LC-HRMS-based Multi-Attribute Method for Oligonucleotides (MAMO): Characterization and Impurity Profiling

Kui Yang, Ph.D.
Sr. Research Scientist
Office of Pharmaceutical Quality Research | CDER | US FDA

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The authors declare no competing financial interest.

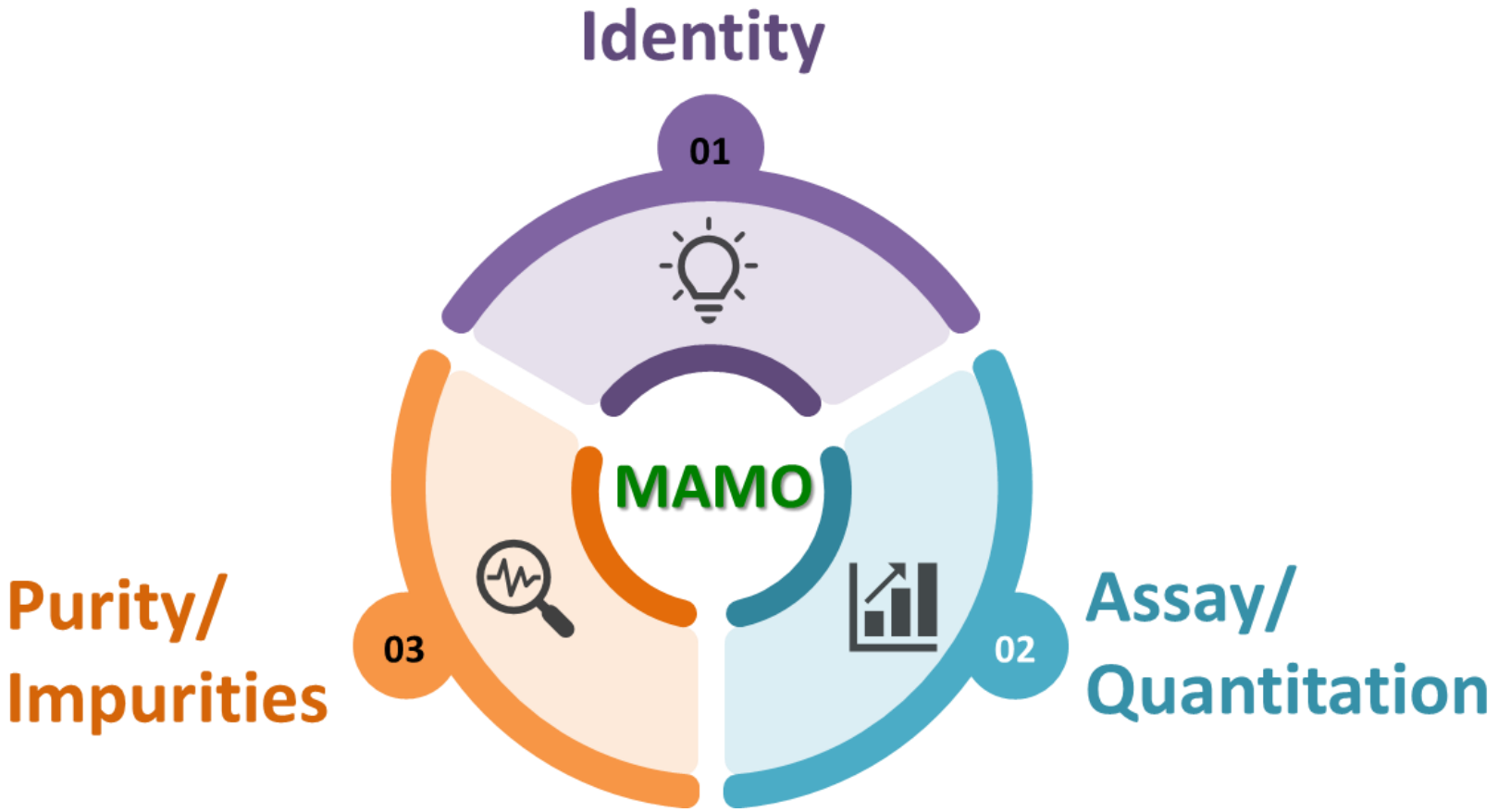
Every patient deserves confidence in their *every* and *next* dose of medicine.

Pharmaceutical quality assures the availability, safety and efficacy of *every* dose.

Outline

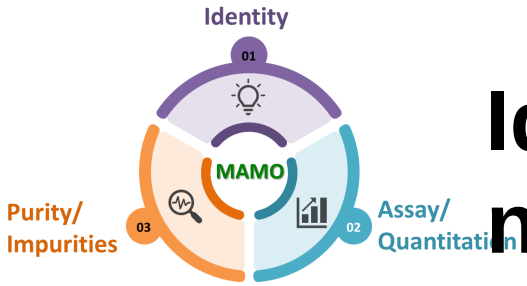
- LC-HRMS-based **M**ulti-**A**ttribute **M**ethod for **O**ligonucleotides (MAMO) analytical platform
- LC-HRMS method validation
- When separation by LC or MS fails

MAMO Analytical Platform



LC-HRMS-based

Multi-Attribute Method for Oligonucleotides



Identity/characterization

Reference standard

Reference standard

Theoretical average mass

Theoretical masses and distribution of isotopic peaks

Intact mass by deconvolution

Sequence mapping coverage

Software

RETENTION TIME

AVERAGE MASS

ACCURATE MASS + ISOTOPIC ENVELOPE

SEQUENCE CONFIRMATION BY SEQUENCE-INDICATING FRAGMENT IONS

LC

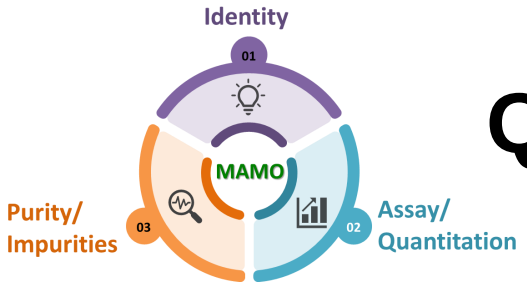
MS

HRMS

HRMS/MS

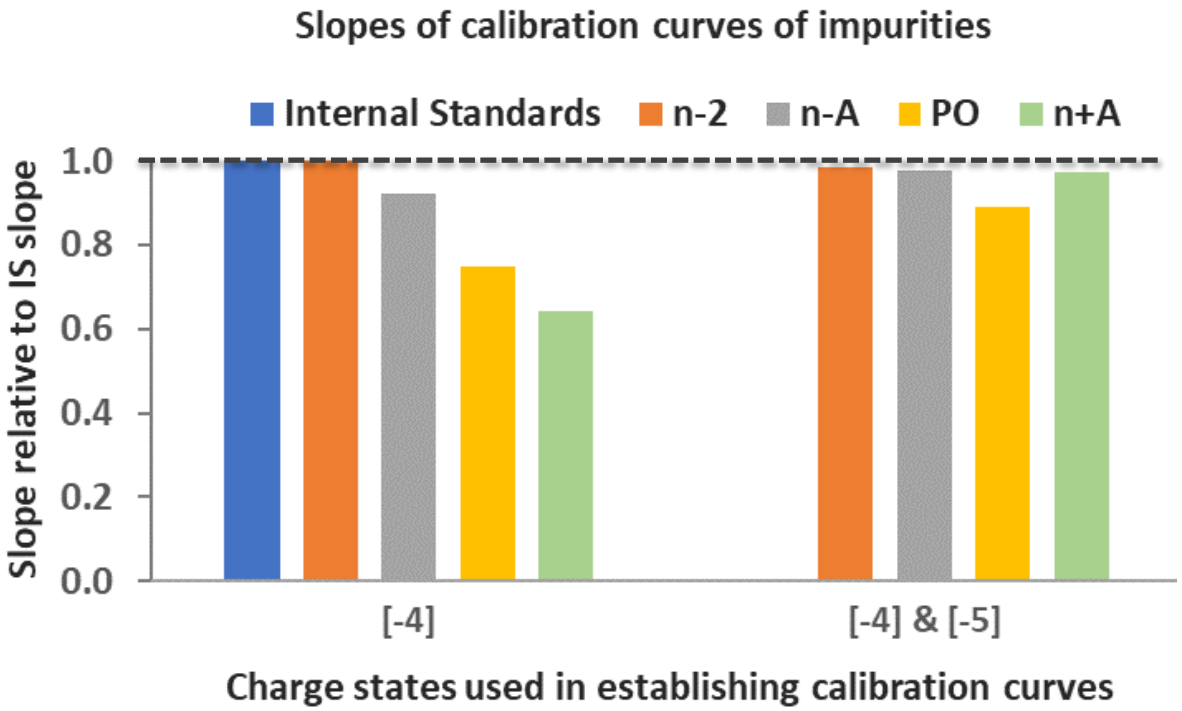
Identity Confidence Improved

Abdullah, AM; Sommers, C.; Hawes, J.; Rodriguez J.; Yang, K. Tandem Mass Spectrometric Sequence Characterization of Synthetic Thymidine-rich Oligonucleotides. Journal of Mass Spectrometry. 2022, 57 (4), e4819

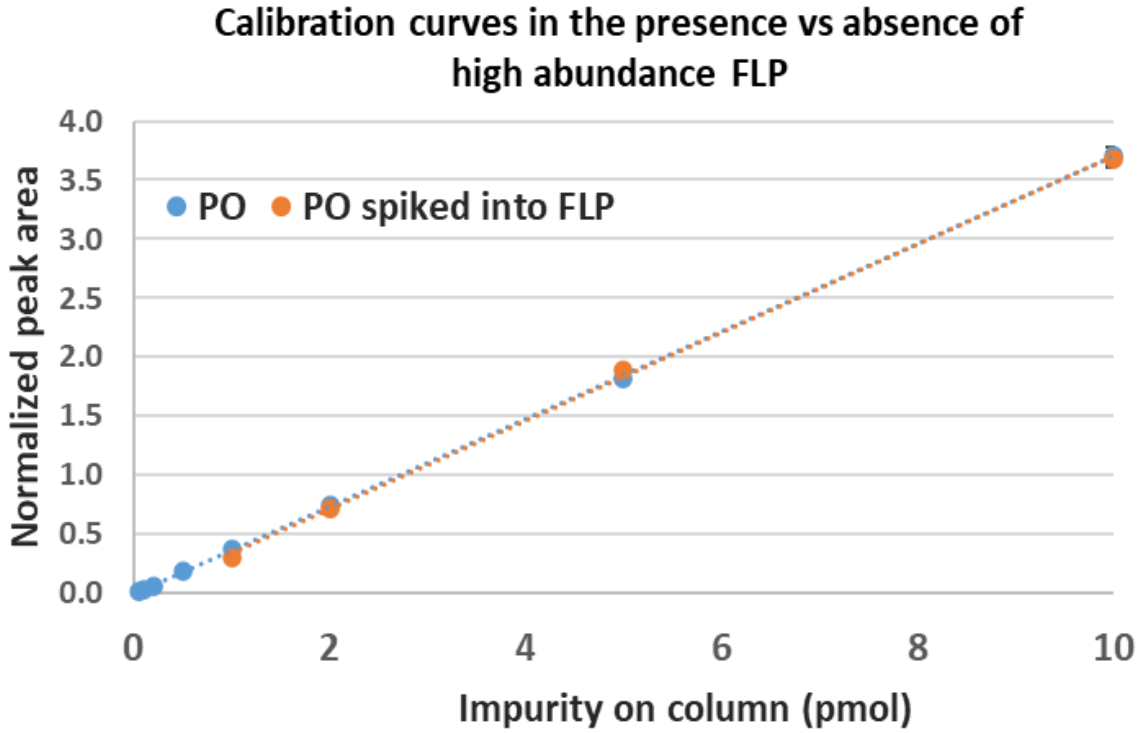


Quantification: Assay, Purity, Impurities

➢ Ionization efficiency



➢ Ion suppression



n-2, n-A, 1PO, n+A: common impurities of nusinersen (18-mer)
ISs: U_{15}, A_{15}, C_{15}

FLP: full-length product (nusinersen sequence)

LC-HRMS Method Validation

- ✓ Hydrophilic interaction liquid chromatography (HILIC)-HRMS
  - System suitability testing
  - Specificity
  - Linearity
  - Range
  - Precision
  - Accuracy
  - LLOQ
  - Robustness
  - Excipient

Q2(R2) Validation of Analytical Procedures

Guidance for Industry

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

March 2024
ICH-Quality
Revision 2

M10 BIOANALYTICAL METHOD VALIDATION AND STUDY SAMPLE ANALYSIS

Guidance for Industry

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

November 2022
ICH

System suitability testing

- Sensitivity check
  - LLOQ (peak area % CV)
  - Before and after sample assay
- Precision
  - Injector performance (peak area % CV)
  - Pump performance (retention time % CV)
  - Column performance (peak symmetry factor)
- Mass accuracy compared to theoretical value
  - ppm
- Linearity of injection accuracy and MS response
  - R^2
  - Before and after sample assay

Method validation

Specificity, Linearity, Range, **Precision**, **Accuracy**, LLOQ, Robustness, Excipient

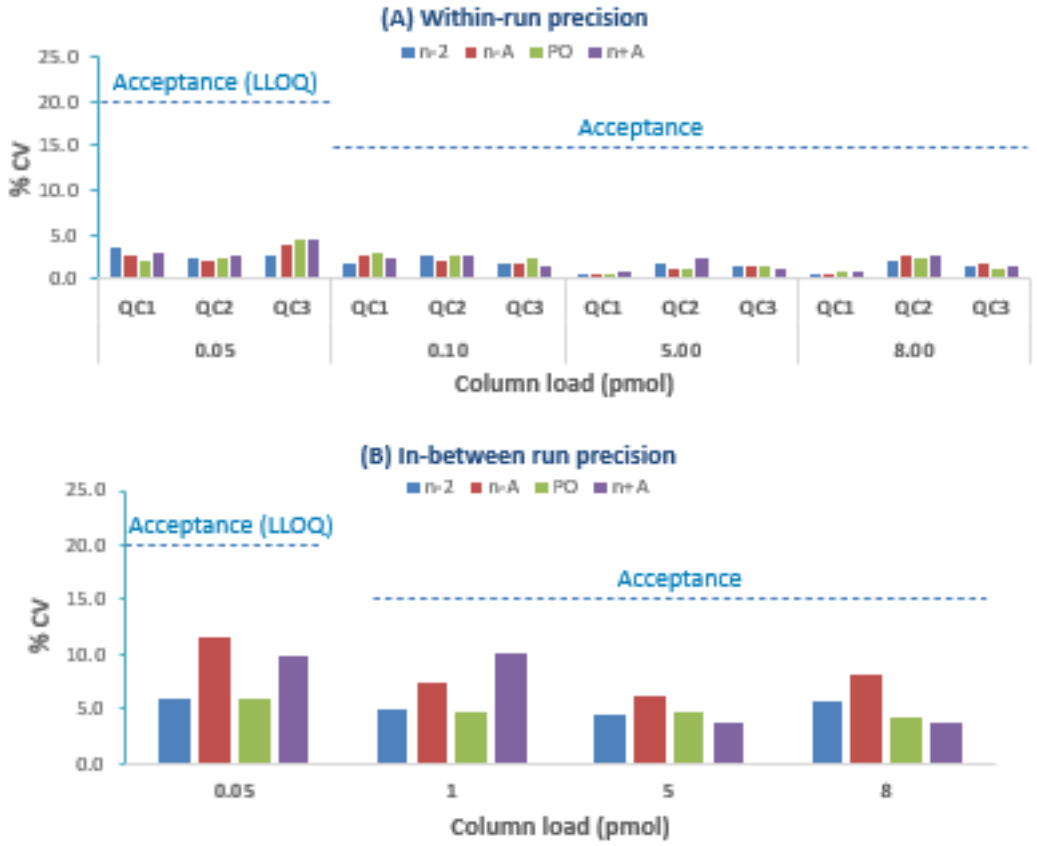


Fig. Precision (% CV) evaluated using 4 QC levels (0.05, 0.1, 5 and 8 pmol) run 3 different days (QC1, QC2 and QC3). (A) Within-run; and (B) In-between run

Table. % Recovery for three QC runs (QC1, QC2 and QC3) each comprising 4 QC levels

(A) % Recovery for n-2

Column load (pmol)	QC1	QC2	QC3
0.05	**93.69**	102.37	94.42
0.10	96.87	99.33	99.87
5.00	**104.47**	98.67	100.53
8.00	101.74	101.25	97.91

(B) % Recovery for n-A

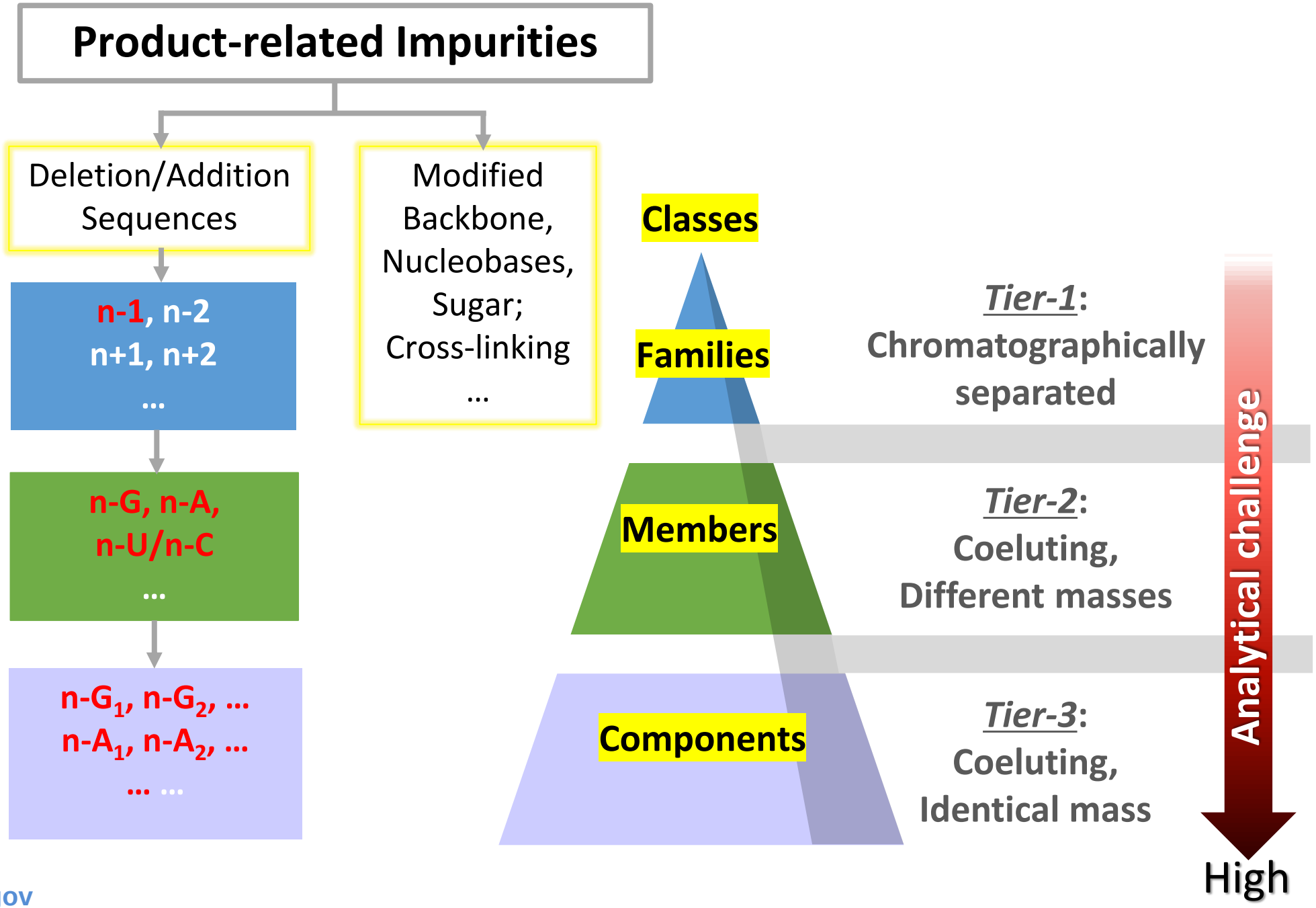
Column load (pmol)	QC1	QC2	QC3
0.05	**86.57**	**104.40**	87.61
0.10	94.20	100.68	103.79
5.00	98.91	101.80	99.66
8.00	95.97	104.29	96.86

(C) % Recovery for PO

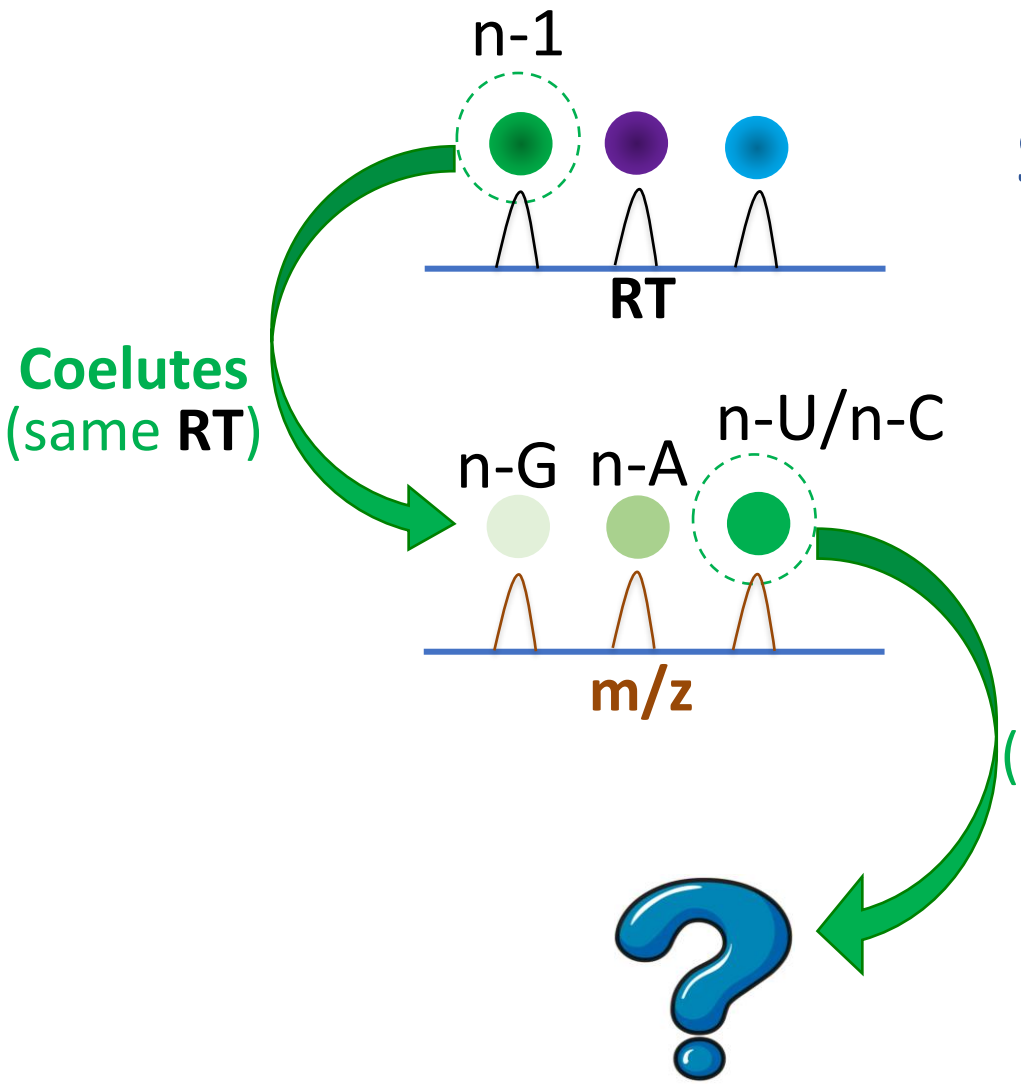
Column load (pmol)	QC1	QC2	QC3
0.05	89.31	**104.77**	95.55
0.10	**88.34**	97.00	96.97
5.00	99.24	94.92	104.31
8.00	95.95	97.08	100.79

(D) % Recovery for n+A

Column load (pmol)	QC1	QC2	QC3
0.05	**86.40**	101.06	97.08
0.10	81.37	96.19	105.34
5.00	100.92	97.40	108.96
8.00	97.86	98.77	**105.70**


Product-related Impurities
Deletion/Addition Sequences
Modified Backbone, Nucleobases, Sugar; Cross-linking …
n-1, n-2
n+1, n+2
…
n-G, n-A,
n-U/n-C
…
n-G1, n-G2, …
n-A1, n-A2, …
… …
Classes
Families
Members
Components
Tier-1: Chromatographically separated
Tier-2: Coeluting, Different masses
Tier-3: Coeluting, Identical mass
Analytical challenge
High

When Separation by LC or MS Fails



Separable by **LC**

Separable by **MS**

Isobars/Isomers
(same or similar **m/z**)

Inseparable by either **LC** or **MS**

Cases: n-U/n-C, positional isomers

Coeluting isobaric impurity ions

analytical chemistry

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Article

Decoding Complexity in Synthetic Oligonucleotides: Unraveling Coeluting Isobaric Impurity Ions by High Resolution Mass Spectrometry

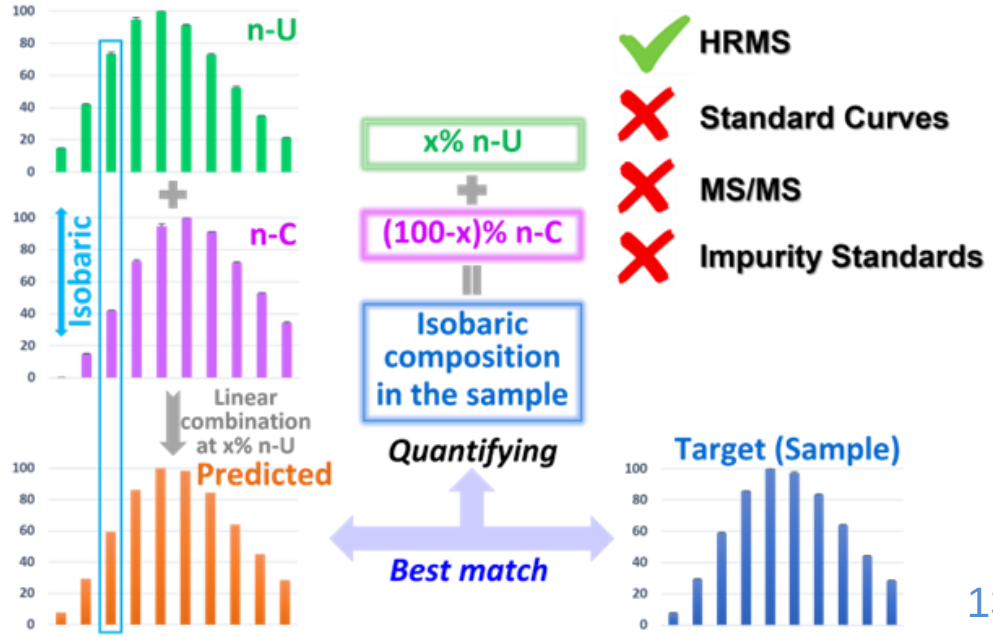
A. M. Abdullah, Cynthia Sommers, Jason D. Rodriguez, Deyi Zhang, Darby Kozak, Jessica Hawes, Mohan Sapru, and Kui Yang*

Cite This: *Anal. Chem.* 2024, 96, 904–909

Read Online

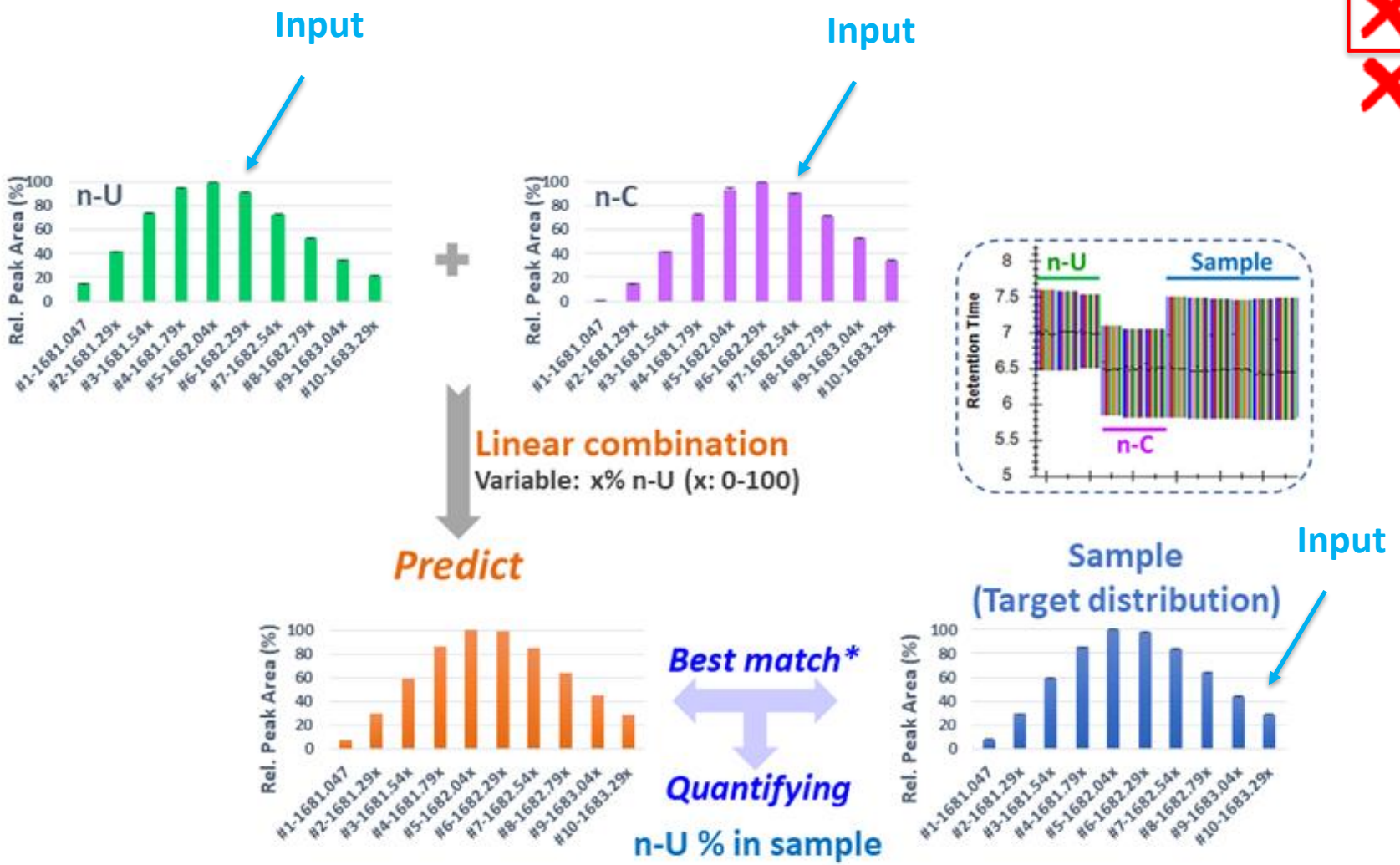
Nusinersen sequence:

MOEmU*MOEmC*MOEA*MOEmC*MOEmU*MOEmU*MOEmU*MOEmC*MOEA*MOEmU*MOEA*MOEA*MOEmU*MOEG*MOEmC*MOEmU*MOEG*MOEG



Coeluting isobaric impurity ions

- ✓ HRMS
- ✗ Standard Curves
- ✗ MS/MS
- ✗ Impurity Standards



Workflow

Coeluting isobaric impurity ions

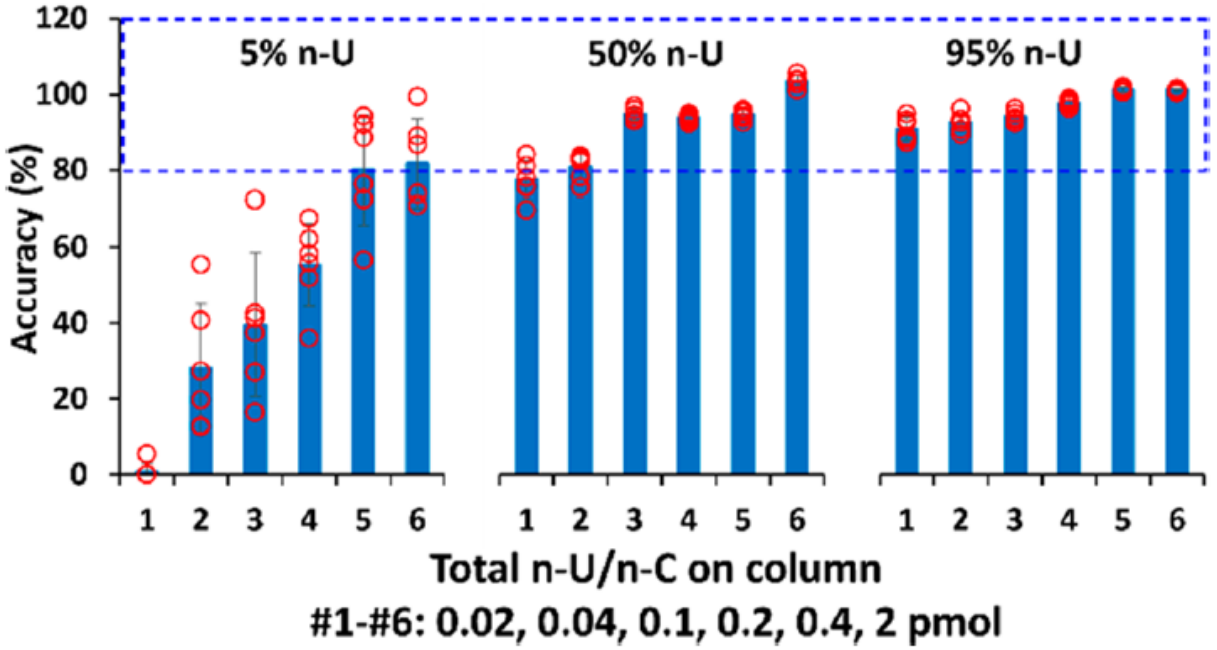


Figure 3. Quantified isobaric compositions of n-U/n-C mixtures at varied concentrations. The bar represents the mean and standard deviation (SD) values of replicate data points (red circles). The frame represents the same as that in Figure 2.

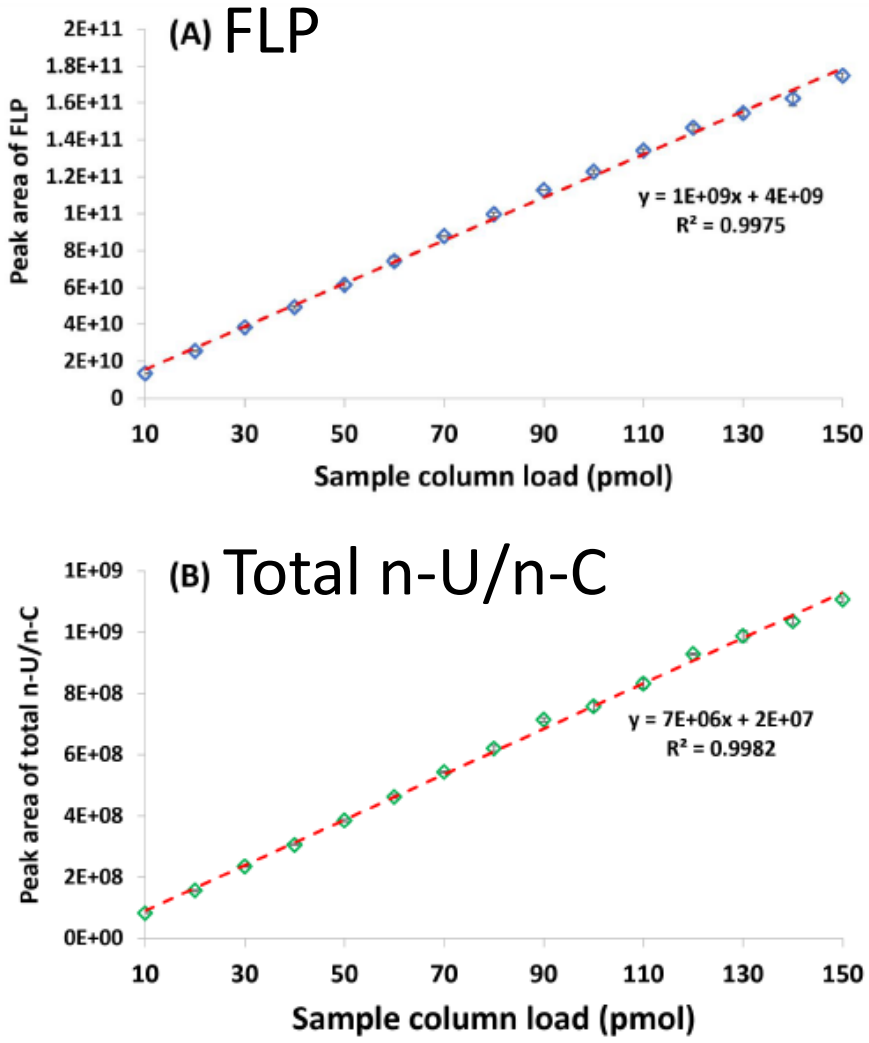


Figure S5. Linear responses in quantitation for FLP and total n-U/n-C impurity within the tested range of sample column loads ranging from 10 to 150 pmol. Quantitation was performed using full MS by total EIC peak area of 10 isotopic peaks for FLP (A) and n-U/n-C (B). Dashed line represents the linear regression.

Coeluting isobaric impurity ions

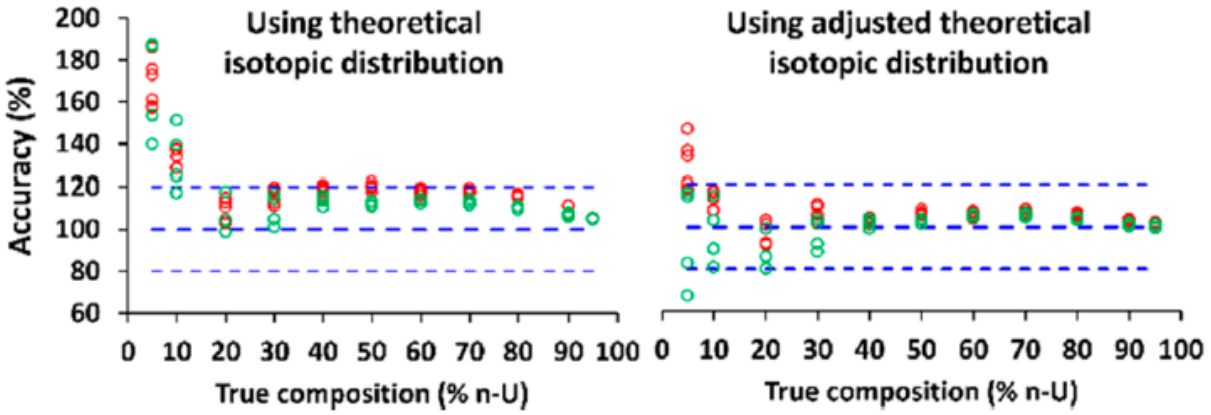


Figure 5. Quantified isobaric compositions of n-U/n-C mixtures using the theoretical isotopic distributions of standards. The same HRMS data as in Figure 2 were reprocessed by replacing the measured isotopic distributions with the theoretical (left panel) or the adjusted theoretical (right panel) distributions of n-U and n-C standards. The quantification results are displayed using the same accuracy plots as denoted in Figure 2A.

Coeluting isobaric impurity ions

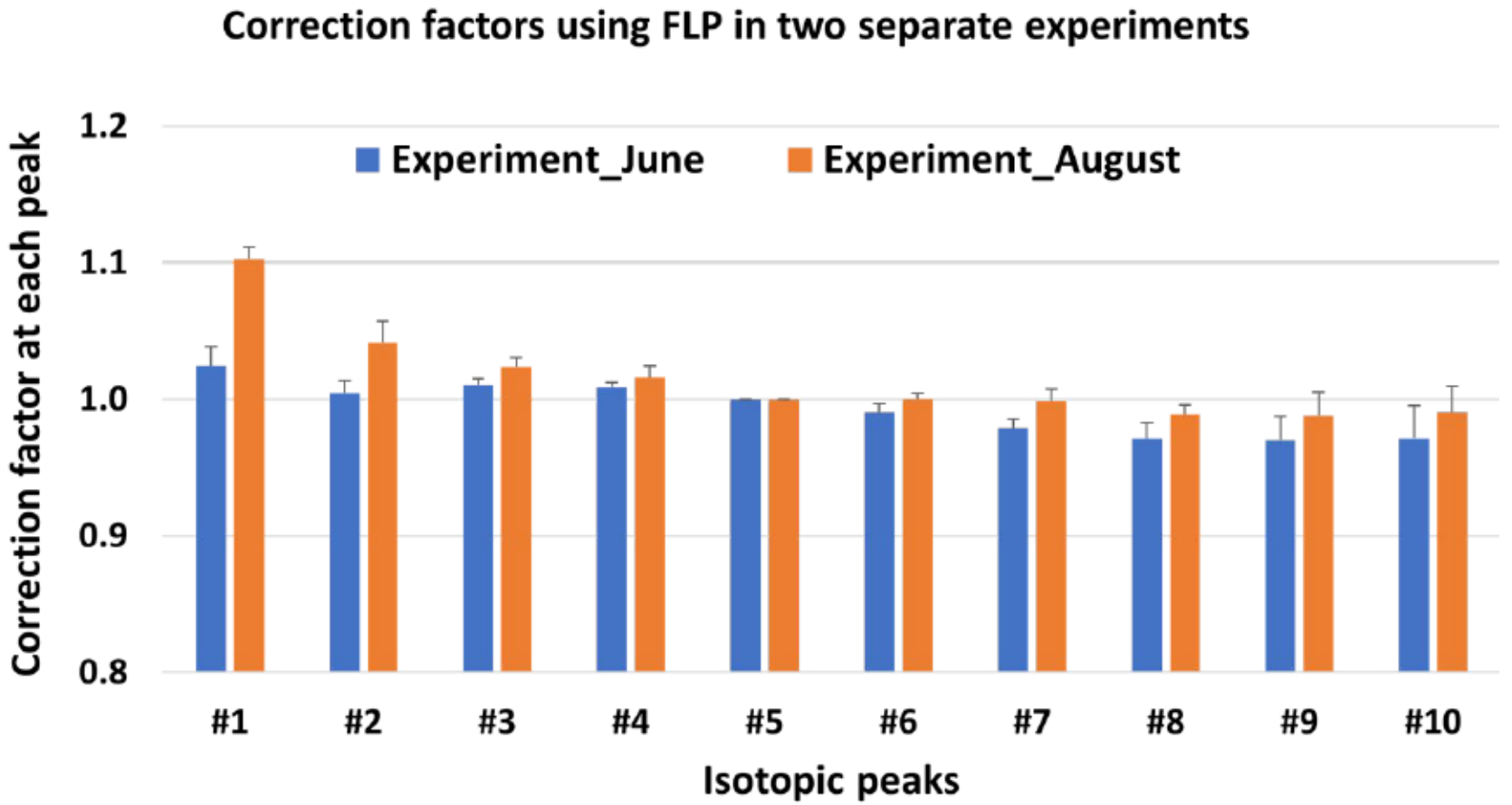


Figure S9. Comparison of correction factors generated by the ratio of the measured *vs* the theoretical isotopic distributions of FLP from two separate experiments. The measured isotopic distributions were from multiple FLP solution preparations in two separate experiments (Table S4B and Table S4C). The experiment-specific correction factors may be attributed to the instrument's performance while operating under its current tune and calibration settings.

Coeluting isobaric impurity ions

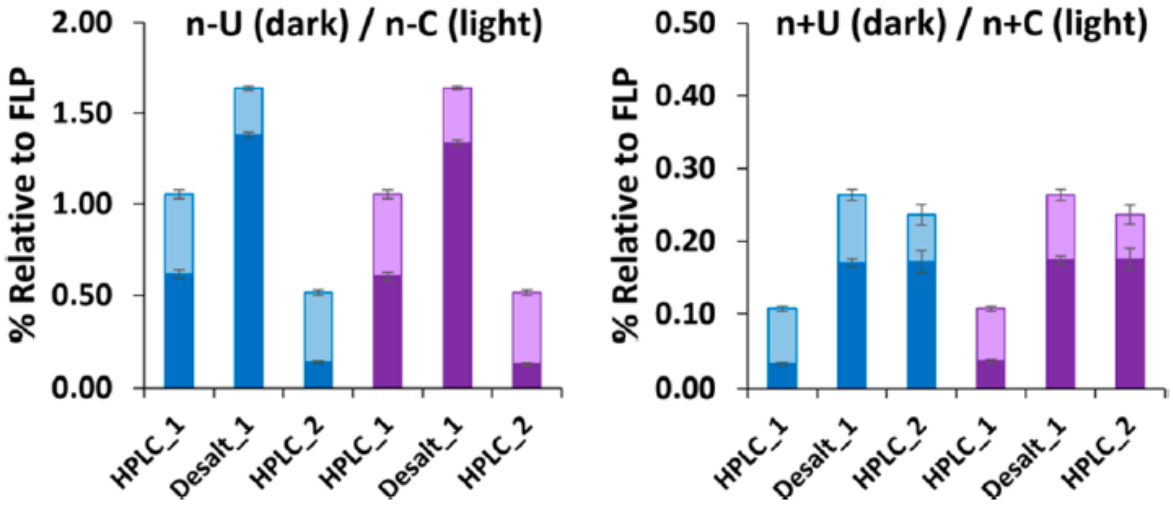
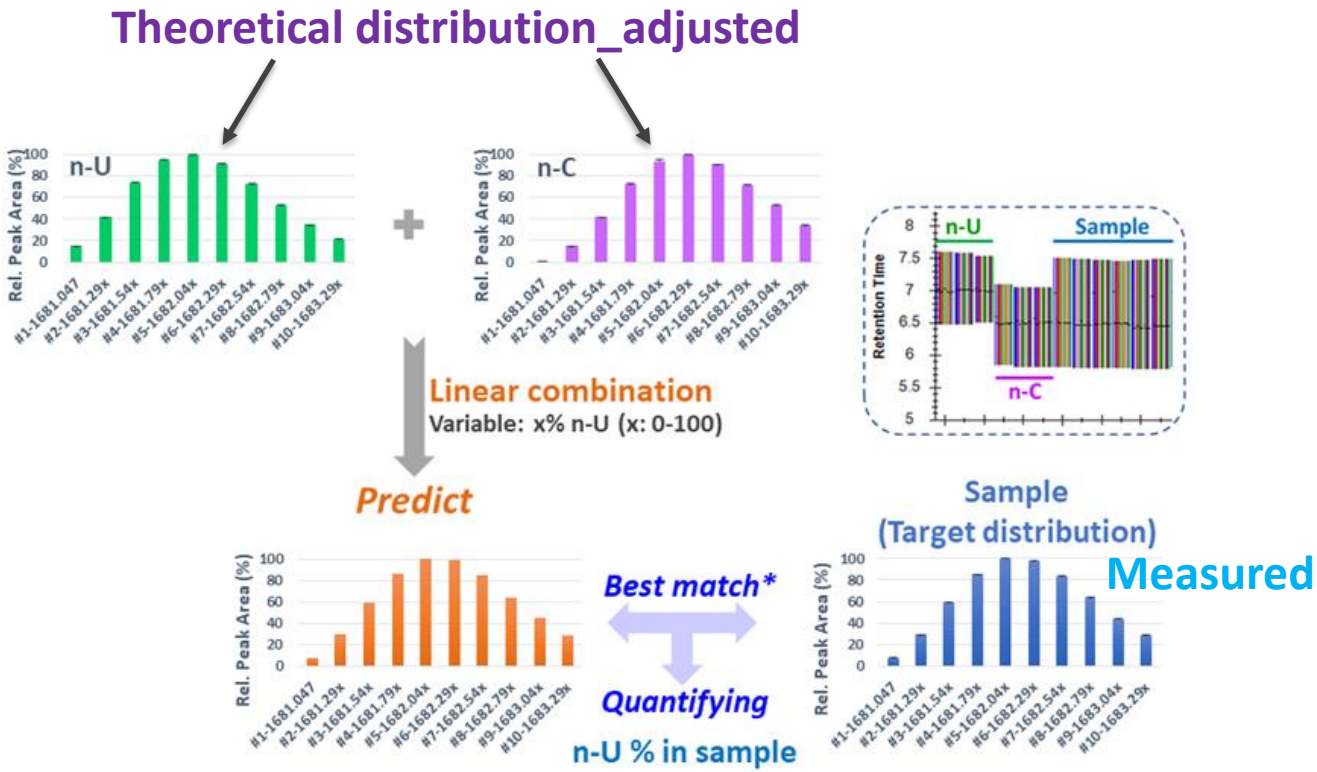
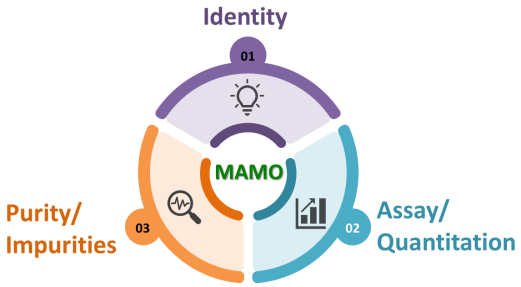


Figure 6. Quantification of U/C deletion and addition impurities using the measured or adjusted theoretical isotopic distributions of n-U/n-C and n+U/n+C. (Left panel) n-U% (dark in color) and n-C% (light). (Right panel) n+U% (dark) and n+C% (light). The *y*-axis represents quantified impurity amounts relative to FLP. Quantification was performed using the measured (in blue) or adjusted theoretical (in purple) isotopic distribution of n-U/n-C or n+U/n+C. The correction factors used for adjusting the theoretical distributions were generated using the measured vs theoretical isotopic distributions of FLP. Three batches of sample were tested, including two HPLC-purified batches and one desalted batch.



Summary



LC-HRMS-based MAMO platform:

Identity/characterization: RT, m/z, MS/MS

Quantification: Assay, Purity, Impurities

LC-HRMS method validated for:

Specificity, Linearity, Range, Precision, Accuracy, LLOQ, Robustness, Excipient

When impurities inseparable by either LC or HRMS:

Coeluting isobaric impurity ion case –
Fully resolved isotopic envelopes enabled by HRMS

Acknowledgement

- **Office of Pharmaceutical Quality Research (OPQR)**
  - ✓OPQR-St. Louis
  - ✓ Oligo research team (including **ORISEs***)
- **Office of Generic Drugs (OGD)**
  - ✓ORISE funds
  - ✓ORS – ORISE co-mentoring

*** FDA ORISE Fellowship Program at CDER** through an agreement between the U.S. DOE and FDA

- **OPQ-OGD complex product PSG development Oligo SME triage team**
  - ✓ Lead: OPQR
  - ✓ Team: OPQ, OGD, OND
- **FDA Research Grants (Critical Path, CSG grant)**
  - ✓ PI: OPQR
  - ✓ Co-PIs and collaborators: OPQ, OGD, OND, NCTR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