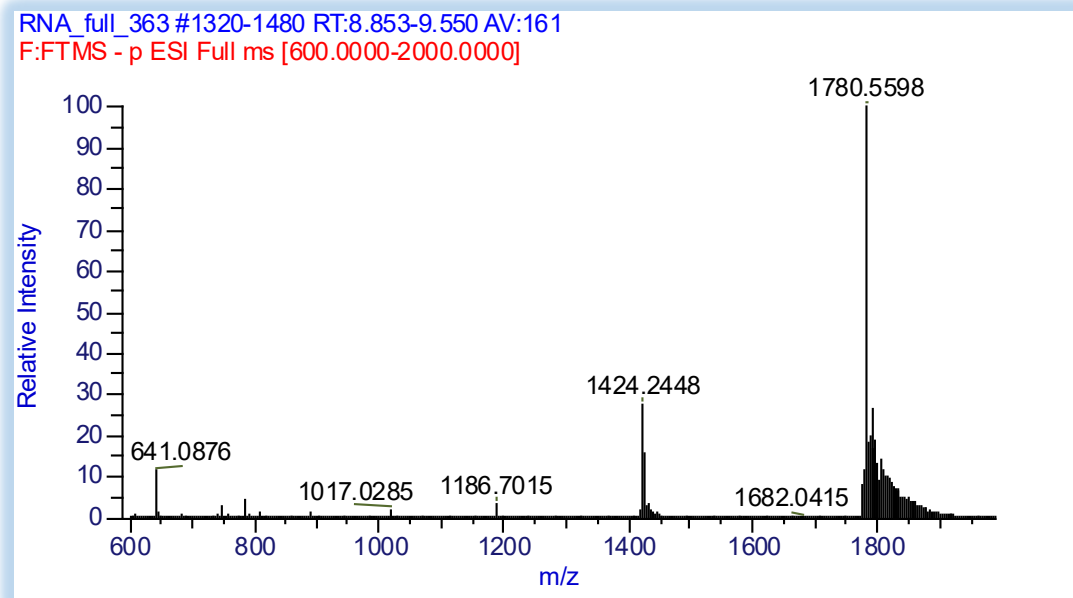
Source Spectrum

Intact Mass Analysis workflow

Biopharma Finder software

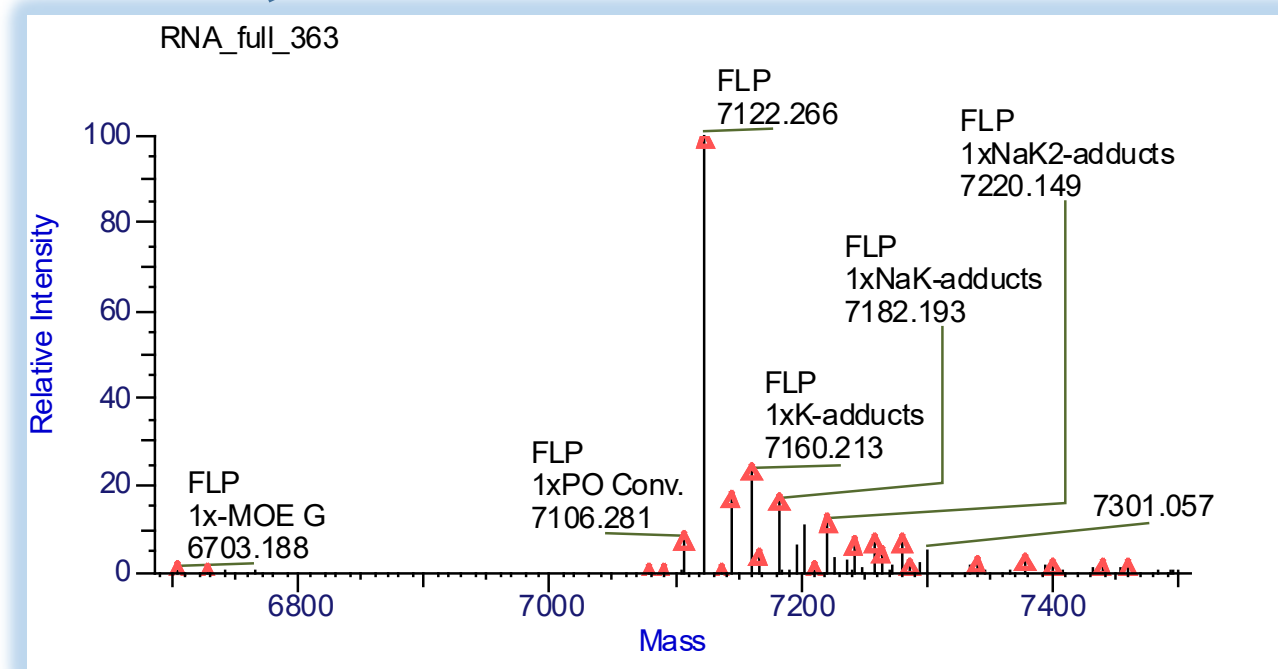


○ Intact Mass Analysis workflow



ESI mass spectrum of FLP

Intact Mass Analysis workflow

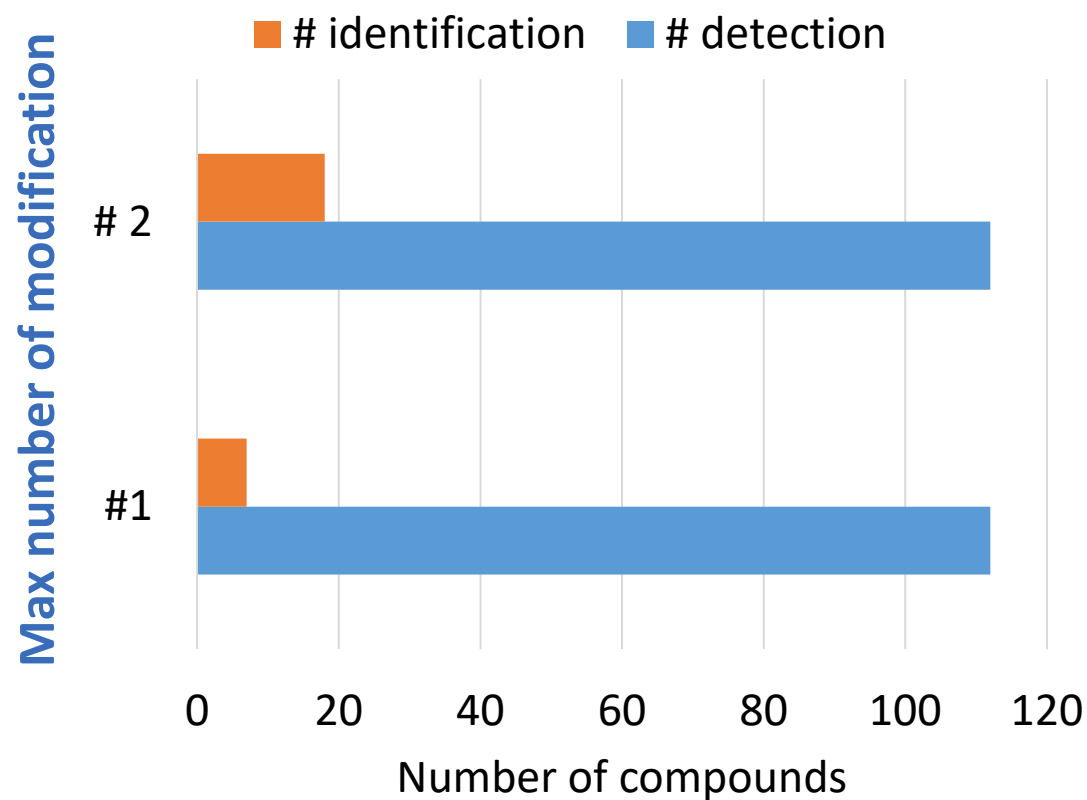


Deconvoluted mass spectrum of FLP₁₀

Intact Mass Analysis Parameter Optimization



○ Sequence Manager



Mass (mono.)	Identified compounds	
	Up to 1 Modif.	Up to 2 Modif.
7122.266	FLP	2'-O-Butyl + Deaminx2
7106.281	PO	2'-O-Ethyl + 2'-O-EOE
6703.188	n-G	(n-2G) + (n+G)
6728.180	n-T	2,6-DAP + (n-C)
7136.260	2'-O-EOE	Isobutyl + Fluorination
7090.279	2xPO	2'-O-Ethyl + 2'-O-Butyl
7078.231	2'-O-Methyl	2'-O-Methyl

Combination of 2 modifications likely lead to incorrect assignments

Intact Mass Analysis Parameter Optimization



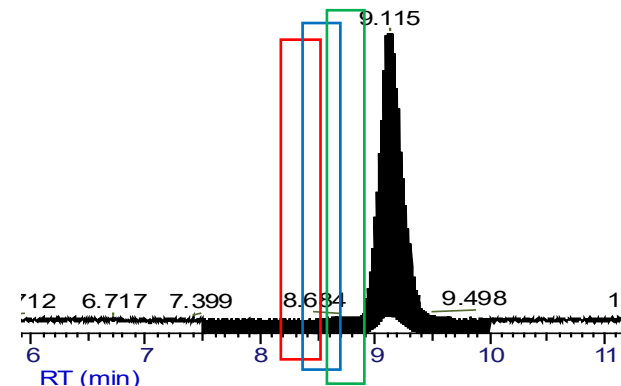
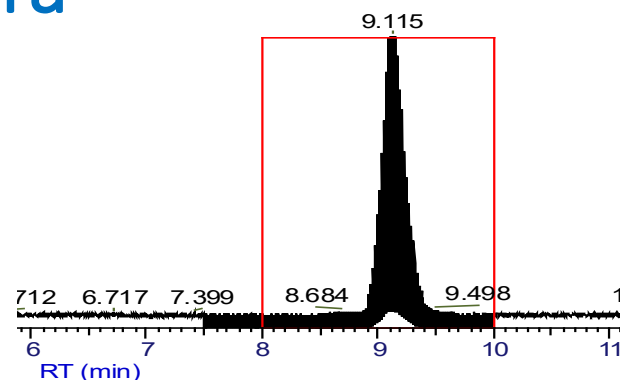
○ Sequence Manager

Identified compounds	Unenabled Truncation	Truncation from 5' Terminal	Truncation from 3' Terminal
FLP	X	X	-
PO	X	X	-
n-G	X	X	-
n-U	X	X	X
2'-O-EOE	X	X	X
2xPO	X	X	X
2'-O-Methyl	X	X	X
1x5'T, 2,6-DAP	-	X	-
1x5'T, 2'-O-Butyl	-	X	-
1x3'T, n+G	-	-	X
1x3'T, n+A	-	-	X
2x3'T, n+G	-	-	X
1x3'T, Cyclic Phosphoryl.	-	-	X
2x3'T, Unylinker dimer (442)	-	-	X

Intact Mass Analysis Parameter Optimization



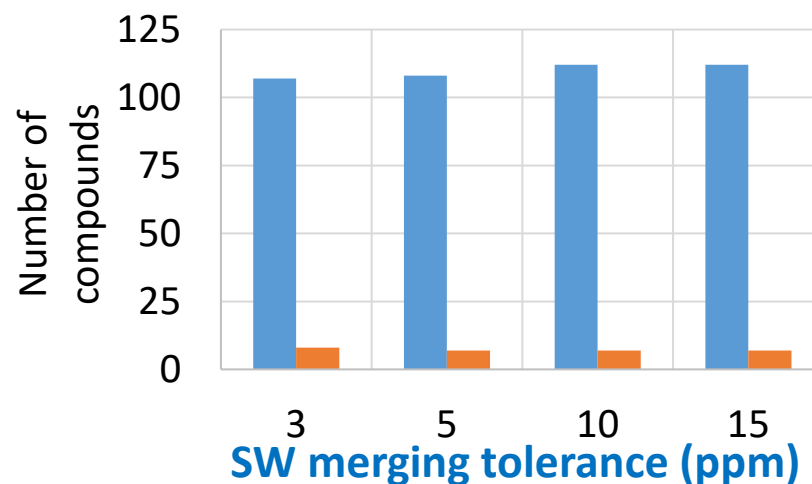
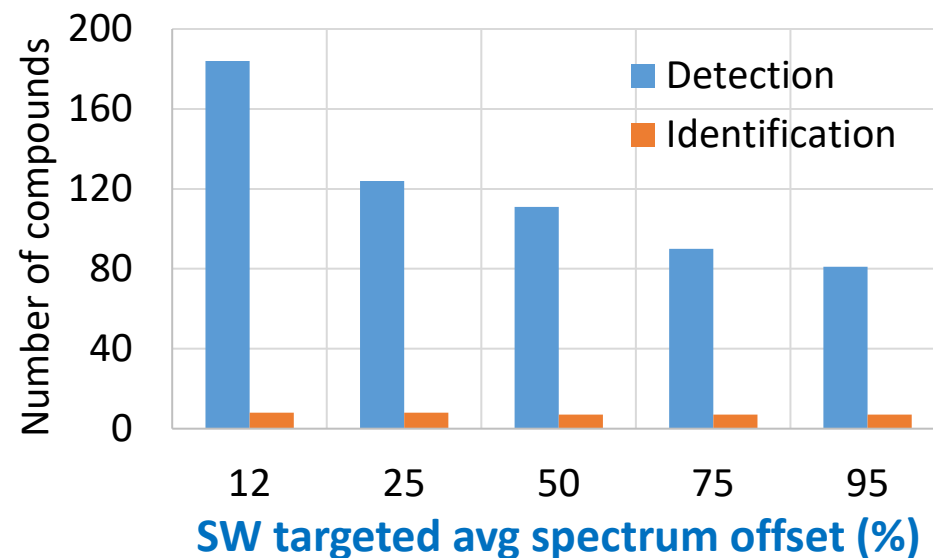
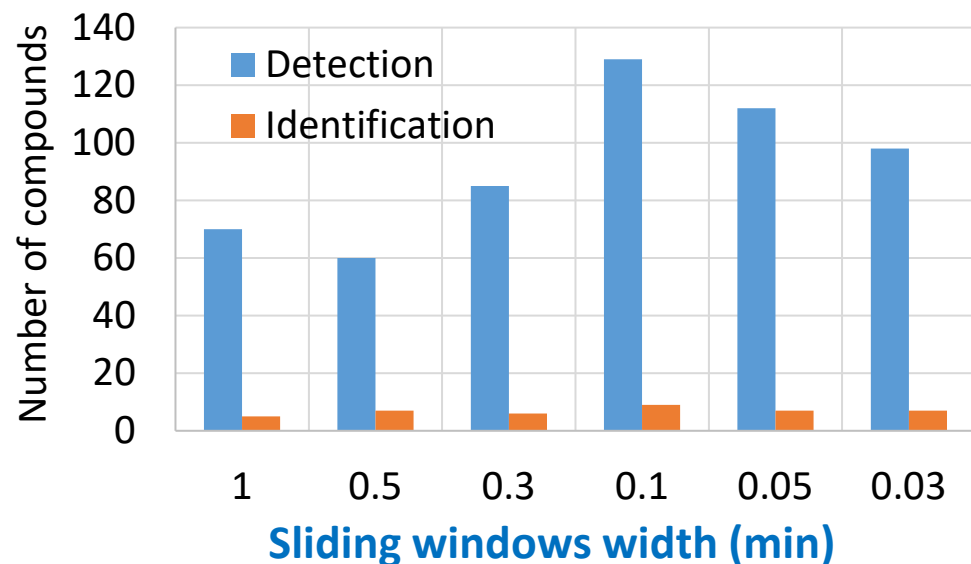
○ Source Spectra



Retention time range (min)	Average Over Selected Retention Time		Sliding Windows	
	Detection/Identification	Identified Compounds	Detection/Identification	Identified Compounds
2-12	66/2	FLP, PO	112/7	FLP, PO n-G, n-U, 2'-O-EOE, 2xPO, 2'-O-Methyl
6-12	100/3	FLP, PO, n-G	84/7	FLP, PO, n-G, n-U, 2'-O-EOE, 2xPO, 2'-O-Methyl
8-12	109/4	FLP, PO, n-G, n-U	84/7	FLP, PO, n-G, n-U 2'-O-EOE, 2xPO, 2'-O-Methyl
1-8 (Early eluting)	3/0	-	29/0	-
8-10 (Coeluting)	116/6	FLP, PO, n-G, n-U, 2'-O-EOE, 2xPO	84/7	FLP, PO, n-G, n-U, 2'-O-EOE, 2xPO 2'-O-Methyl
10-12 (Late eluting)	4/1	FLP	1/1	2,6-DAP

Intact Mass Analysis Parameter Optimization

○ Sliding Windows (SW) Settings



Intact Mass Analysis Parameter Optimization

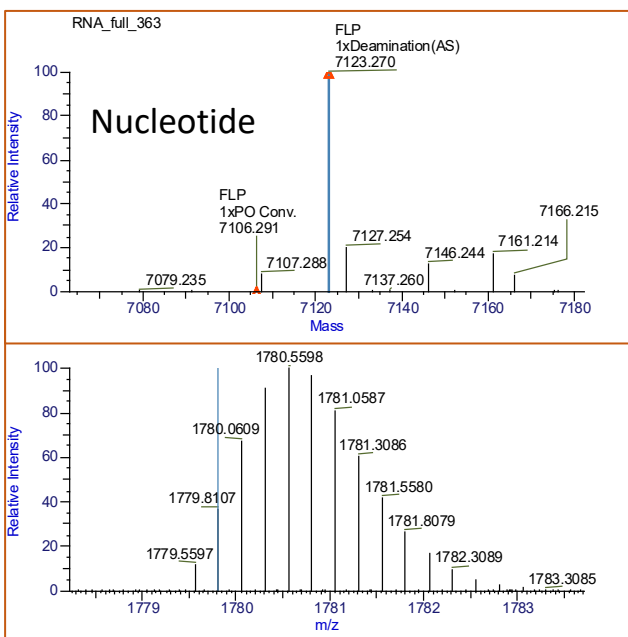
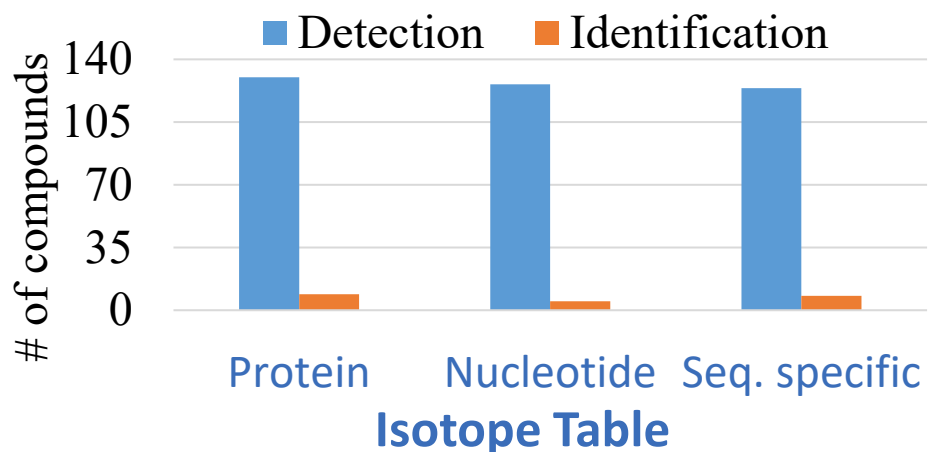


○ Sliding Windows (SW) Settings

Identified compounds (Rel. abundance)	Minimum # of detected intervals				
	3	5	10	20	30
FLP (100%)	X	X	X	X	X
PO (7.8%)	X	X	X	X	X
n-G (0.8%)	X	X	X	X	-
n-U (0.4%)	X	X	X	X	-
2'-O-EOE (0.3%)	X	X	X	-	-
2xPO (0.2%)	X	X	X	-	-
2-O-Methyl (0.2%)	X	X	X	-	-
Total detection	112	69	47	29	12

Intact Mass Analysis Parameter Optimization

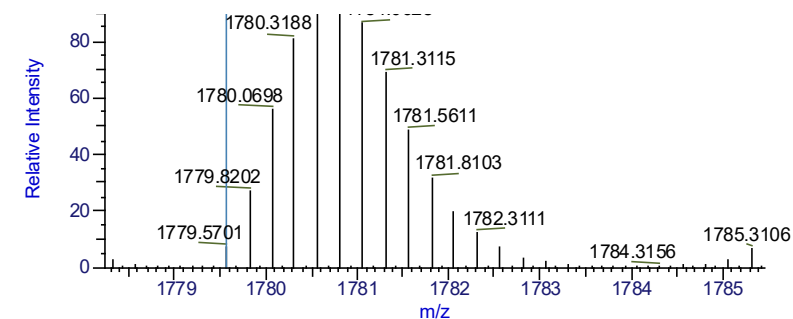
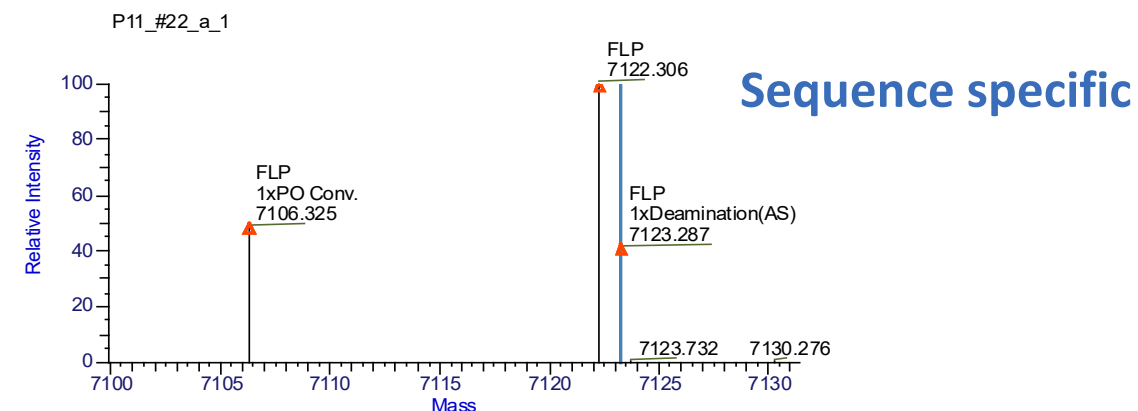
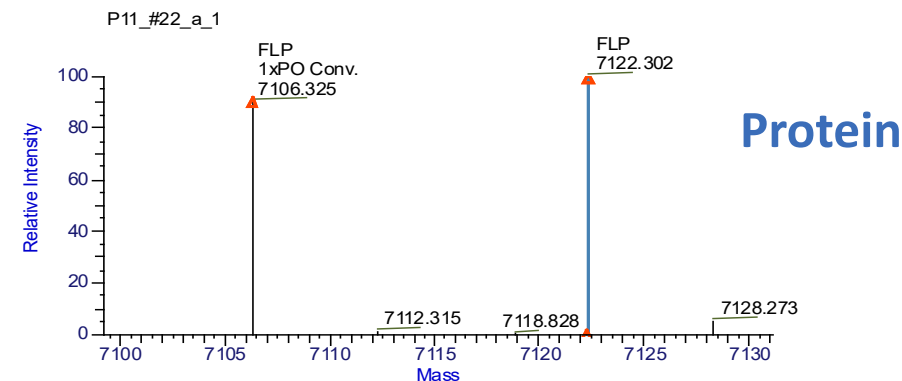
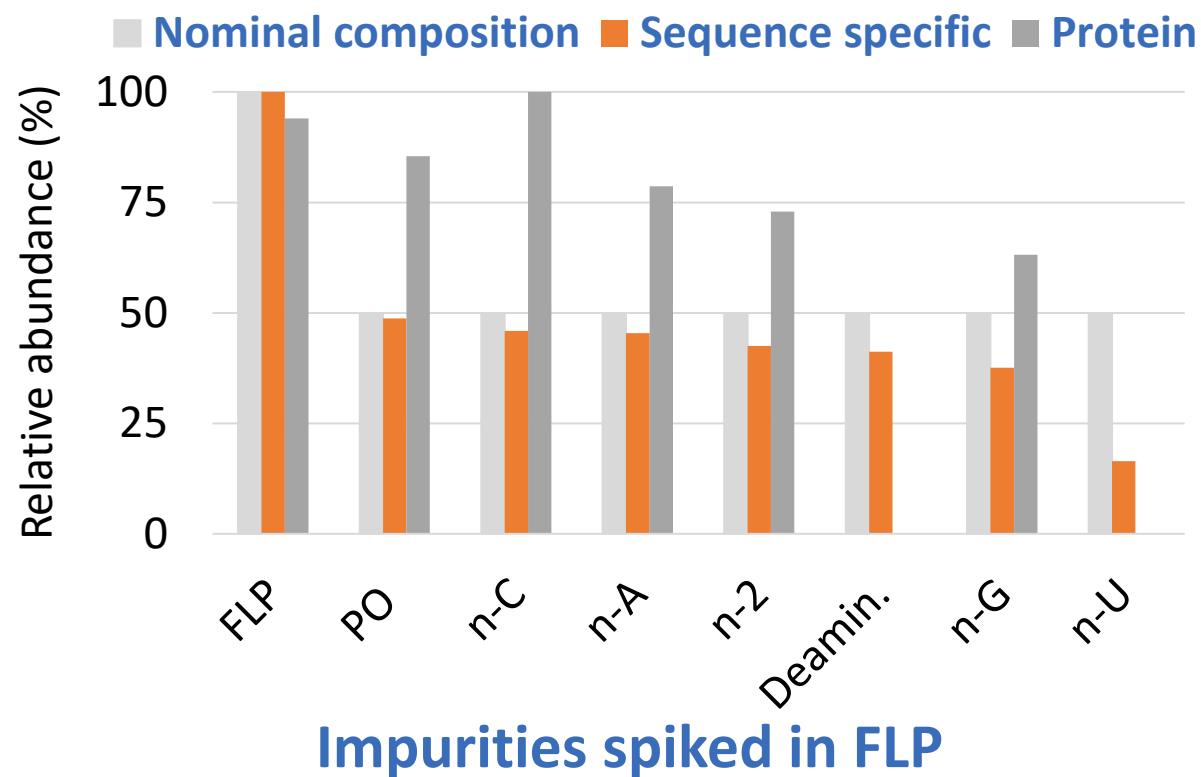
○ Deconvolution Isotope Table



Identified compounds	Rel. abundance		
	Protein	Nucleotide	Sequence-specific
FLP	100 %	-	100%
PO	8.0 %	0.2 %	7.8 %
n-G	0.8 %	-	0.8 %
n-U	0.4 %	0.1 %	0.4 %
2'-O-Methyl	0.3 %	-	0.2 %
Dithioate	0.3 %	-	-
2xPO	0.1 %	-	0.2 %
n-C	0.1 %	0.3 %	-
2'-O-EOE	0.03%	-	0.3 %
Deamination	-	100 %	-
Cyclic phosphoryl.	-	11.3 %	-

Intact Mass Analysis Parameter Optimization

○ Deconvolution Isotope Table



Case study

○ Impurity analysis of different batches

	Batch-1	Batch-2
Manufacturer	GenScript	
Manufacturing date	2022-03-02	2022-06-27
Batch number	U8427HB160	U3805HF200

Sample analysis:

- Column load: 100 pmol. Replicate injections: 5

System suitability test:

- Sensitivity check at 0.05 pmol column load, before and after sample analysis

MS data acquisition methods:

- Experiment 1: HRAM-MS at 120,000 K resolution
- Experiment 2: ddMS2 (MS at 60,000 K and MS² at 30,000 K)

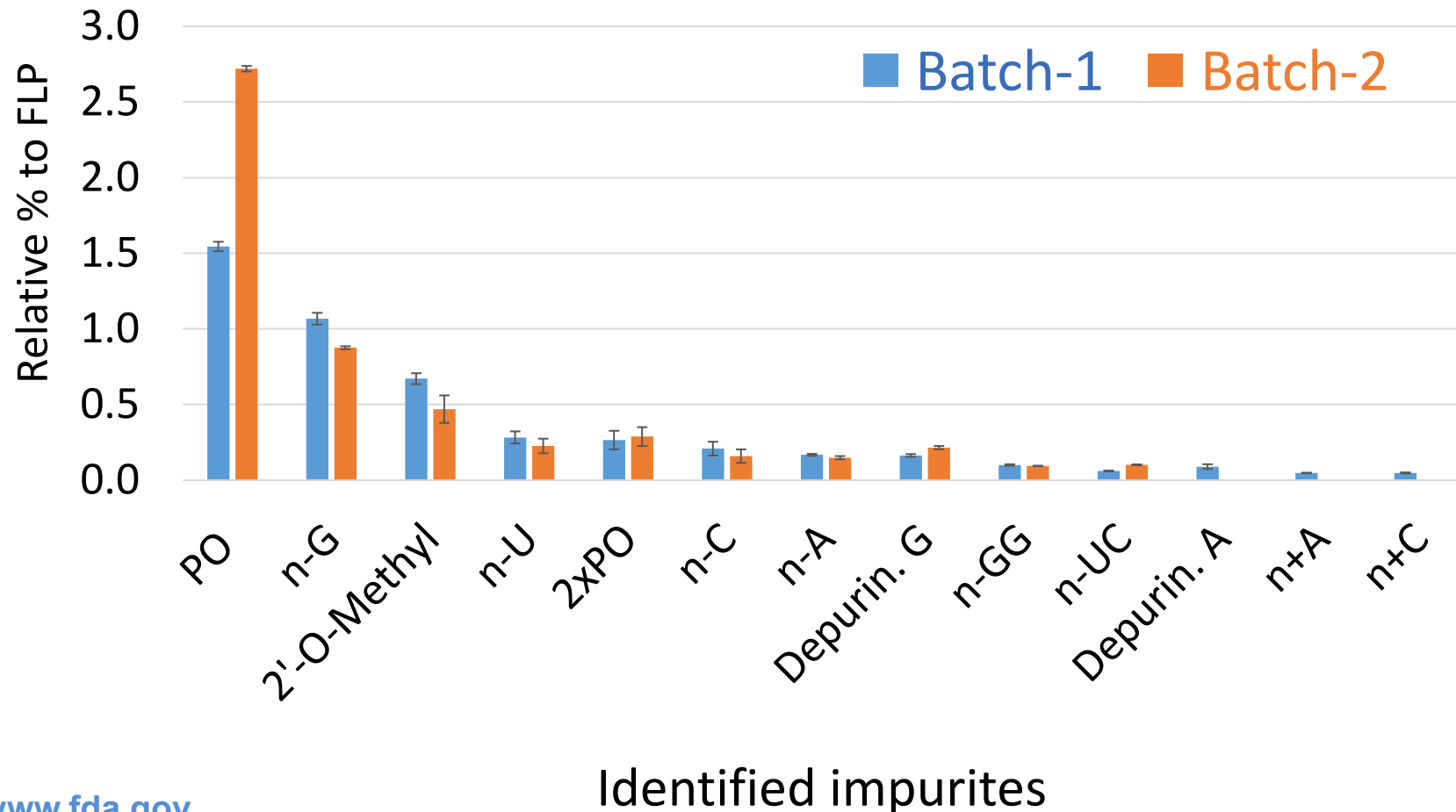
Data processing:

- BPF Intact Mass workflow using optimized parameters

Case study

○ Impurity analysis of different batches

➤ Experiment 1: HRAM-MS



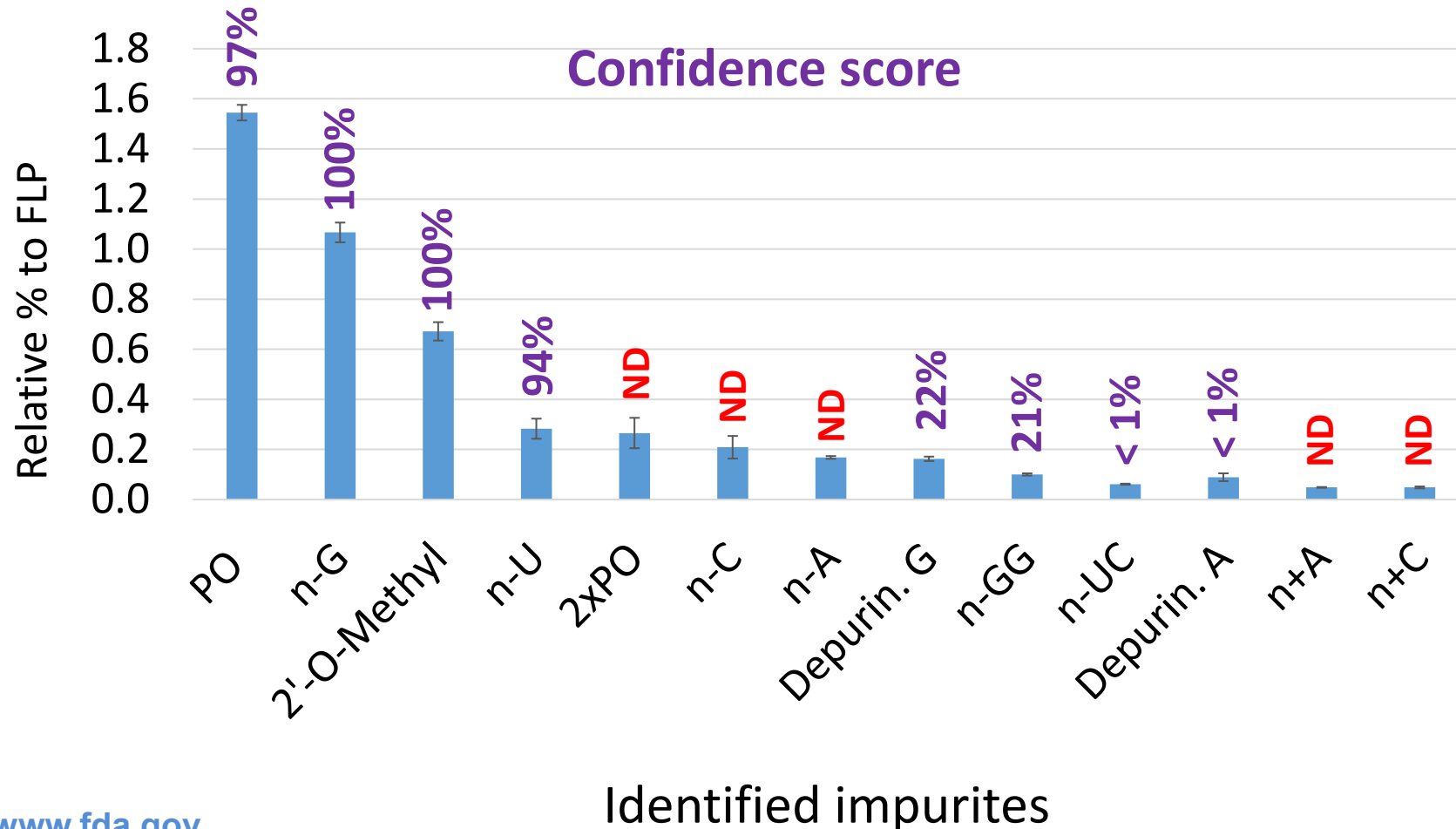
Impurity reporting criteria:

- Relative abundance $\geq 0.05\%$
- Detected in at least 4 out of 5 replicates

Case study

○ Impurity analysis of different batches

➤ Experiment 2: ddMS2



BPF Oligonucleotide Analysis workflow

- Sequence confirmation
- Modification site localization

Conclusions

- HRMS and intact mass data processing offer a promising option to automate impurity profiling of synthetic oligonucleotides.
- Parameters in BioPharma Finder Intact Mass Analysis workflow greatly impact impurity identification and quantitation.
- Critical parameters include maximum number of modifications, sliding windows settings and isotope table selection.
- In combination with Oligonucleotide Analysis workflow, enabled MS/MS fragment sequencing provides an additional level of structural confirmation.



Acknowledgement

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- **DCDA/OTR/OPQ/CDER**
 - Dr. Kui Yang
 - Dr. Cynthia Sommers
 - Dr. Jason Rodriguez
 - MS team
- **DTP/ORS/OGD/CDER**
 - Dr. Deyi Zhang
 - Dr. Darby Kozak
- **FDA Critical Path Grants**
 - PI – Kui Yang (OTR/OPQ)
 - Collaboration –OPQ, OGD, NCTR
- **FDA ORISE Fellowship Program at CDER**
 - Dr. AM Abdullah (ORISE fellow 2020-2022)
 - Mentors: Kui Yang (OTR/OPQ), Deyi Zhang (ORS/OGD)