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

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Disclaimer

This presentation reflects the views of the author and should not be construed to represent FDA's views or policies.

The mention of trade names, commercial products, or organizations is for clarification of the methods used and should not be interpreted as an endorsement of a product or manufacturer.

Pharmaceutical quality is

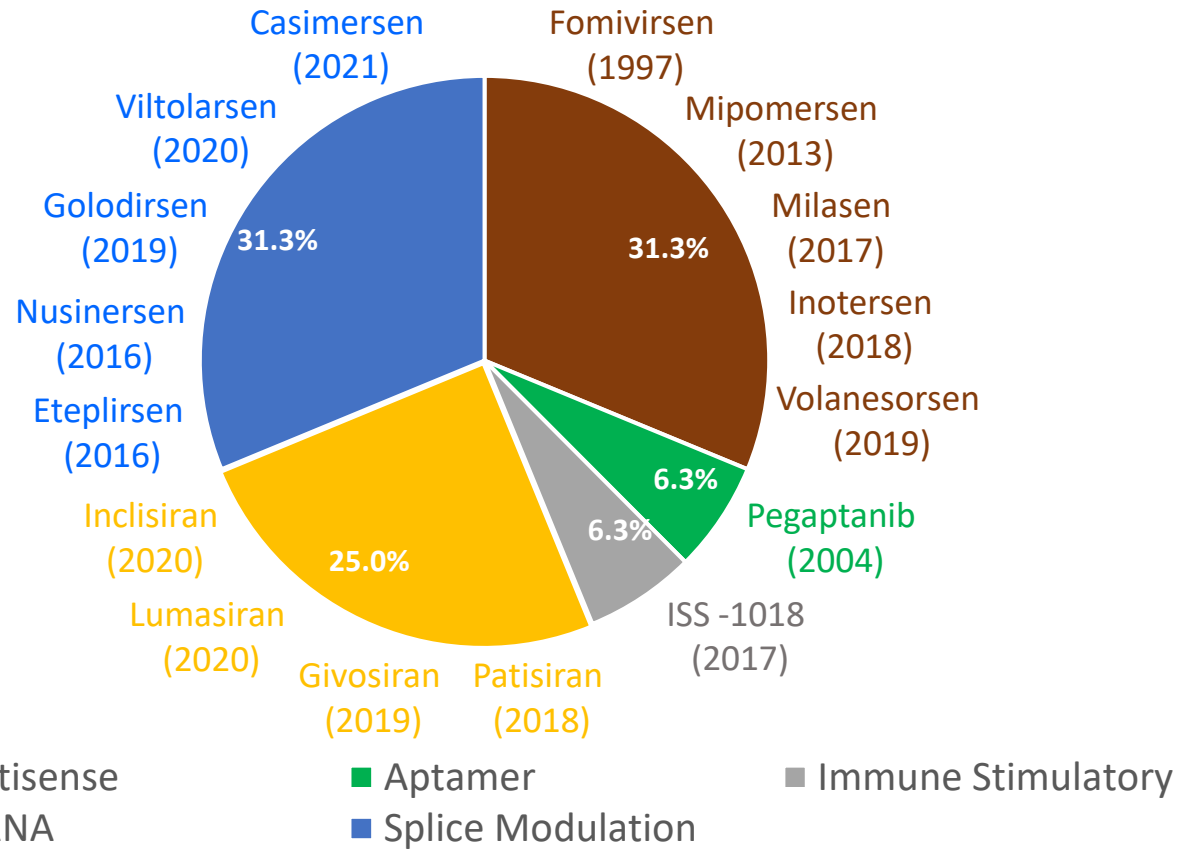
assuring *every* dose is safe and effective, free of contamination and defects.

Outline

- 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)

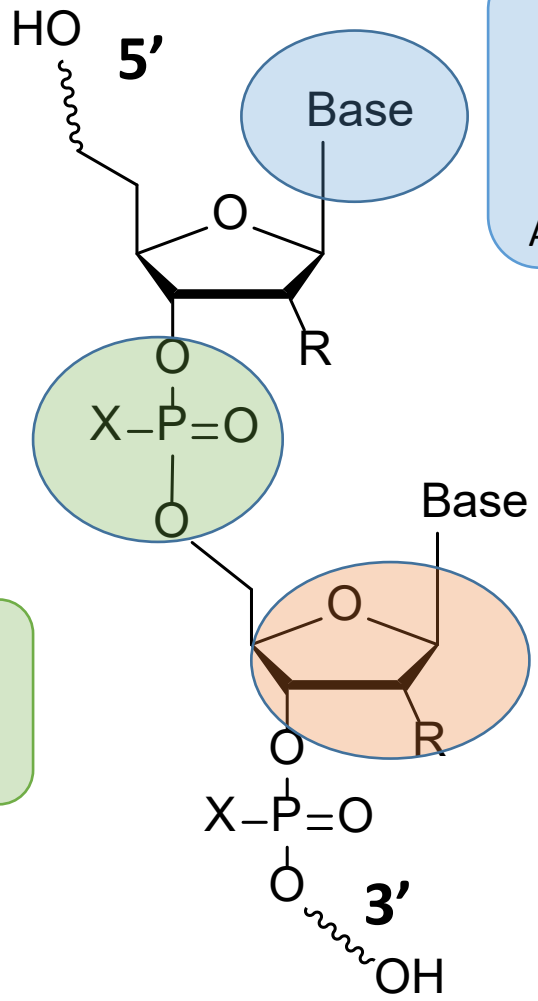


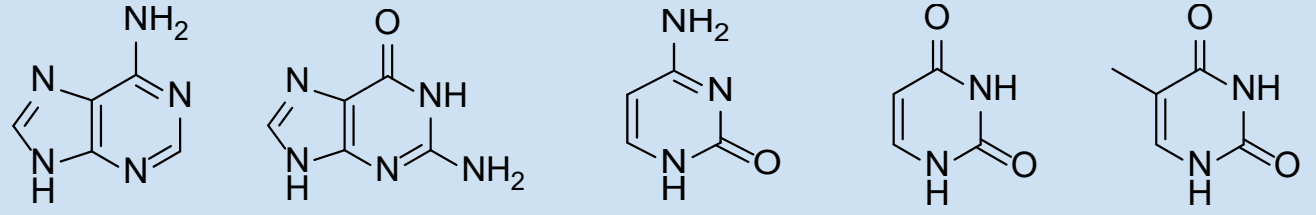
- Potential to treat a broad spectrum of indications from rare/life-threating to common diseases
- Impurity profiling is critical for ensuring quality, efficacy and safety of complex ONT drugs

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
CH_3	Methylphosphonate

R = OCH_3	2’-O-methyl
OCH_2CH_3	2’-O-ethyl
$OCH_2CH_2OCH_3$	2’-O-methoxyethyl (MOE)
$OCH_2CH_2OC_2H_5$	2’-O-ethoxyethyl (EOE)
$OCH_2CH{=}CH_2$	2’-O-allyl

 Chemical modifications on backbone, bases, and sugar

Introduction

- Product-related impurities of ONTs
  - Deletion / addition sequences

    n-1, n-2, n+1, n+2…

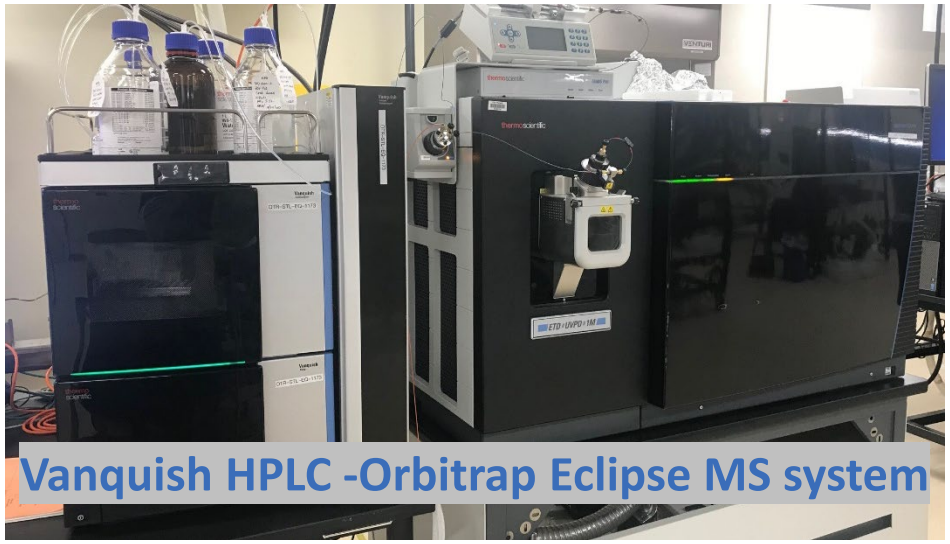
    

    - n-A
    - n-T
    - n-C
    - n-G
  - Truncations (terminal deletion)
  - Internucleotide linkage modifications
  - Sugar moiety modifications
  - Base modifications
  - High-molecular-weight impurities

Capaldi et al., Nucleic Acid Therapeutics 2017, 309-322

Introduction

- HILIC-HRMS for oligonucleotide impurity profiling



Vanquish HPLC -Orbitrap Eclipse MS system

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 NH_4Ac, pH 5.5
MPB: 70% ACN & 30% H2O, 20 mM NH_4Ac, 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)

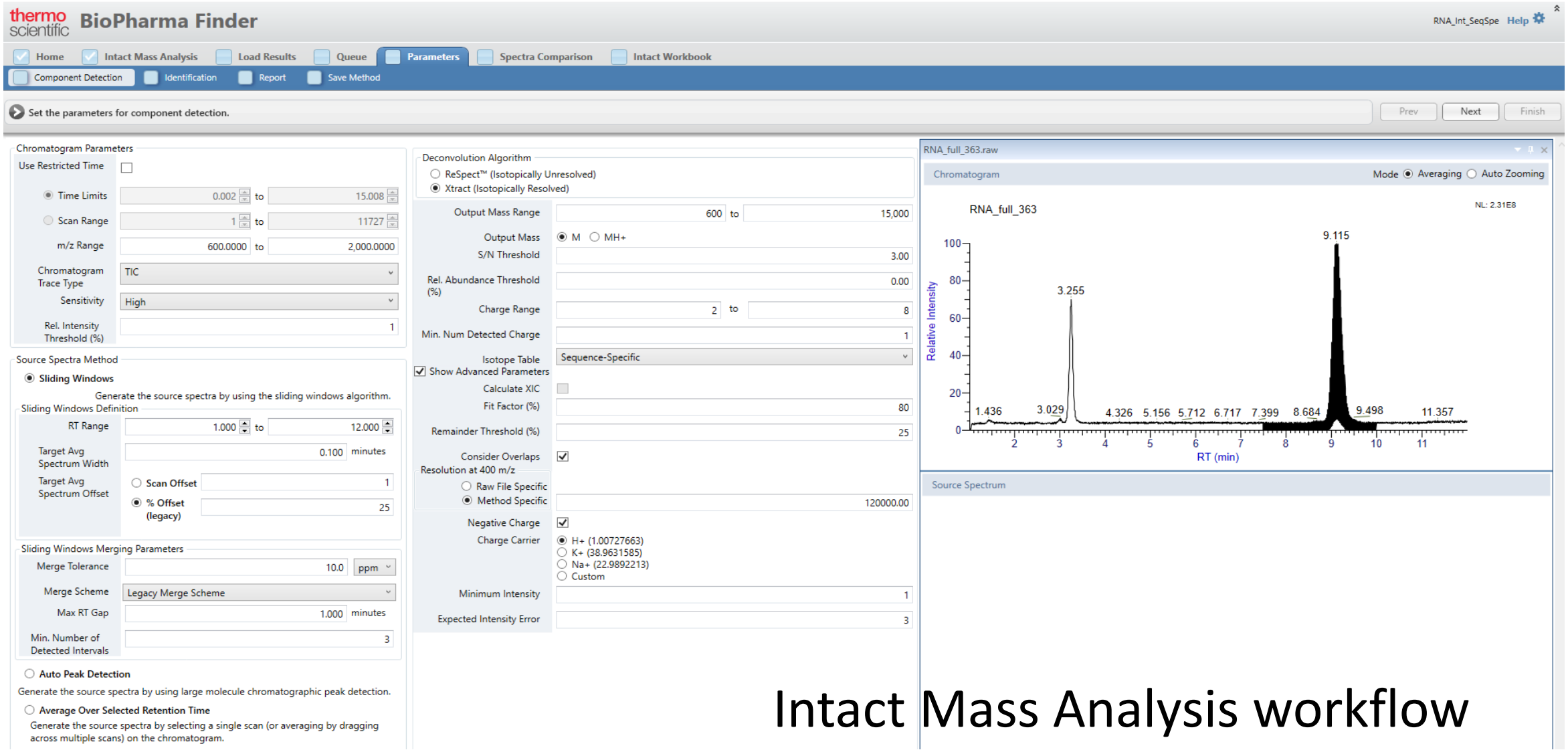
U-C-A-C-U-U-U-C-A-U-A-A-U-G-C-U-G-G (C and U are methylated)

Manufacturer	Manuf. date	Batch
GenScript	2020 July	U670TFG080

Used as is.

Biopharma Finder software

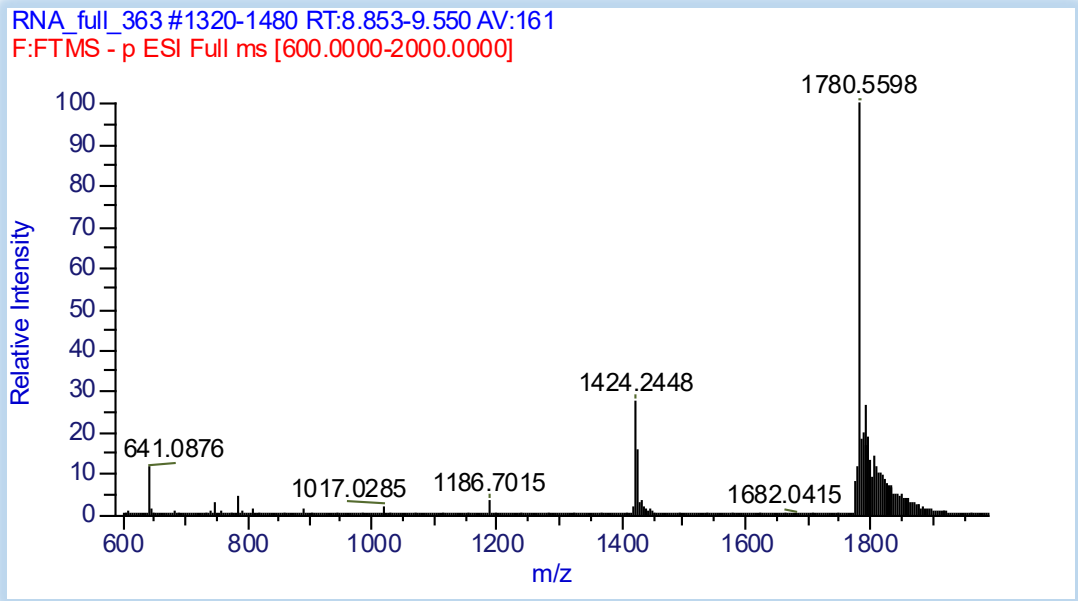
- Intact Mass Analysis workflow



Intact Mass Analysis workflow

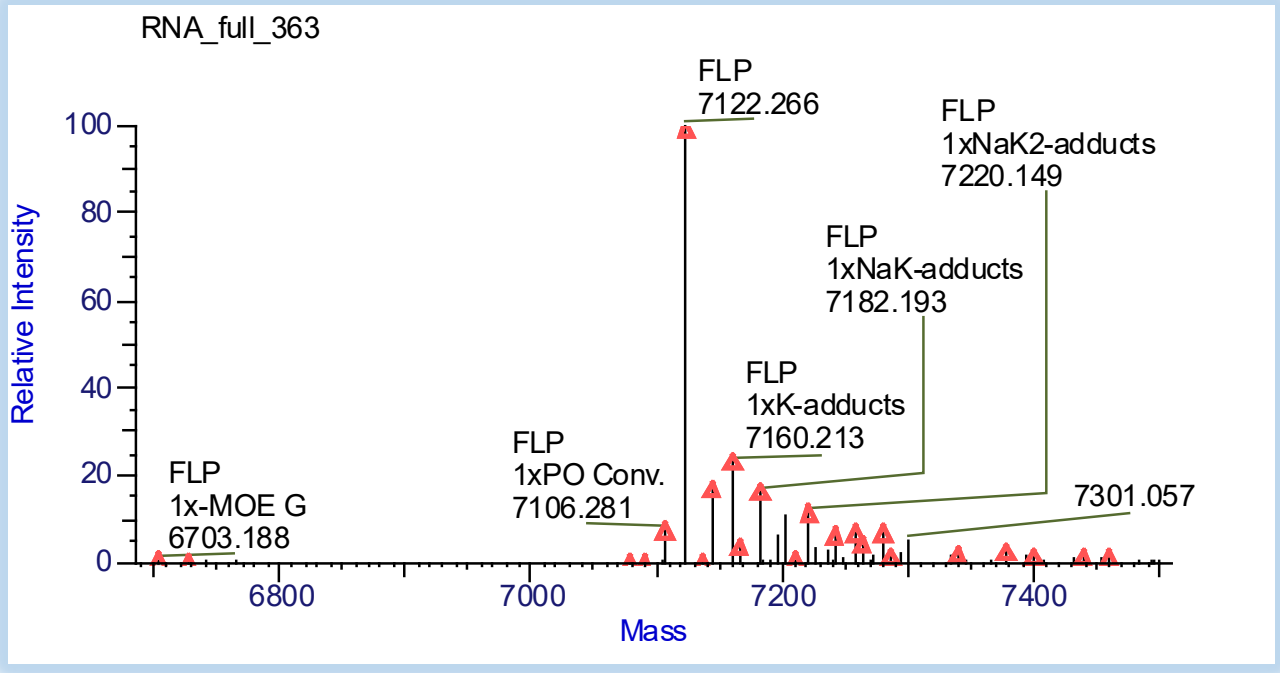
Biopharma Finder software

○ Intact Mass Analysis workflow



ESI mass spectrum of FLP

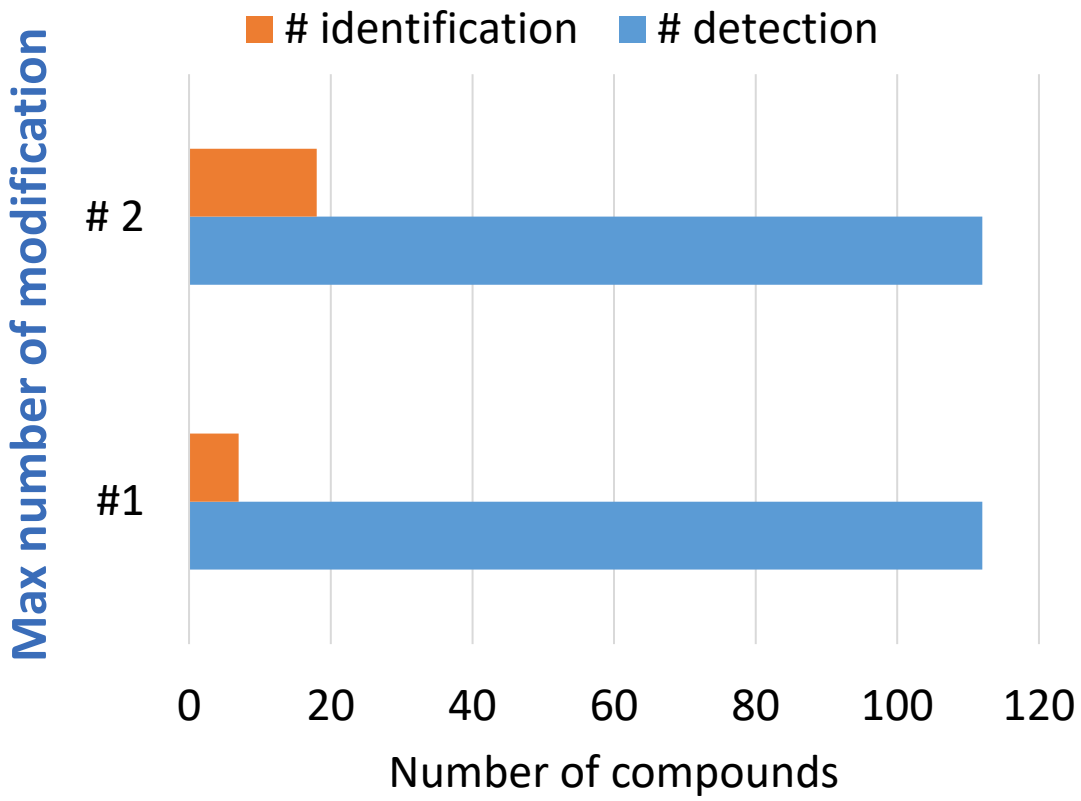
Intact Mass Analysis workflow



Deconvoluted mass spectrum of FLP

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

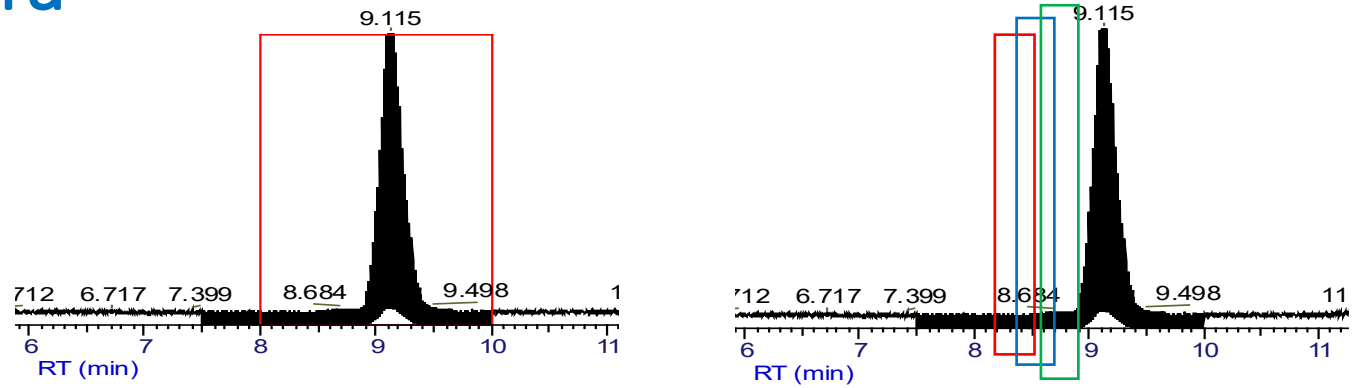
Intact Mass Analysis Parameter Optimization

○ 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

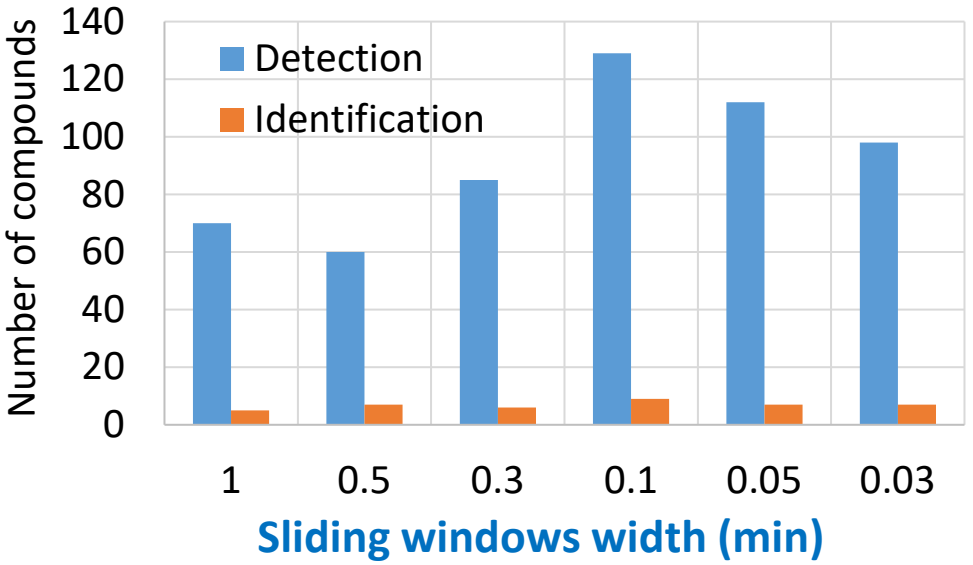
- Source Spectra

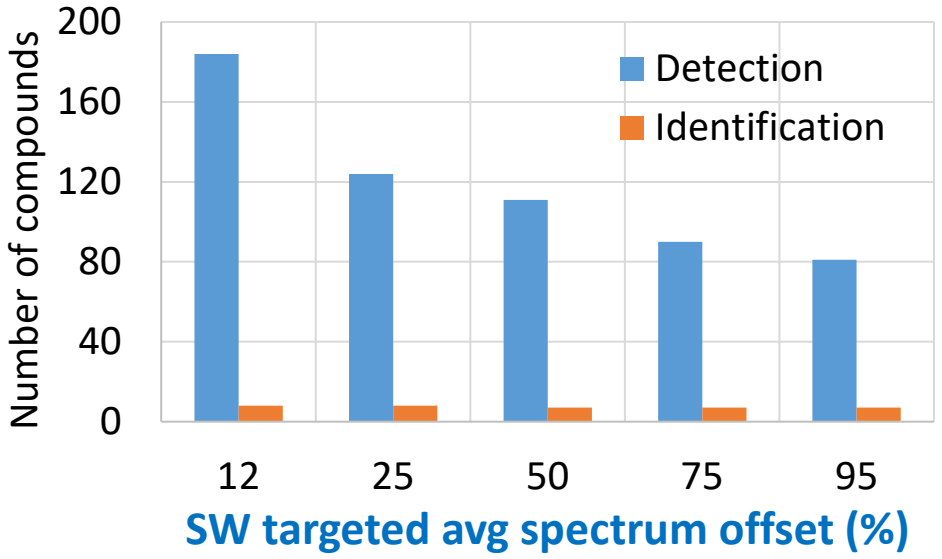


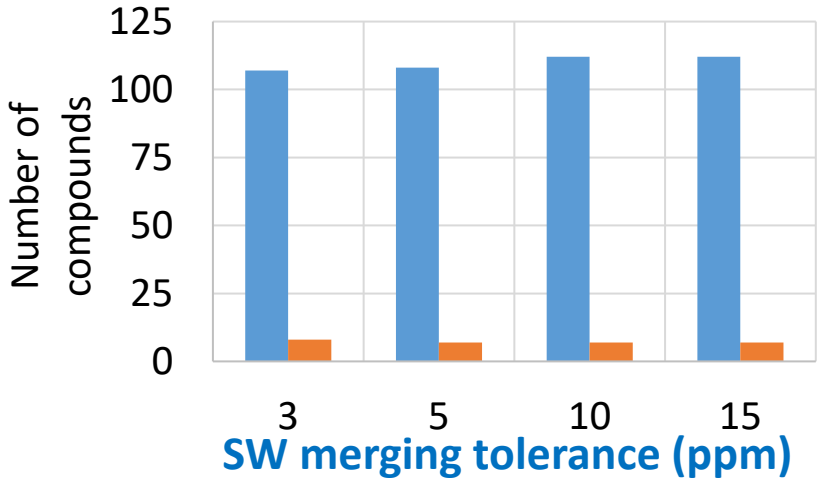
Retention time range (min)	Average Over Selected Retention Time		Sliding Windows	
	Detection/ Identification	Identified Compounds	Detection/ Identification	Identified Compounds
2-12	66/2	FLP, PO	112/7	FLP, PO **n-G, n-U, 2'-O-EOE, 2xPO, 2'-O-Methyl**
6-12	100/3	FLP, PO, n-G	84/7	FLP, PO, n-G, **n-U, 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







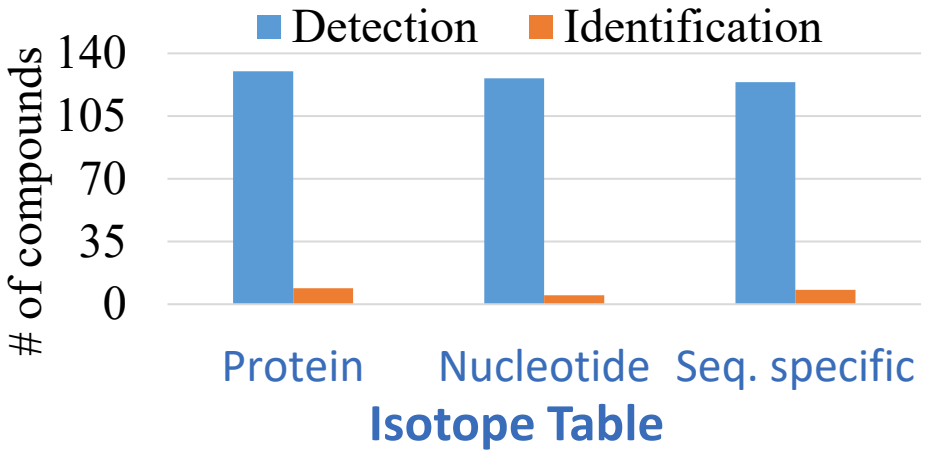
Intact Mass Analysis Parameter Optimization

○ Sliding Windows (SW) Settings

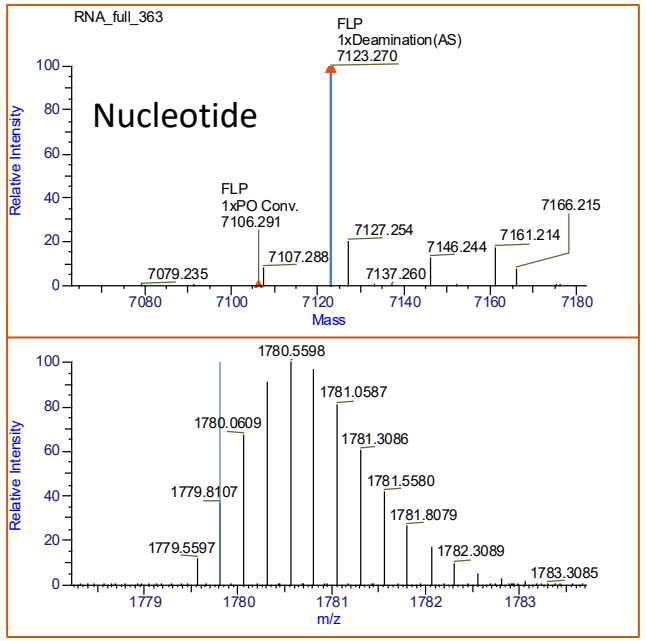
Identified compounds (Rel. abundance)	Minimum # of detected intervals				
	3	5	10	20	30
FLP (100%)	X	X	X	X	X
PO (7.8%)	X	X	X	X	X
n-G (0.8%)	X	X	X	X	-
n-U (0.4%)	X	X	X	X	-
2'-O-EOE (0.3%)	X	X	X	-	-
2xPO (0.2%)	X	X	X	-	-
2-O-Methyl (0.2%)	X	X	X	-	-
Total detection	112	69	47	29	12

Intact Mass Analysis Parameter Optimization

○ Deconvolution Isotope Table



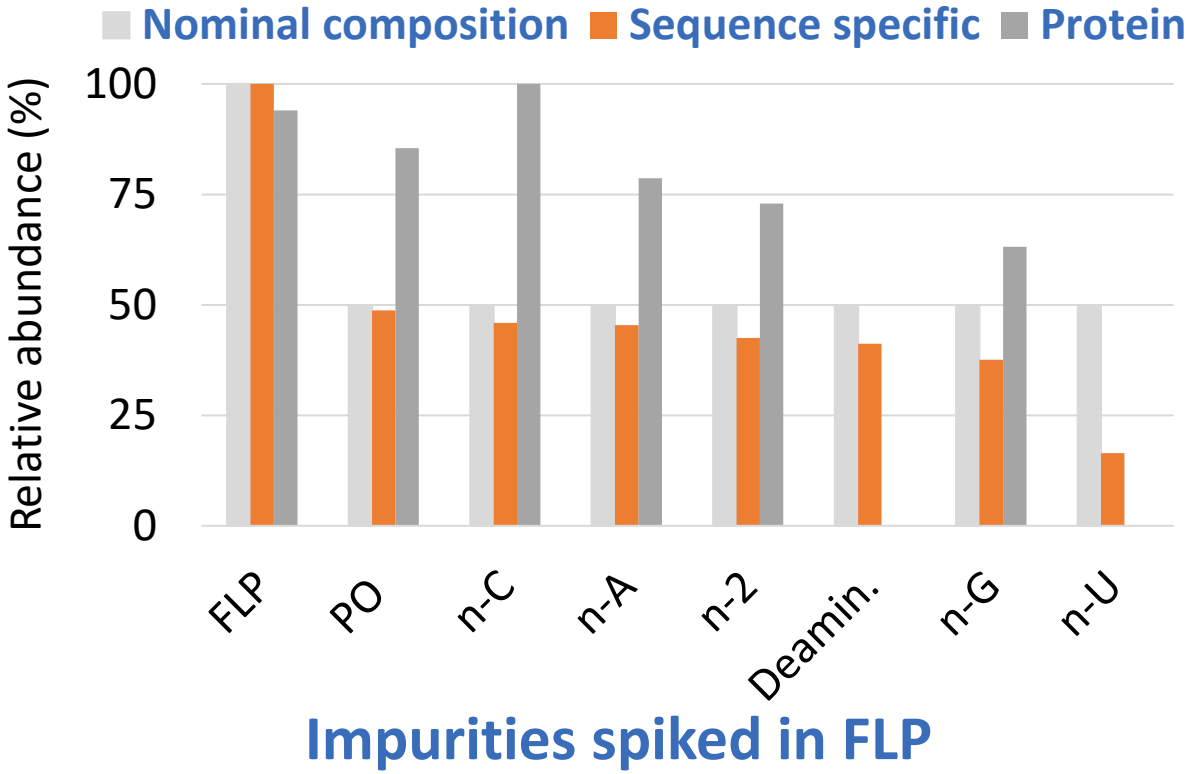
Isotope Table

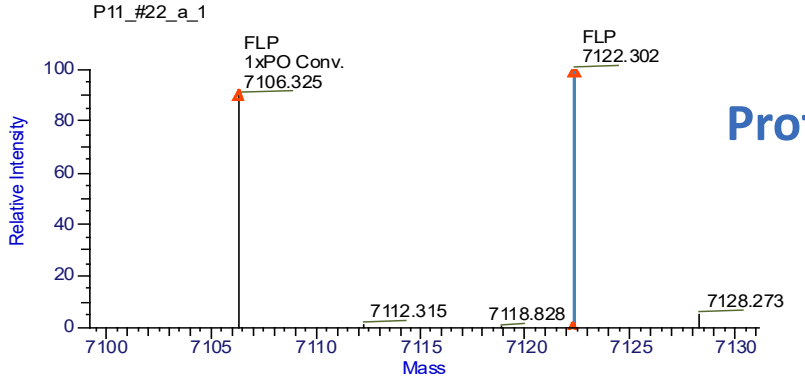


Identified compounds	Rel. abundance		
	Protein	Nucleotide	Sequence-specific
FLP	100 %	-	100%
PO	8.0 %	0.2 %	7.8 %
n-G	0.8 %	-	0.8 %
n-U	0.4 %	0.1 %	0.4 %
2’-O-Methyl	0.3 %	-	0.2 %
Dithioate	0.3 %	-	-
2xPO	0.1 %	-	0.2 %
n-C	0.1 %	0.3 %	-
2’-O-EOE	0.03%	-	0.3 %
Deamination	-	100 %	-
Cyclic phosphoryl.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

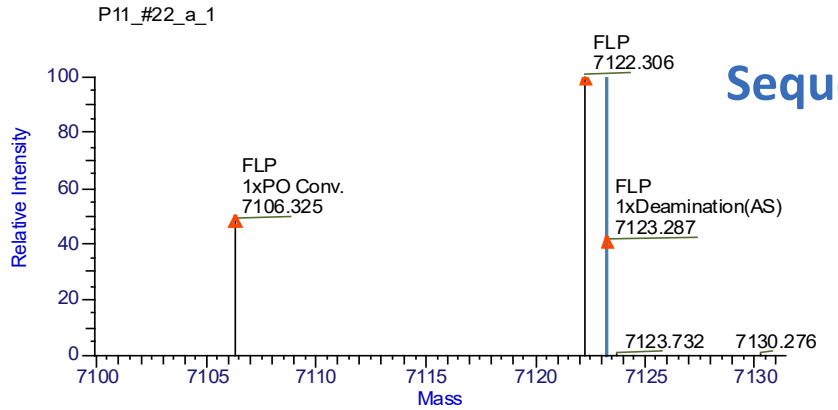
Intact Mass Analysis Parameter Optimization

○ Deconvolution Isotope Table

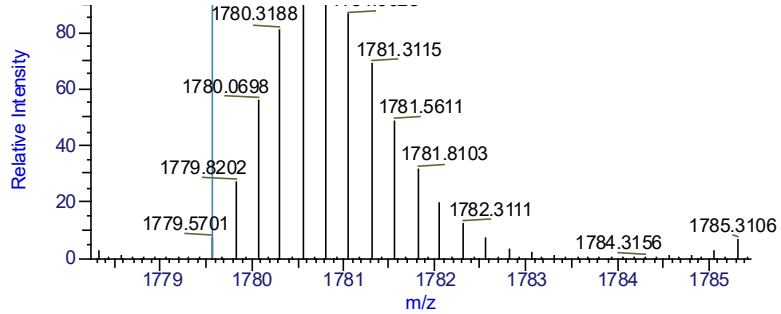




Protein



Sequence specific



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 MS^2 at 30,000 K)

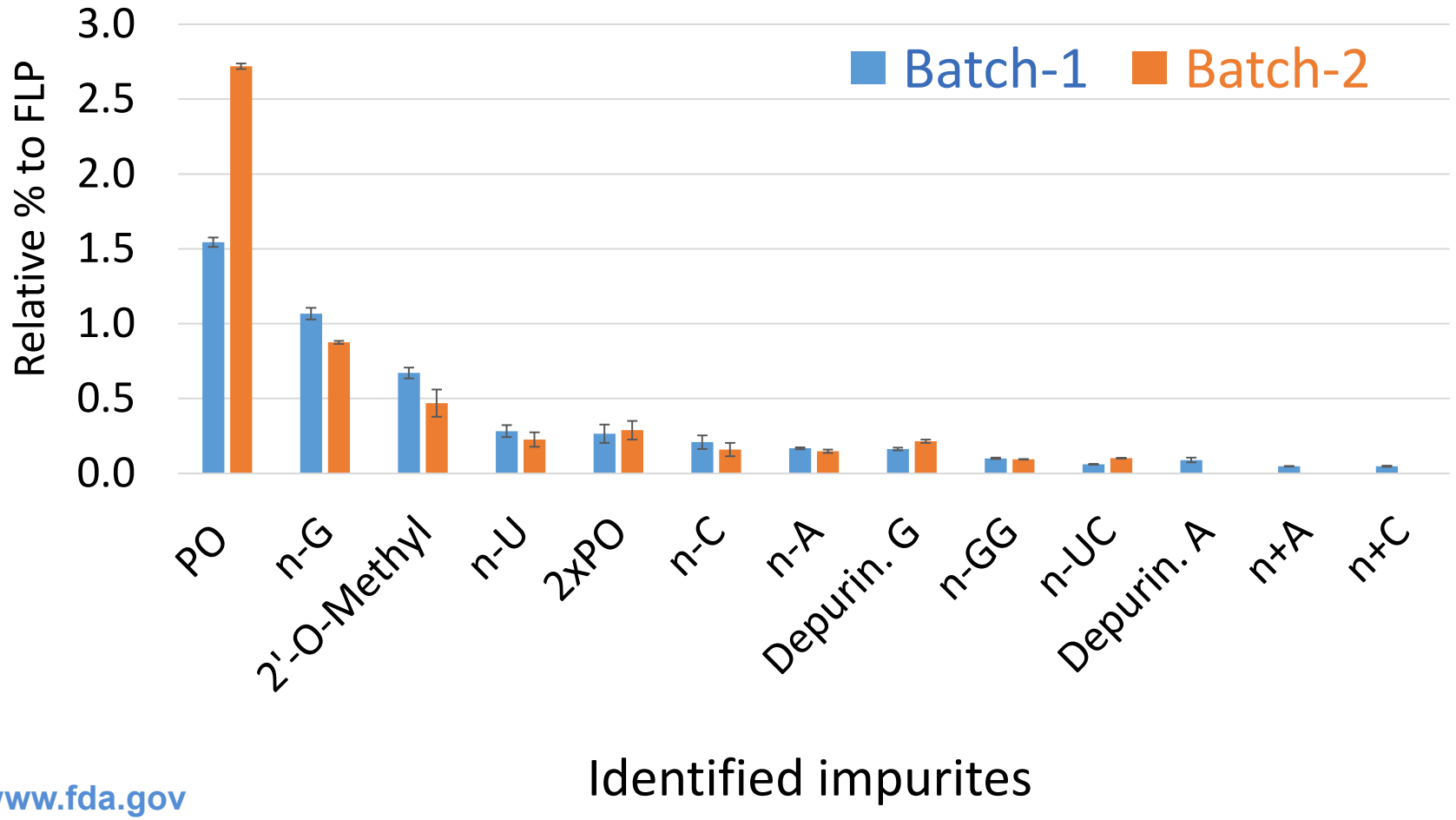
Data processing:

- BPF Intact Mass workflow using optimized parameters

Case study

○ Impurity analysis of different batches

➢ Experiment 1: HRAM-MS



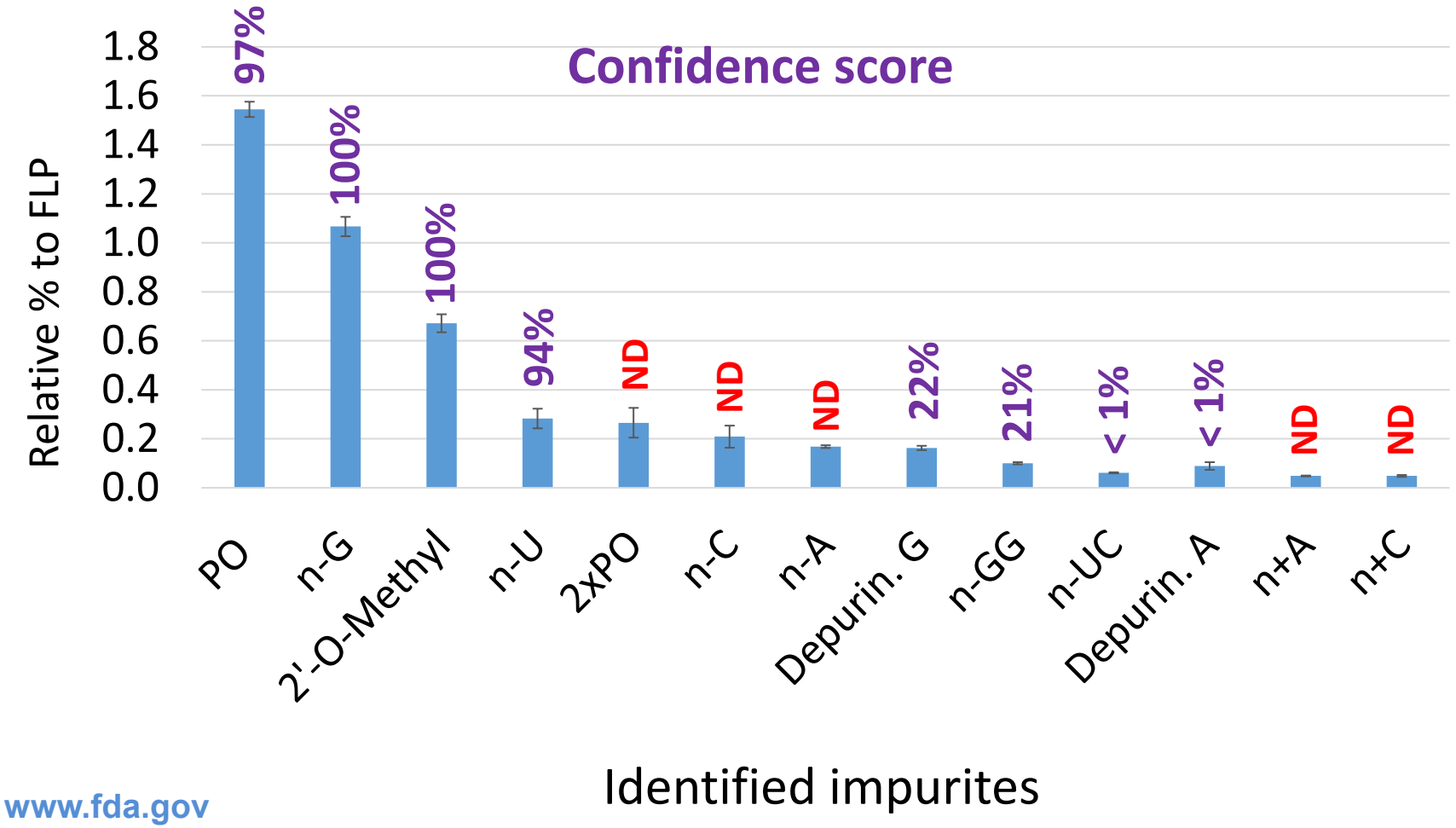
Impurity reporting criteria:

- Relative abundance >= 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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  - Dr. Darby Kozak
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  - Collaboration –OPQ, OGD, NCTR
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