High-Resolution Ion Mobility Mass Spectrometry for Oligonucleotide Impurity Analysis

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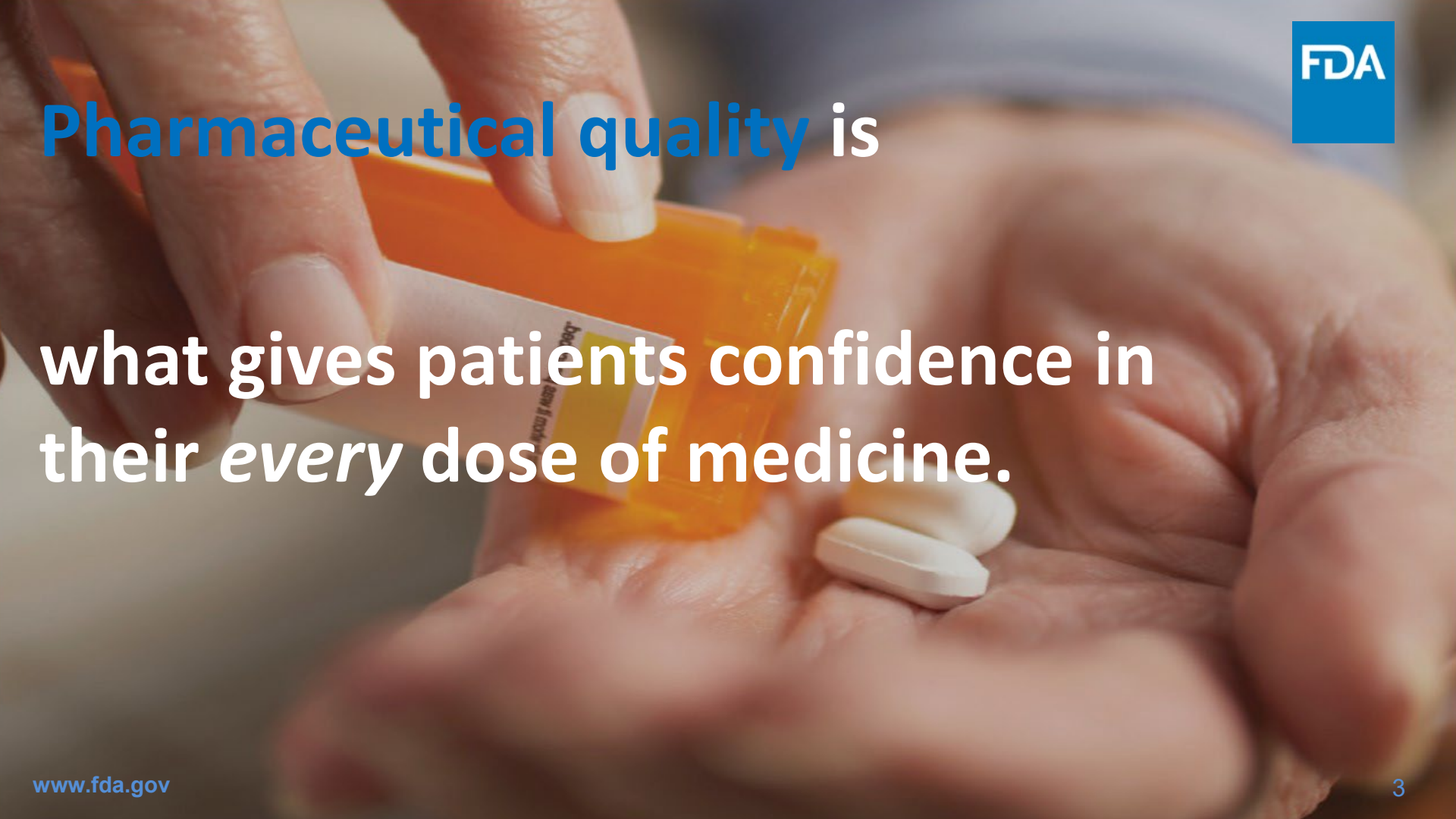
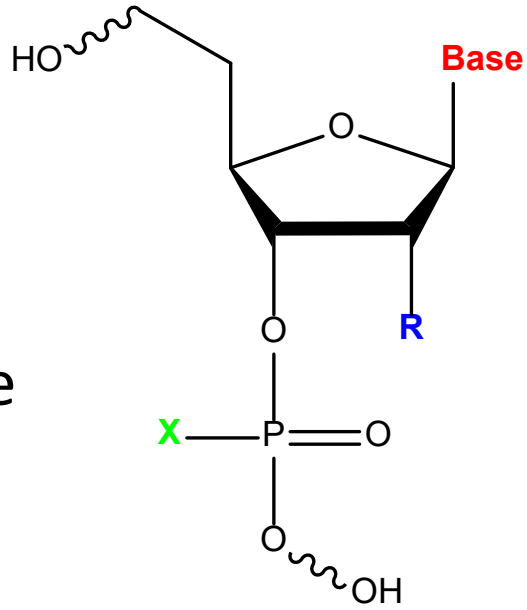

Pharmaceutical quality is
what gives patients confidence in their *every* dose of medicine.

Oligonucleotide Therapeutics (ONTs)

- An evolving class of drugs that can modulate gene expression or hinder protein function by binding to specific mRNA targets.
- Modification can occur at the nucleobase, sugar, and internucleotide linkage.
- Solid-phase synthesis consists of repetitive synthetic cycles, each cycle including multiple steps. Failure in any step may lead to formation of impurities



Analytical Challenges of ONTs

- Product-related impurities produced during synthesis can be structurally closely related. Examples include:
  - Deletion sequences
  - Addition sequences
  - P=O impurities
  - Deamination impurities
- Separation of impurities from desired product or from each other can be challenging, particularly for LC or MS-inseparable isomeric and isobaric molecules.

Limitations of Current Analytical Approach

IP-RP LC/UV-MS

- Ion-pair reversed phase (IP-RP) LC involves ion pair reagents that can lead to MS signal suppression.
- Structurally similar impurities may not be fully LC resolved.
- Isobaric and isomeric molecules may be inseparable by either LC or MS dimension.

Recent development of methods free of IP reagents: HILIC-MS

- ASMS poster **#313427** (Rabiul Islam) – method validation
- ASMS WOD pm **#312952** (AM Abdullah) – data processing
- ASMS ThOH am **#313595** (Kui Yang) – MAMO platform

An Orthogonal Dimension to LC/MS

Ion mobility

- Adds an extra dimension of separation by separating molecules based off size, shape, and charge.
- Potential of separating LC-MS inseparable molecules.
- Provides collision cross section (CCS) of a molecule as a molecular characteristic.

Collision Cross Section (CCS)

- CCS describes the collision between the ion and the buffer gas, and gives direct information about the conformation of the ion travelling through the drift region.
- Mobility or drift time (**DT**) is measured and converted to CCS using the Mason-Schamp equation for drift tube ion mobility.
- Cyclic-IM requires calibration to extract CCS values.

$$\Omega = \frac{\frac{3}{16}\left(\frac{2\pi}{\mu k_b T}\right)^{\frac{1}{2}} ze}{N_0 K_0}$$

e , charge of an electron
z , ion charge
N_0 , buffer gas density
μ , reduced mass of collision partners
K_0, mobility
T , drift region temperature
k_b , Boltzmann's constant

Materials and Methods

- Instrumentation: Cyclic IMS (Waters)
- Software: MassLynx, DriftScope
- Model molecules: full-length product (FLP), and impurities

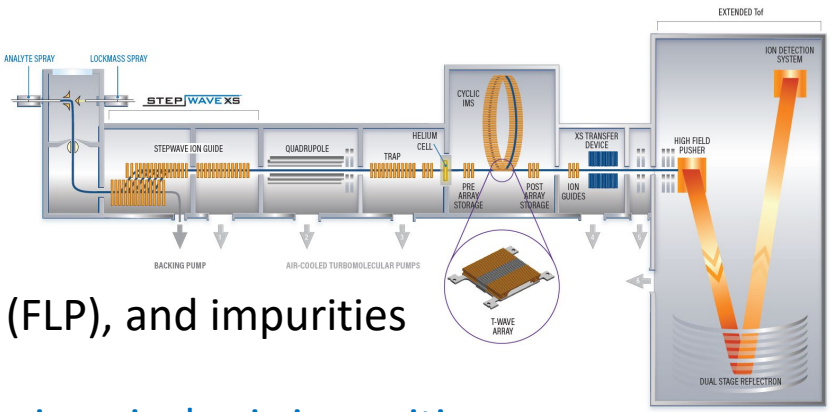
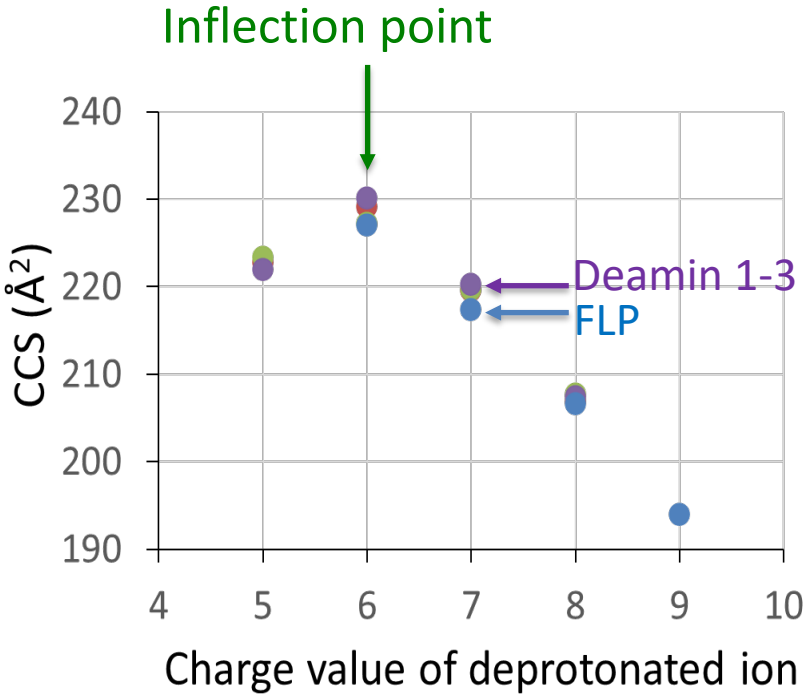


Table 1. Custom-synthesized FLP and isomeric or isobaric impurities

Name	Sequence	Theoretical Molecular Weight (g/mol)
FLP*	UCACUUUCAUAAUGCUGG (nusinersen)	7126.2
Deamin_1	UUACUUUCAUAAUGCUGG	7127.2
Deamin_2	UCAUUUUCAUAAUGCUGG	
Deamin_3	UCACUUUUAUAAUGCUGG	
n-G_1	UCACUUUCAUAAU~~G~~CUGG	6706.9
n-G_2	UCACUUUCAUAAUGCUG~~G~~	
n-U_1	~~U~~CACUUUCAUAAUGCUGG	6732.0
n-U_2	UCAC~~U~~UUCAUAAUGCUGG	
n-U_3	UCACUUUCA~~U~~AAUGCUGG	
n-U_4	UCACUUUCAUAA~~U~~GCUGG	
n-U_5	UCACUUUCAUAAUGC~~U~~GG	

*FLP has the same sequence and modifications as nusinersen.

FLP and Deamination Products: CCS



FLP*	UCACUUUCAUAAUGCUGG
Deamin_1	UUACUUUCAUAAUGCUGG
Deamin_2	UCAUUUUCAUAAUGCUGG
Deamin_3	UCACUUUUAUAAUGCUGG

- Deamin 1
- Deamin 2
- Deamin 3
- FLP

FLP is separated slightly from the deamination products.

FLP and Deamination Products: Relative DT shift

	RSD (%) of DT		
Reference:	*-6*	*-7*	*-8*
FLP	0.79	0.64	0.66

	% Relative DT shift		
	-6	*-7*	*-8*
Deamin_1	0.71	0.86	0.08
Deamin_2	0.33	0.92	0.13
Deamin_3	1.10	1.09	0.07

	-6	*-7*	*-8*
Deamin_all	0.40	0.9366	0.02

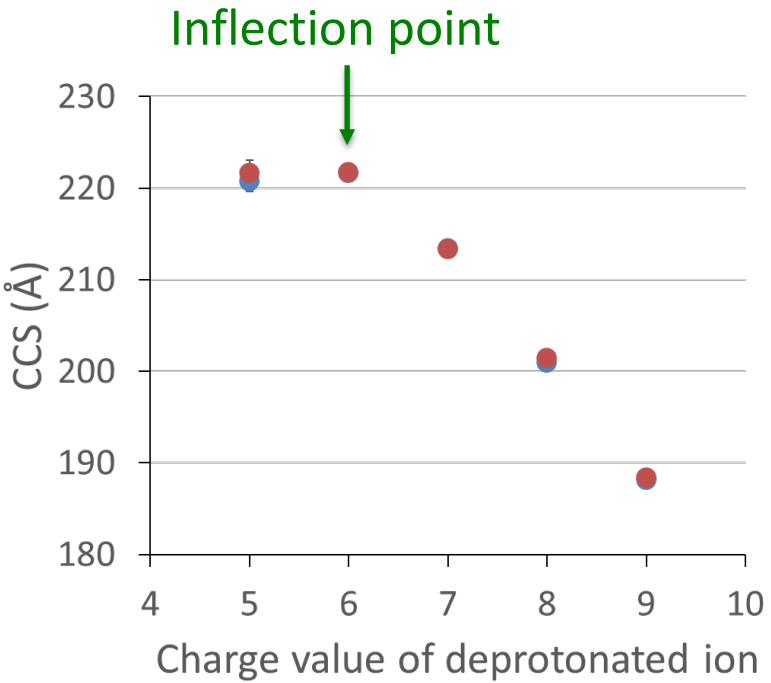
n-fold: ≤ 1 > 1 > 2 > 3

n = **% Relative DT shift / RSD (%) of DT of reference**

**n > 1 or above indicates a detected difference in mobility between a tested compound vs reference.*

% Relative DT shift of all deamination products for $[M - 7H]^{7-}$ exceeds the DT RSD (%) of the FLP, *i.e.*, yellow coded for n > 1.

n-G Impurities (Isomers): CCS

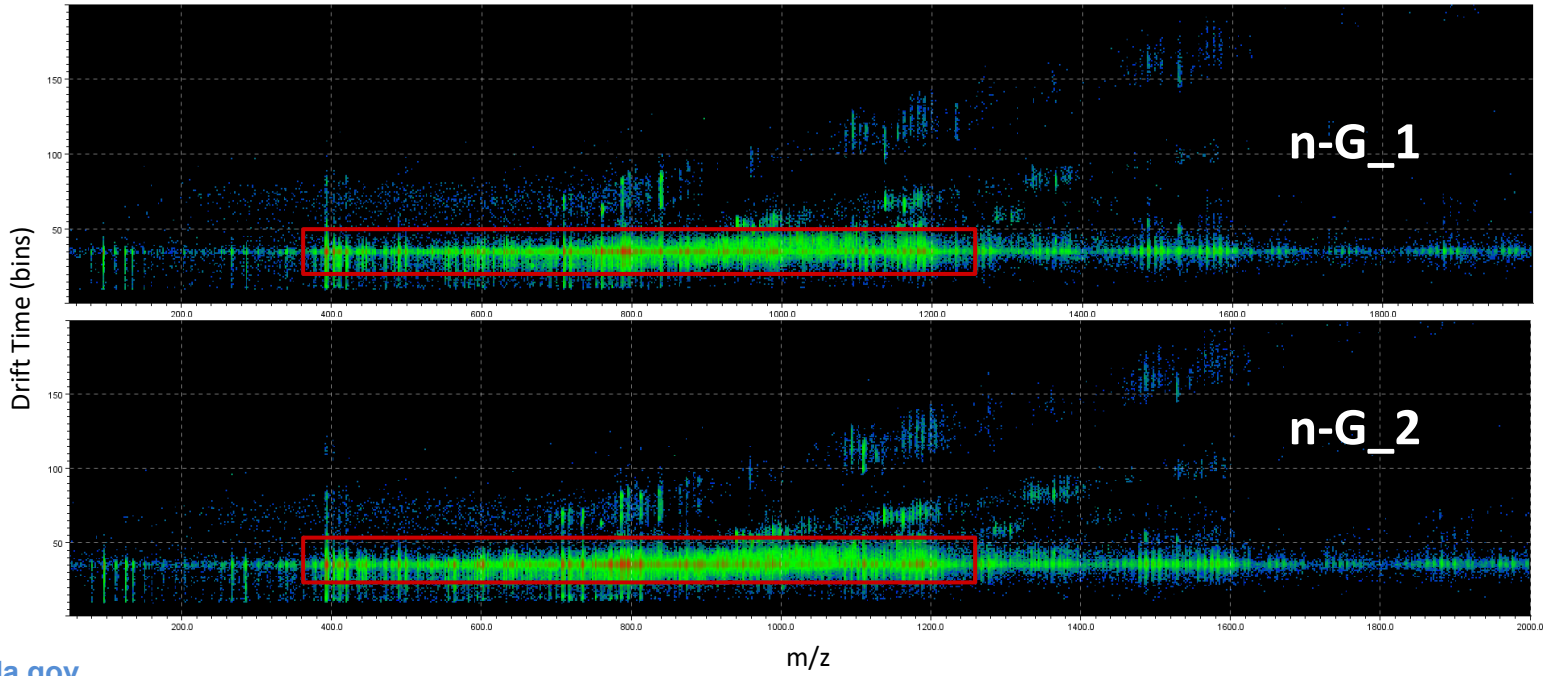


n-G_1	UCACUUUCAUAAUGCUGG
n-G_2	UCACUUUCAUAAUGCUGG

Indistinguishable by IM regardless of charge state.

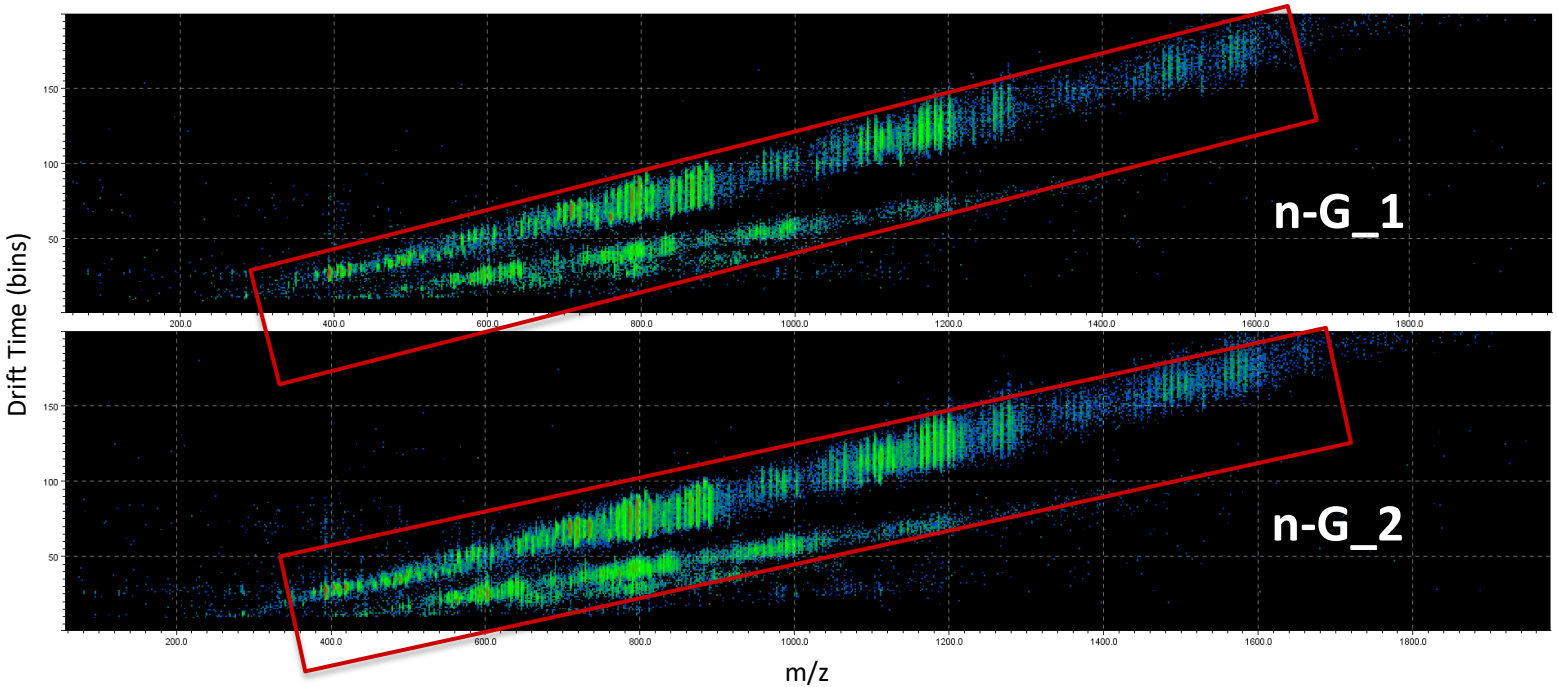
n-G Impurities (Isomers): MS/MS After IM Separation

- Fragments have identical DT that aligns with the DT of precursor ions

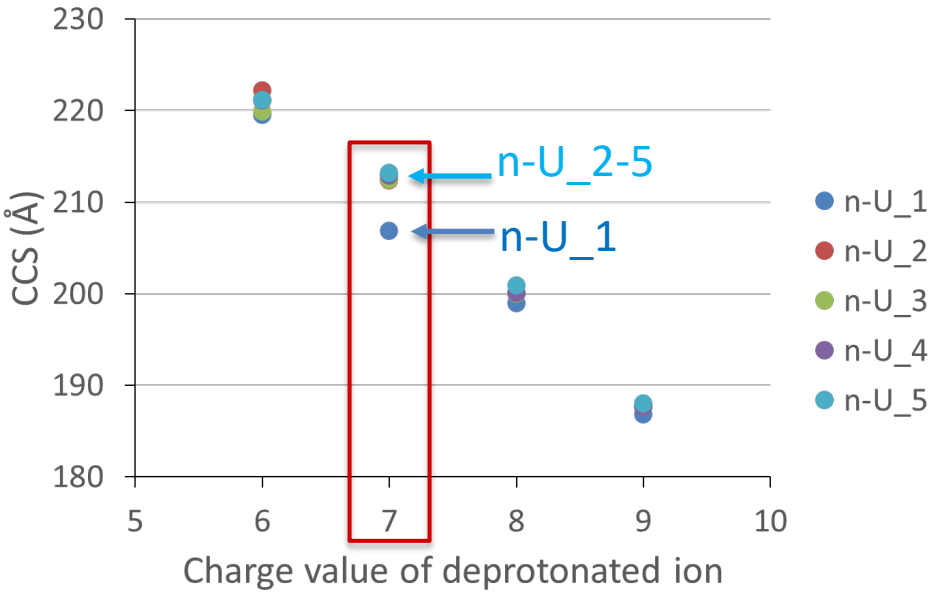


n-G Impurities (Isomers): MS/MS Before IM Separation

- Fragments undergo IM separation. Distinguishable fragments may help differentiate isomeric precursors.



n-U Impurities (Isomers): CCS



n-U_1	~~U~~CACUUUCAUAAUGCUGG
n-U_2	UCAC~~U~~UUCAUAAUGCUGG
n-U_3	UCACUUUCA~~U~~AAUGCUGG
n-U_4	UCACUUUCAUAA~~U~~GCUGG
n-U_5	UCACUUUCAUAAUGC~~U~~GG

n-U_1 (terminal deletion) is separated from the others.

n-U Impurities (Isomers): Relative DT Shift

RSD (%) of DT

Reference:	*-6*	*-7*	*-8*	*-9*
n-U_1	1.1	1.0	1.1	1.1

% Relative DT shift

	-6	*-7*	*-8*	*-9*
n-U_2	1.5	3.3	0.6	0.6
n-U_3	0.2	3.3	0.6	0.5
n-U_4	0.9	3.6	0.7	0.5
n-U_5	0.9	3.8	1.2	0.8

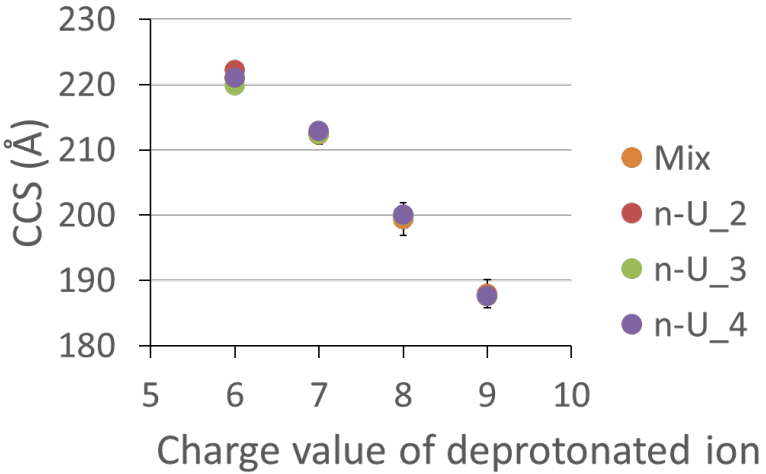
n-fold: ≤ 1 > 1 > 2 > 3

n = **% Relative DT shift / RSD (%) of DT of reference**

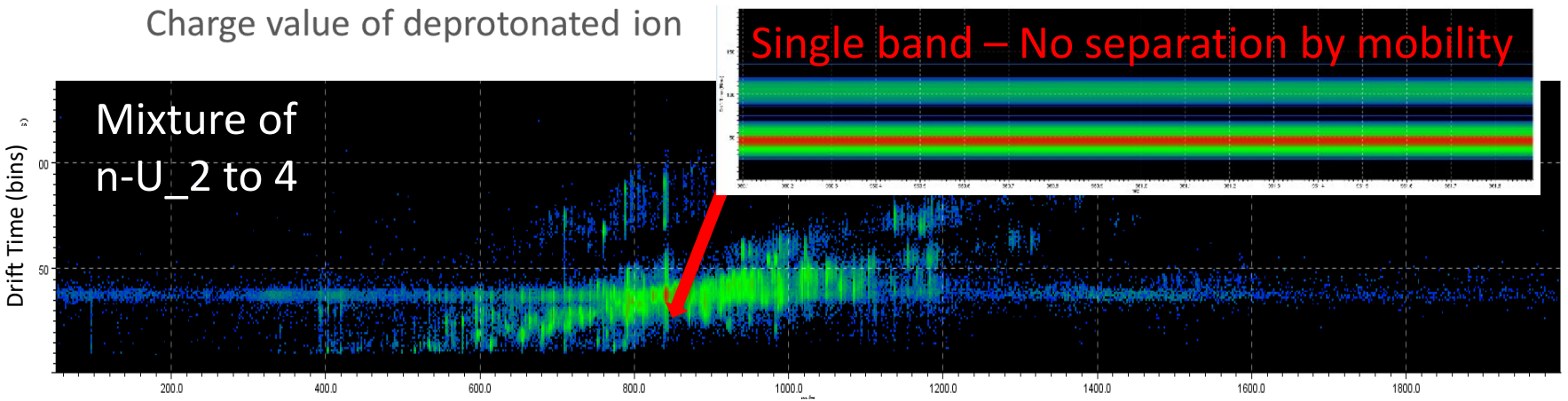
**n > 1 or above indicates a detected difference in mobility between a tested compound vs reference.*

% Relative DT shift of the n-U_2 to 5 isomers exceeds the RSD (%) of n-U_1 by over 3-fold for $[M - 7H]^{7-}$, *i.e.*, red coded for n > 3

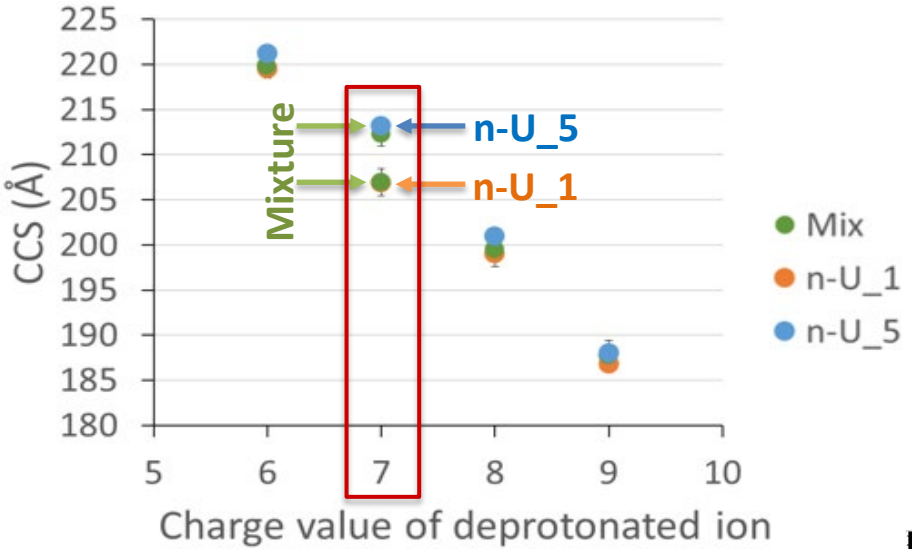
Mixture of n-U Impurities (n-U_2 to 4): CCS



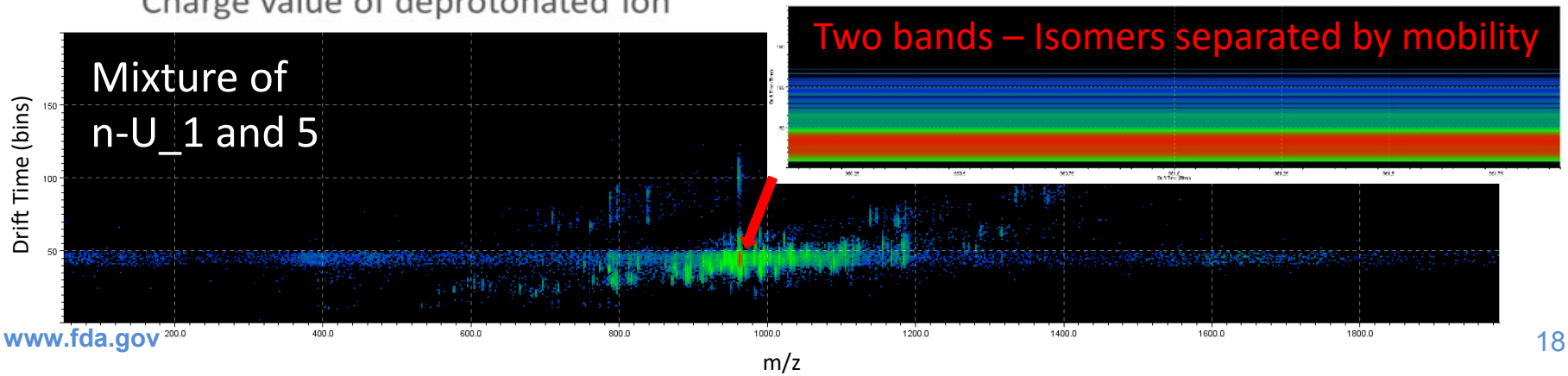
No difference in CCS regardless of charge state.



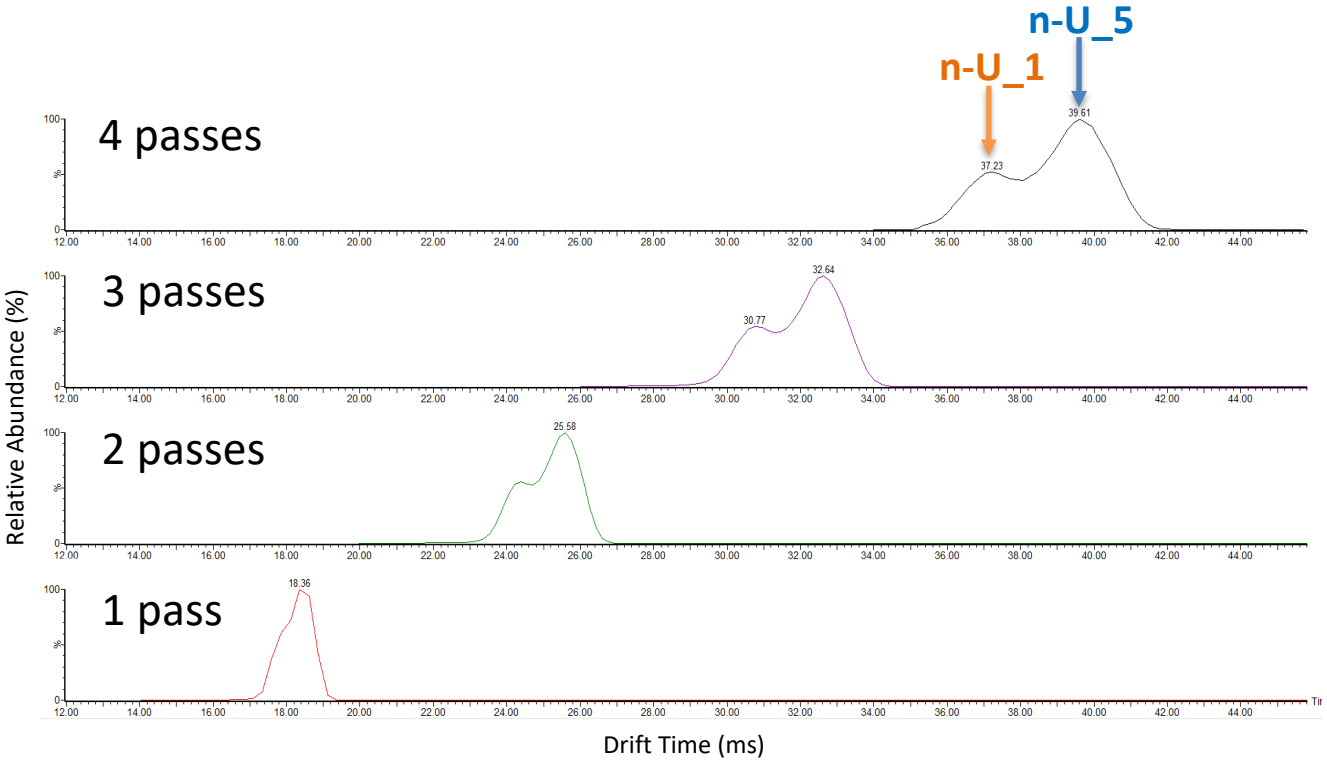
Mixture of n-U Impurities (n-U_1 and 5): CCS



CCS values for the two ion populations of the mixture overlay with those for individual isomers measured separately.



Mixture of n-U Impurities (n-U_1 and 5): cIM Multi-pass



Multi-pass enables better-resolved separation of coeluting isomers.

Summary

- IM provides an additional dimension of separation orthogonal to LC and MS.
- An inflection point is observed in the plot of CCS *vs* charge state of oligonucleotide molecules. CCS decreases inversely with charge state after the point.
- IM separation of isomeric or isobaric molecules may be sequence- and charge state-dependent.
- cIM multi-pass has the potential to improve the separation resolution of structurally similar molecules.

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