# Resolving Complex Impurity Isomers in Synthetic Oligonucleotides by High Resolution Mass Spectrometry

Kui Yang

Division of Pharmaceutical Analysis (DPA)

FDA/CDER/OPQ/OTR

# Pharmaceutical Quality

**A quality product of any kind consistently meets the expectations of the user.**

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**A quality product of any kind consistently meets the expectations of the user.**

**Drugs are no different.**

**Patients expect safe and effective medicine with every dose they take.**

FDA

**Pharmaceutical quality is**

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

FDA

**It is what gives patients confidence in their *next* dose of medicine.**

FDA

# Disclaimer

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

# Outline

❑ **Challenge in impurity analysis of synthetic oligonucleotides – Composite impurity mixtures**

❑ High resolution mass spectrometry in differentiating isomeric impurities

  ➢ High resolution tandem mass spectrometry (HR MS/MS)

  ➢ Ion mobility mass spectrometry (IMMS)

❑ Summary and Q&A

# Synthetic Therapeutic Oligonucleotides – an evolving therapeutic class

- Target a broad range of mRNAs that encode critical cellular proteins ("undruggable")

- "Big small molecules", unique scientific and regulatory challenges

- Currently no ICH or FDA regulatory guidelines

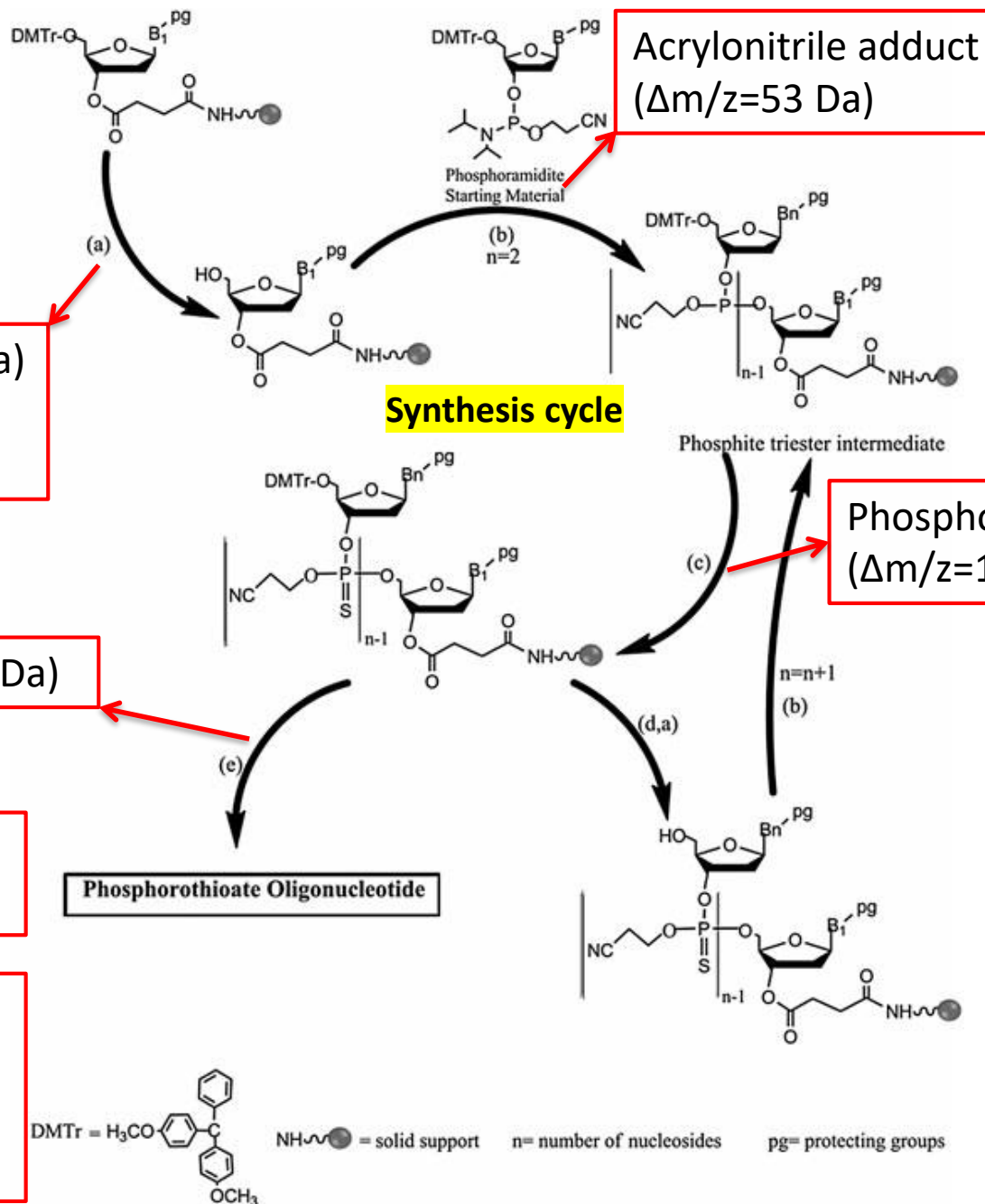# Oligo impurities inherent from synthetic process

Acrylonitrile adduct (Δm/z=53 Da)

**FDA**

Chloral adduct (Δm/z=147 Da)
DMTr adduct (Δm/z=303 Da)
3'-TPT monoester (Δm/z=94)

**Synthesis cycle**

Phosphodiester (Δm/z=16 Da)

Isobutyrul adduct (Δm/z=71 Da)

Shortmers: n-1, n-2, etc.
Longmers:  n+1, n+2, etc.

Depurinated: Δm/z=135 (A)
151 (G)
Deaminated: Δm/z=1
Modified cytosine: Δm/z=80

El Zahar NM et al. Biomedical Chromatography, 2018, 32:e4088

Phosphoramidite Starting Material

Phosphite triester intermediate

Phosphorothioate Oligonucleotide

DMTr = H_3CO—〈phenyl〉C〈phenyl〉—OCH_3        NH~〇 = solid support        n= number of nucleosides        pg= protecting groups

(a) Deprotection, (b) Coupling, (c) Sulfurization, (d) Capping, (e) Cleavage, deprotection, purification, isolation
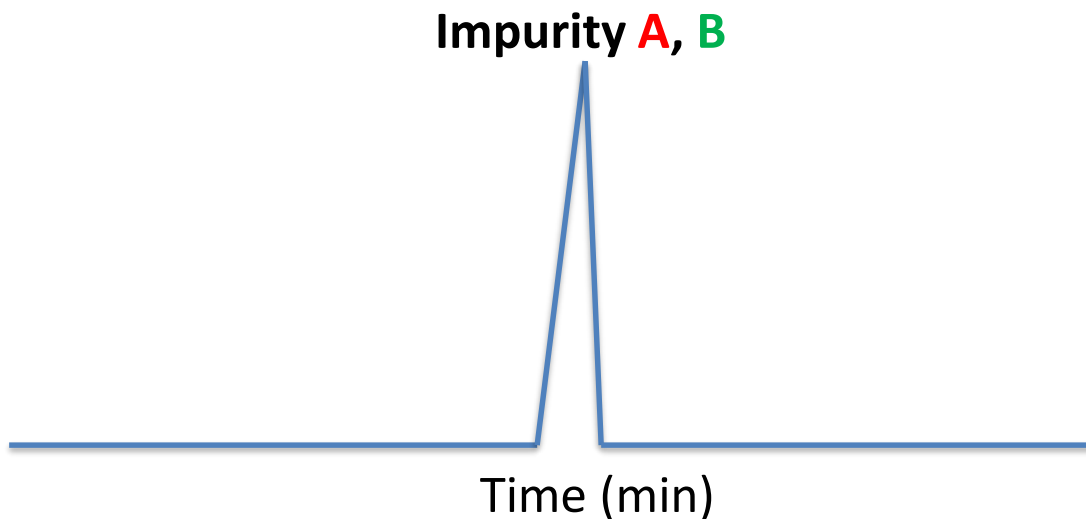
# Importance of impurity profile testing

FDA

Manufacturing ⇄ **Oligo impurity profile** ⇄ Quality and safety
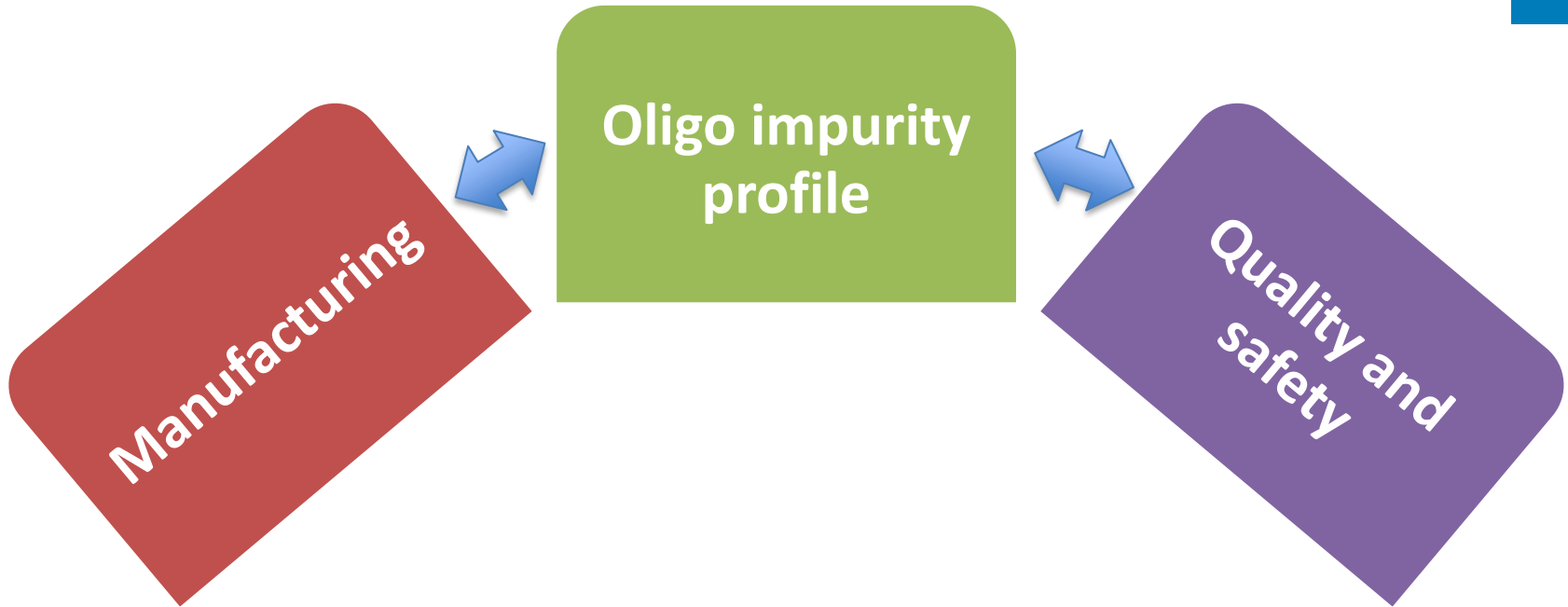
## Challenging:

Structural complexity, large size, high number of negative charges, presence of numerous diastereoisomers
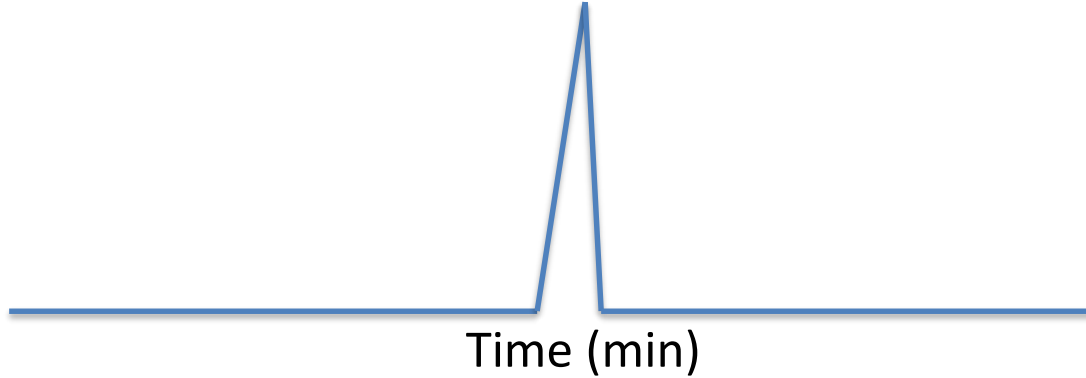
11

# Impurity analysis by LC/MS

**Impurity A**
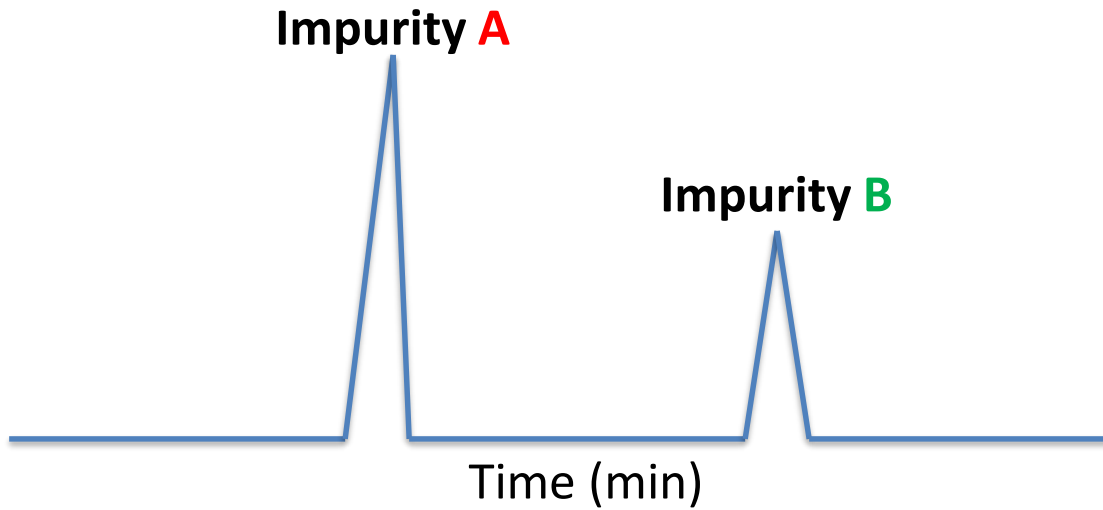
**Impurity B**

**Separated Chromatographically**

Time (min)

**Impurity A, B**

**Coeluting**
(Example: n-1 family)

Time (min)

**Impurity A, B**

Time (min)

**Coeluting**
(Example: n-1 family)

*Case I*

**Impurity A**

**Impurity B**

m/z
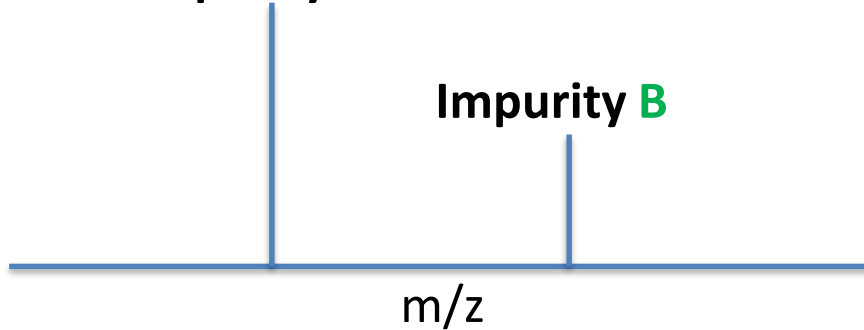
**Mass: Impurity A ≠ Impurity B**
Example: n-T vs n-A

**Mass Spectrometry (MS)**

*Case II*

**Impurities A, B**

m/z
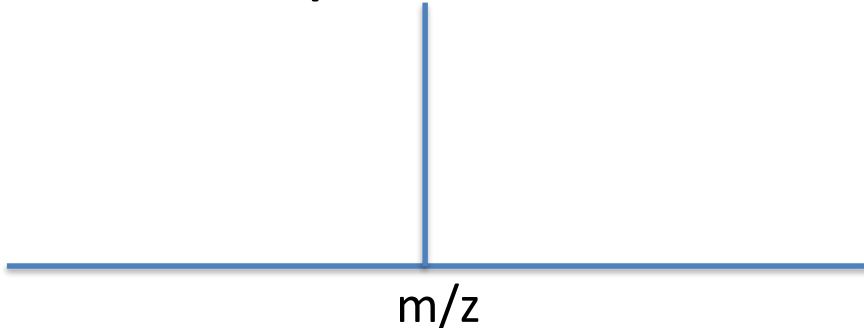
**Mass: Impurity A = Impurity B**
Example: n-T positional isomers

**Challenging**

# Examples of positional isomers

**FDA**

TCACTTTCATAATGCTGG (18-mer)

| Family | Sub-family | max # of isomeric components |
|---|---|---|
| Total n-1 | n-MOE G | 2 |
| | n-MOE A | 3 |
| | n-MOE Me-C | 4 |
| | n-MOE Me-U | 5 |
| Total n+1 | n+MOE G | 2 |
| | n+MOE A | 3 |
| | n+MOE Me-C | 4 |
| | n+MOE Me-U | 5 |
| Full-length (P=O)1 | | 17 |
| Dithioate | | 17 |
| Total Abasic | n-guanine+H2O | 3 |
| | n-adenine+H2O | 4 |
| | n-methylcytosine+H2O | 4 |
| Deaminated | | 4 |
| Total | 14 | 77 |

# Potential applications of resolving composite positional isomers

- Manufacturing process optimization and control

- Batch-to-batch reproducibility assessment

- Impurity formation mechanism, leading to higher purity oligonucleotide drugs

S. Roussis, M Pearce, C. Rentel J. Chromatogr. A (2019), 105-111

# Custom synthesized representative isomers

DNA sequence: TCACTTTCATAATGCTGG (18-mer)

n-T isomers:
n-T_1: TCACTTTCATAATGCTGG
n-T_2: TCACTTTCATAATGCTGG
n-T_3: TCACTTTCATAATGCTGG
n-T_4: TCACTTTCATAATGCTGG
n-T_5: TCACTTTCATAATGCTGG

# **Outline**

❑ Challenge in impurity analysis of synthetic oligonucleotides – Composite impurity mixtures

❑ **High resolution mass spectrometry in differentiating isomeric impurities**

    ➢ **High resolution tandem mass spectrometry (HR MS/MS)**

    ➢ Ion mobility mass spectrometry (IMMS)

❑ Summary and Q&A

# To address isomer challenge
○ **Approach I: HR MS/MS**



intact | pieces

MS1    Collision Cell    MS2

Targeted Precursor m/z Isolation | Precursor m/z Fragmentation | All Fragment m/z Analysis

Q    HCD    Orbitrap

MS/MS spectrum    Quantification
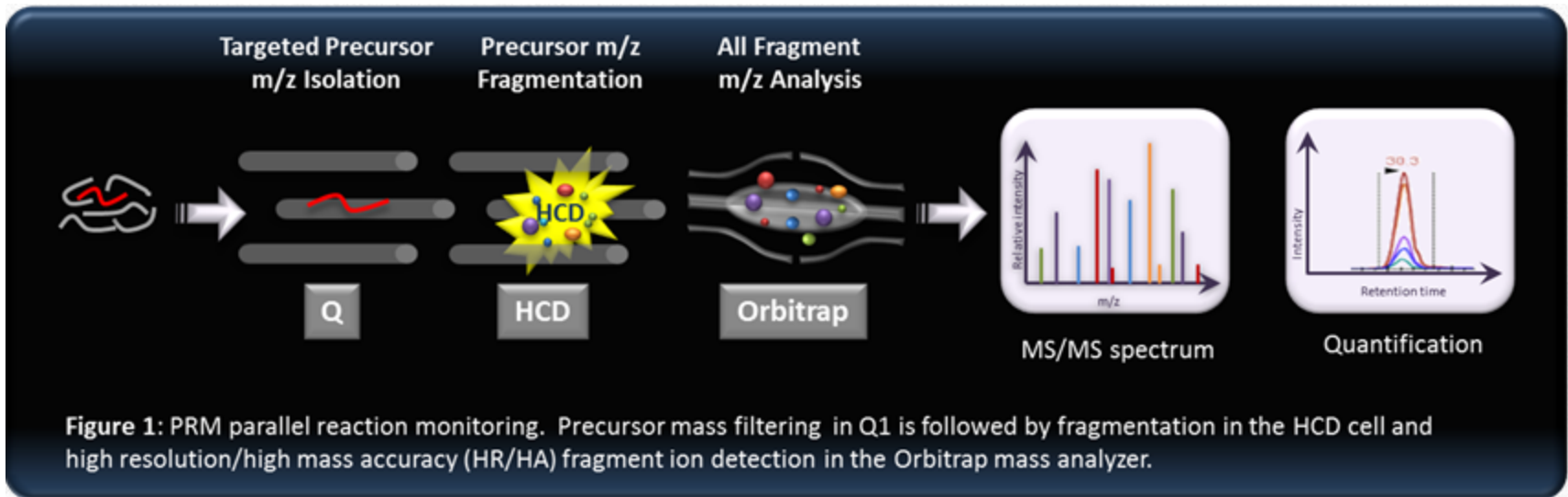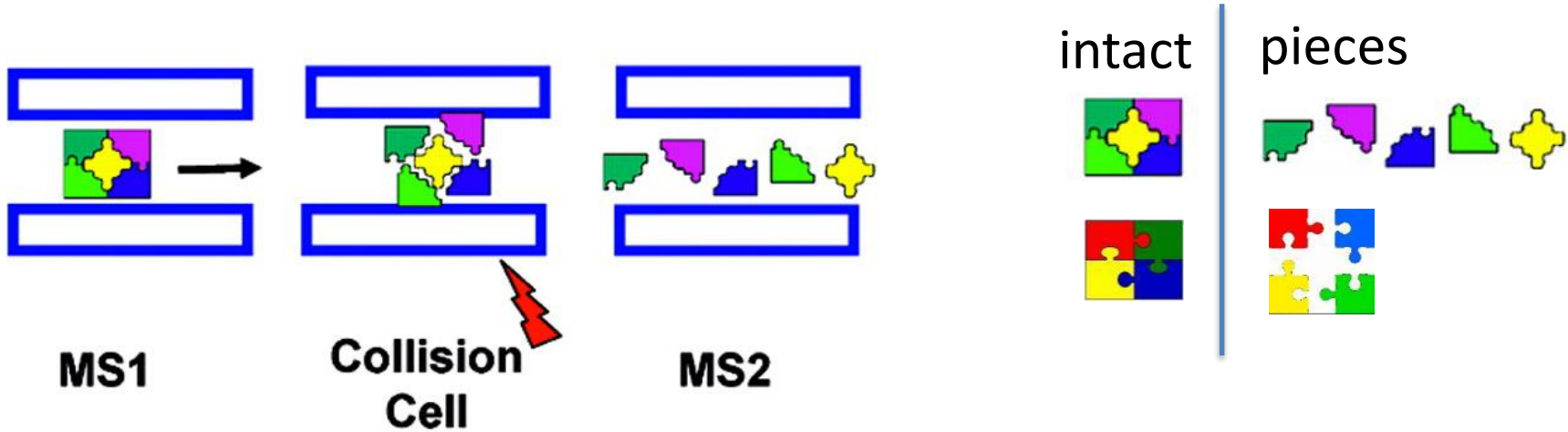
Figure 1: PRM parallel reaction monitoring. Precursor mass filtering in Q1 is followed by fragmentation in the HCD cell and high resolution/high mass accuracy (HR/HA) fragment ion detection in the Orbitrap mass analyzer.
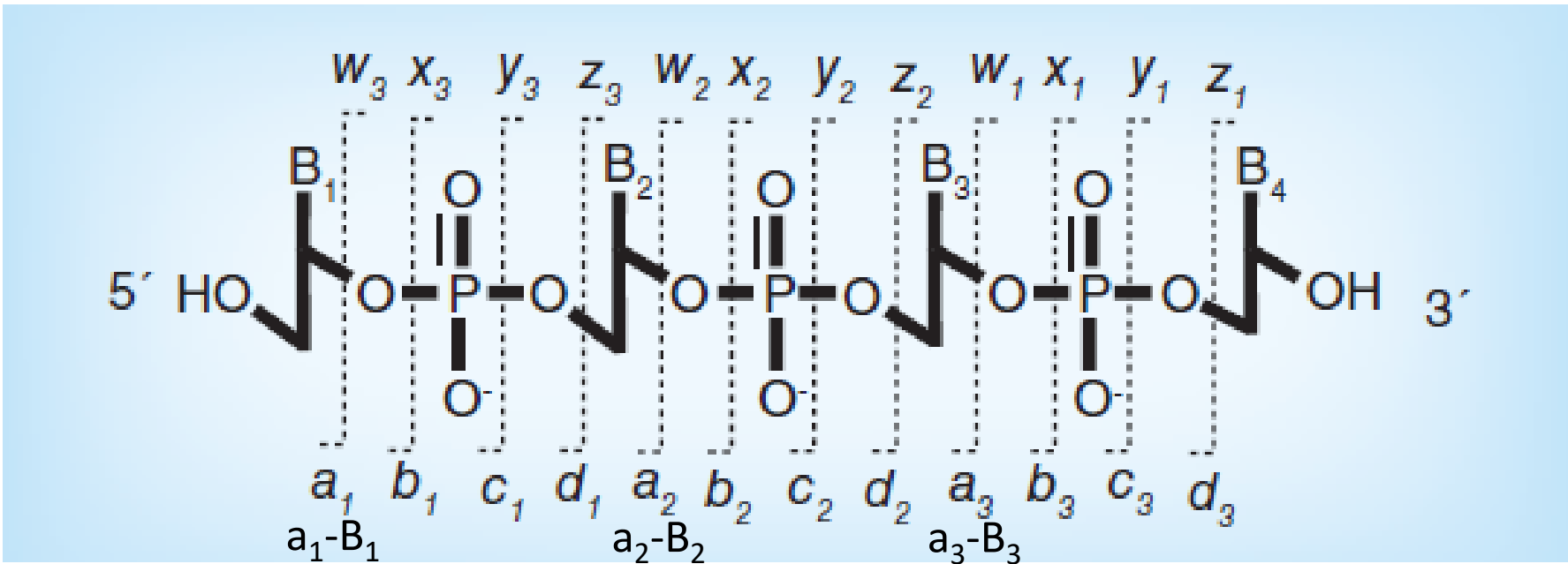
# MS/MS fragments



**Fragmentation pathways of oligonucleotides**

# Characteristic fragments

**n-T isomers:**

| n-T_1 | | | | n-T_2 | | | | | n-T_3 | | | | | n-T_4 | | | | | n-T_5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

5' T-C-A-C-T-T-T-C-A-T-A-A-T-G-C-T-G-G 3'
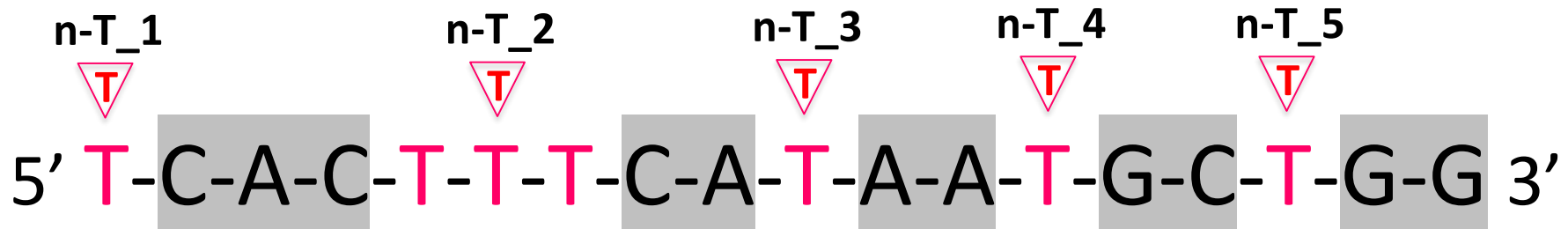
**Optimizing collision conditions**

**- better fragment coverage and intensity**

Parameters tested include:

- ✓ Collision type (CID *vs* HCD)
- ✓ Collision energy (20%, 25%, 30%)
- ✓ Charge states (single *vs* multiplexing)
- ✓ Mass resolution (15K *vs* 30K)

# MS/MS of individual isomers

## - identifying fragments: characteristic and high intensity
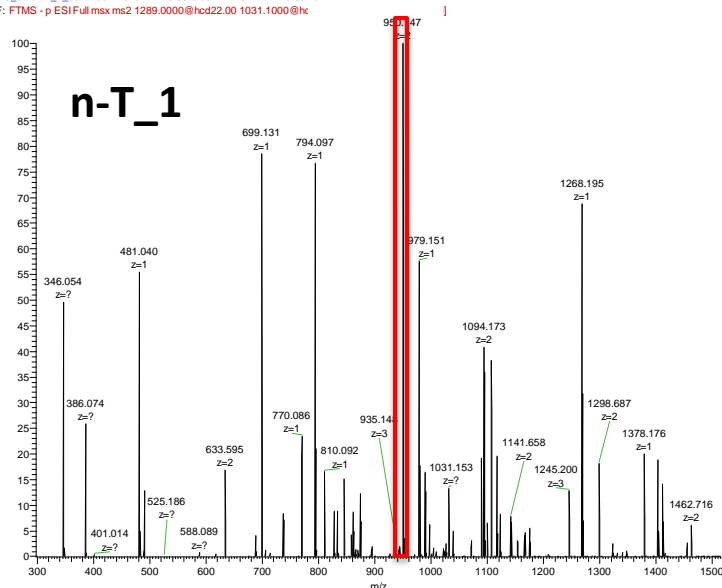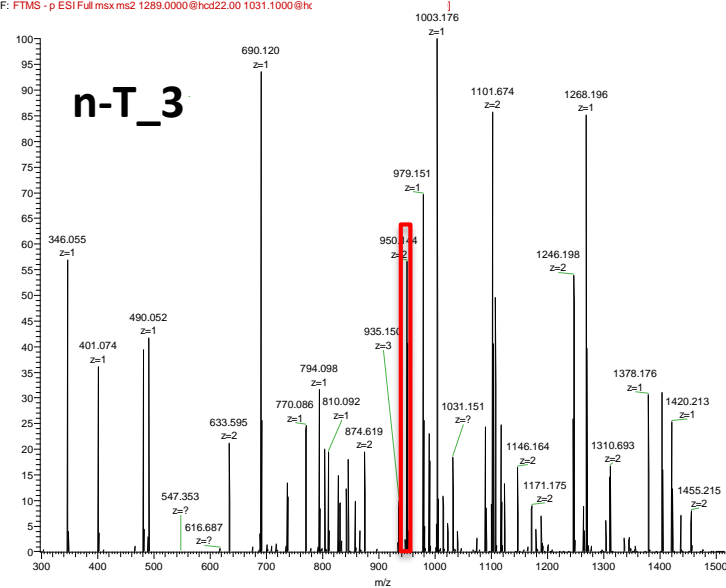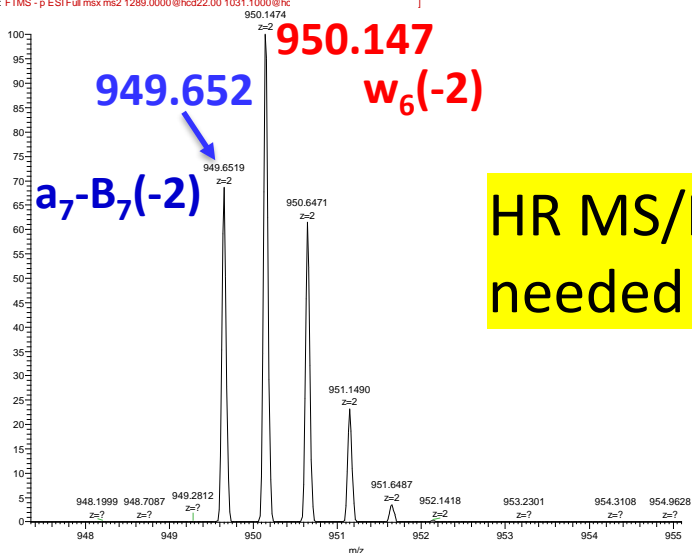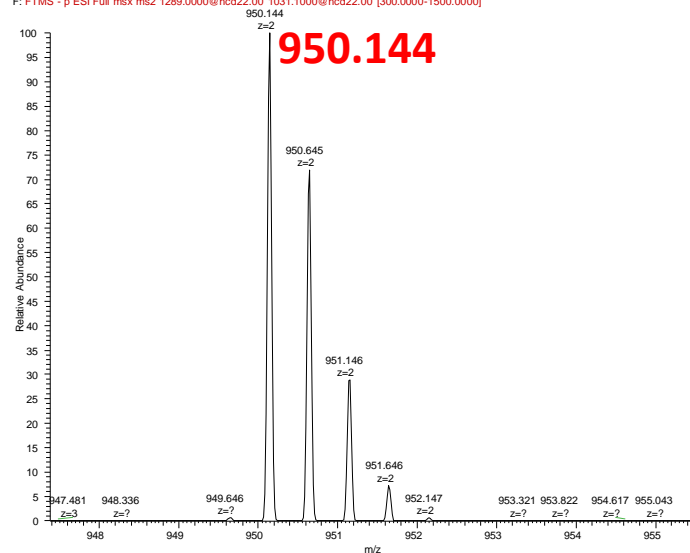
**Identified fragments** (characteristic and high-intensity)

5' T-C-A-C-T-T-T-C-A-T-A-A-T-G-C-T-G-G 3'

5' C-A-C-T-T-T-C-A-T-A-A-T-G-C-T-G-G 3'  **T1**

$a_3$-$B_3$ (-1)

5' T-C-A-C-T-T-C-A-T-A-A-T-G-C-T-G-G 3'
5' T-C-A-C-T-T-C-A-T-A-A-T-G-C-T-G-G 3'  **T2**
5' T-C-A-C-T-T-C-A-T-A-A-T-G-C-T-G-G 3'

$a_8$-$B_8$(-2)

5' T-C-A-C-T-T-T-C-A-A-A-T-G-C-T-G-G 3'  **T3**

$W_6$(-2)

5' T-C-A-C-T-T-T-C-A-T-A-A-G-C-T-G-G 3'  **T4**

$W_3$(-1)

5' T-C-A-C-T-T-T-C-A-T-A-A-T-G-C-G-G 3'  **T5**

$W_4$(-1)  $W_1$(-1)

$a_3$-$B_3$(-1)  $a_8$-$B_8$(-2)

22
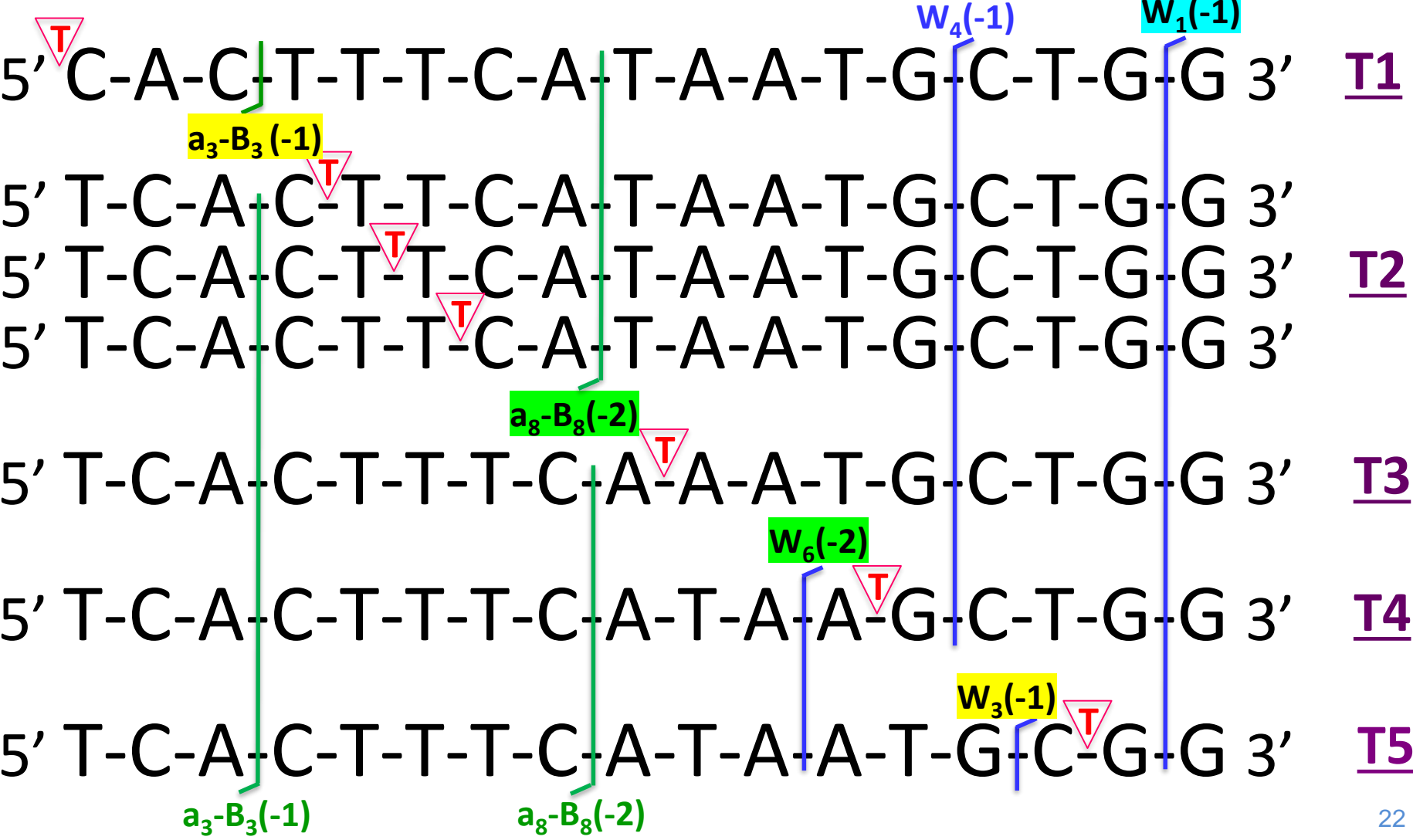
# Extracted ion chromatogram (EIC) of identified fragments

RT: 1.00 - 10.00   SM: 7G

n-T_1
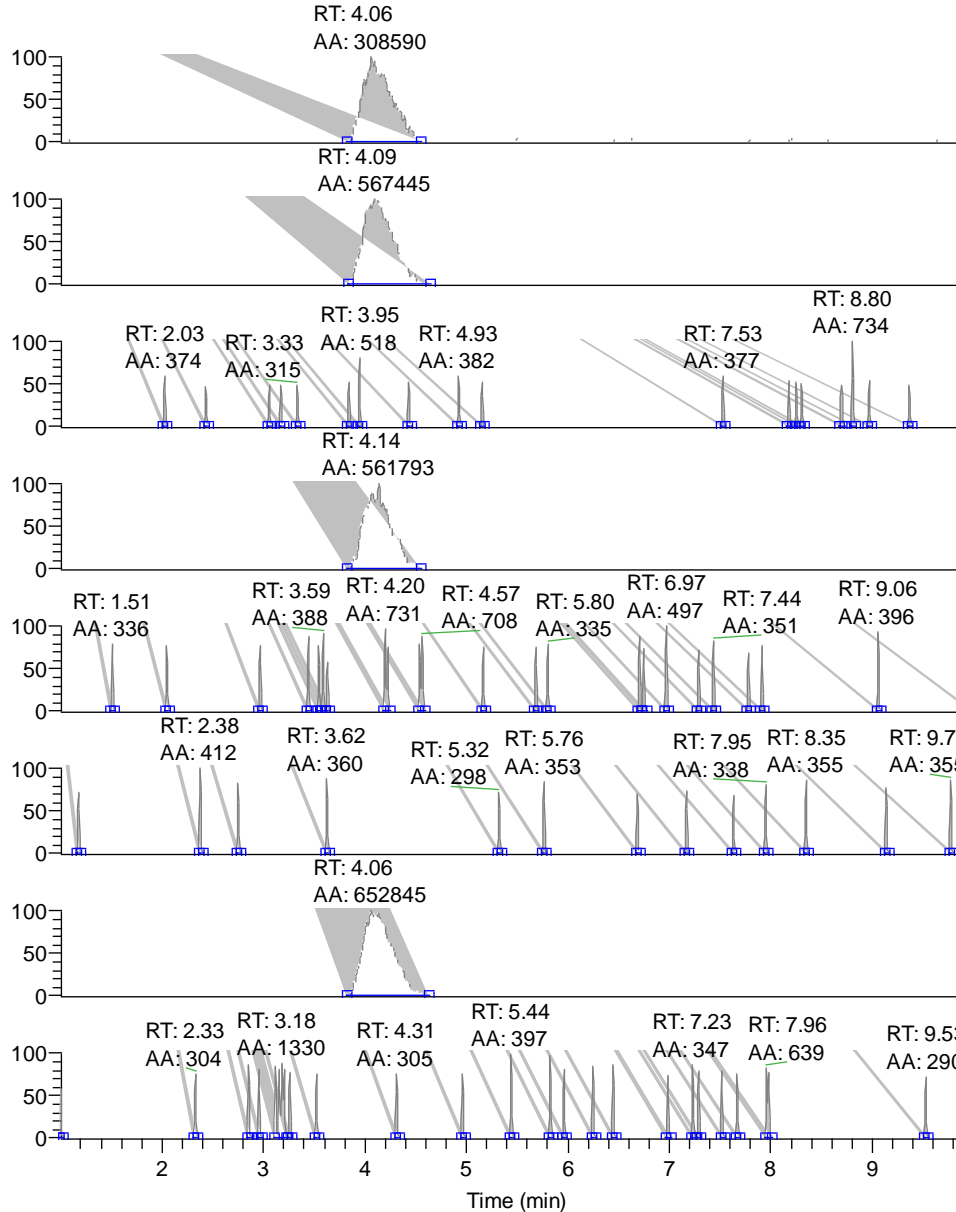
| Label | Description |
|---|---|
| $W_1(-1)$ | Common to **all** |
| $a_3\text{-}B_3(-1)$ | Unique to **T1** |
| $W_3(-1)$ | Unique to T5 |
| $a_8\text{-}B_8(-2)$ | **T1**&T2 |
| $W_6(-2)$ | T4&T5 |
| $a_8\text{-}B_8(-2)$ | T3&T4&T5 |
| $W_4(-1)$ | **T1**&T2&T3&T4 |
| $a_3\text{-}B_3(-1)$ | T2&T3&T4&T5 |

# Extracted ion chromatogram (EIC) of identified fragments



RT: 1.00 - 10.00   SM: 7G

**Mixture of n-T_1 & n-T_5**

RT: 3.96  AA: 725520 — $W_1(-1)$  Common to **all**

RT: 4.07  AA: 539480 — $a_3\text{-}B_3(-1)$  Unique to **T1**

RT: 3.91  AA: 1075801 — $W_3(-1)$  Unique to **T5**

RT: 4.10  AA: 532867 — $a_8\text{-}B_8(-2)$  **T1**&T2

RT: 3.90  AA: 606460 — $W_6(-2)$  T4&**T5**

RT: 3.90  AA: 1457001 — $a_8\text{-}B_8(-2)$  T3&T4&**T5**

RT: 4.09  AA: 605926 — $W_4(-1)$  **T1**&T2&T3&T4

RT: 3.88  AA: 771484 — $a_3\text{-}B_3(-1)$  T2&T3&T4&**T5**

Time (min)

24

# EIC areas of identified fragments:
## - normalized to that of the common fragment



n-T_1 & n-T_5 & mixture
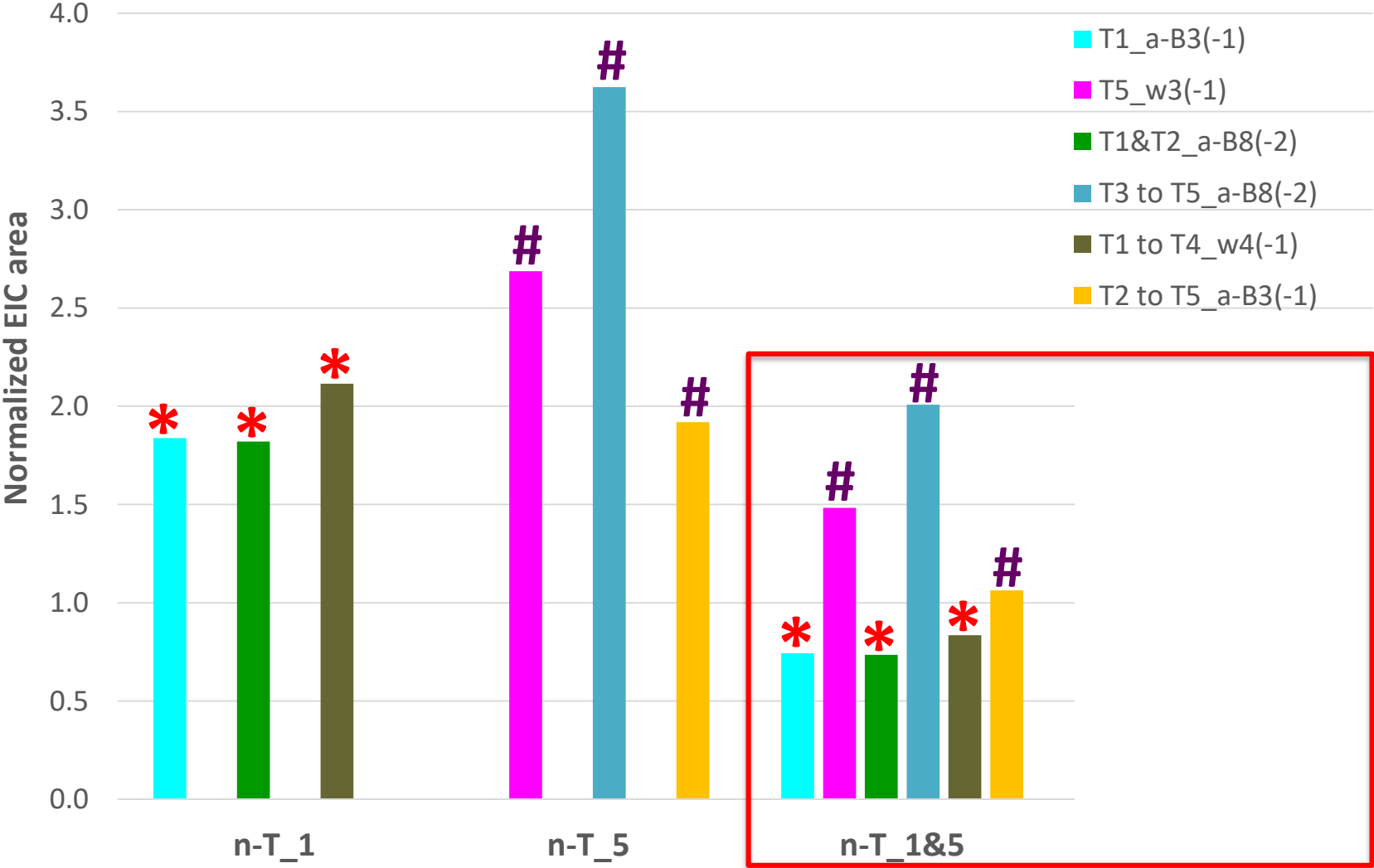
# Normalized EIC areas: Experimental *vs* Calculated

**n-T_2&4, 1:1**

Legend:
- T1_a-B3(-1)
- T5_w3(-1)
- T1&T2_a-B8(-2)
- T3 to T5_a-B8(-2)
- T1 to T4_w4(-1)
- T2 to T5_a-B3(-1)
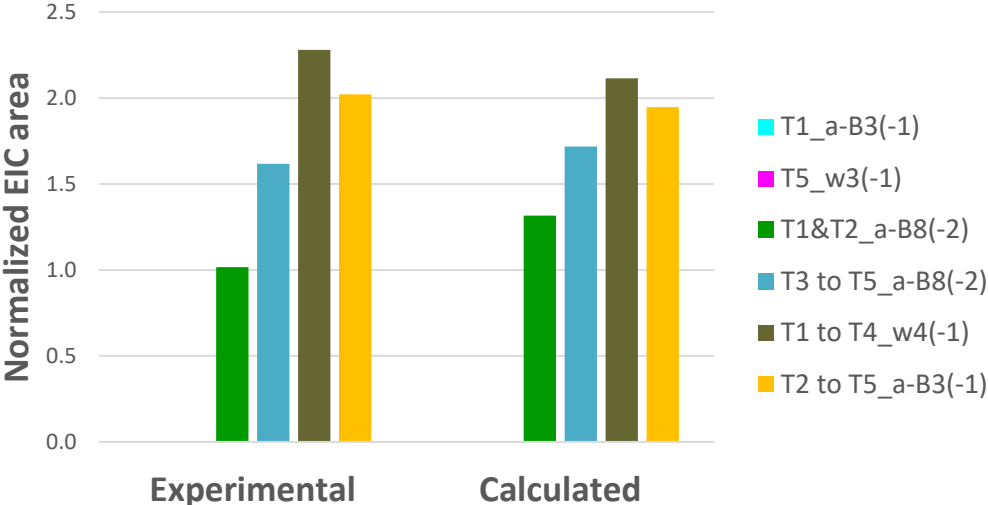
Hold true for binary mixtures of isomers at different ratios

**n-T_2&4, 1:3**

**n-T_2&4_3:1**

n-T_2&4&5

Legend:
- T1_a-B3(-1)
- T5_w3(-1)
- T1&T2_a-B8(-2)
- T3 to T5_a-B8(-2)
- T1 to T4_w4(-1)
- T2 to T5_a-B3(-1)

n-T_1&2&3&4&5

Legend:
- T1_a-B3(-1)
- T5_w3(-1)
- T1&T2_a-B8(-2)
- T3 to T5_a-B8(-2)
- T1 to T4_w4(-1)
- T2 to T5_a-B3(-1)
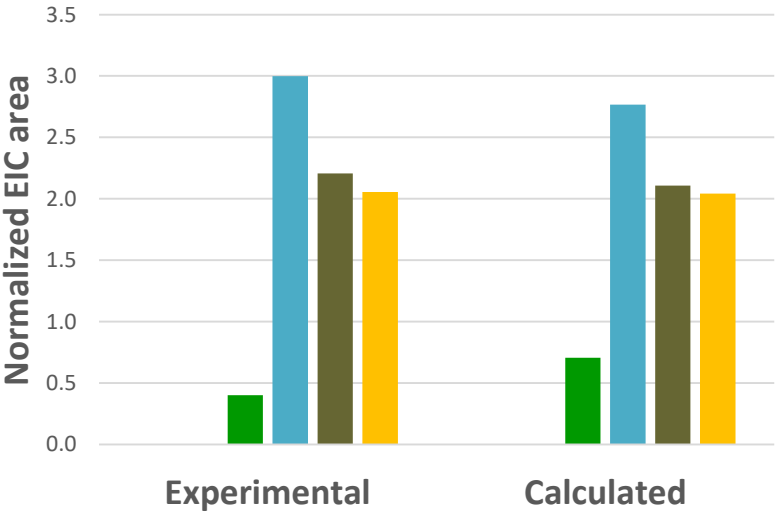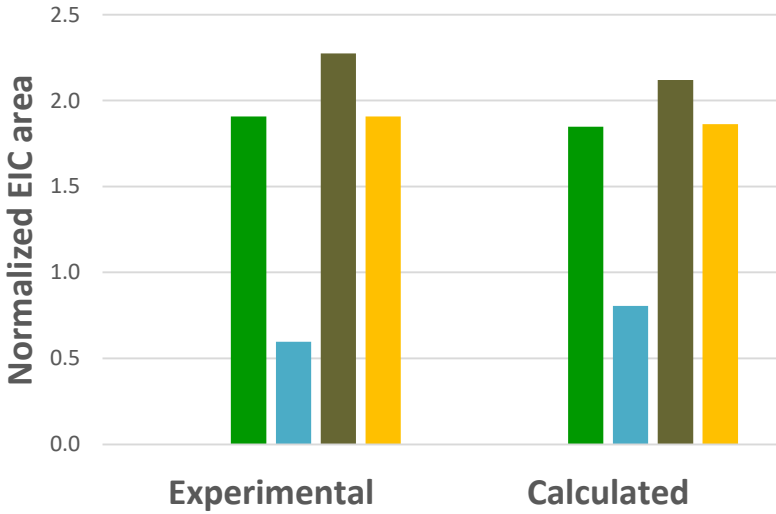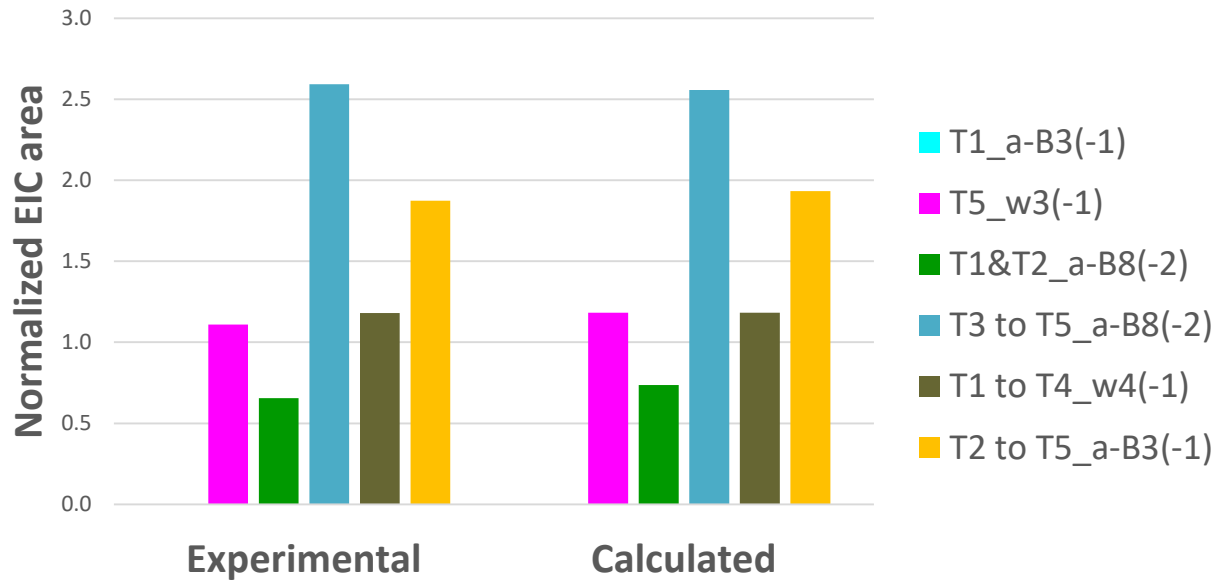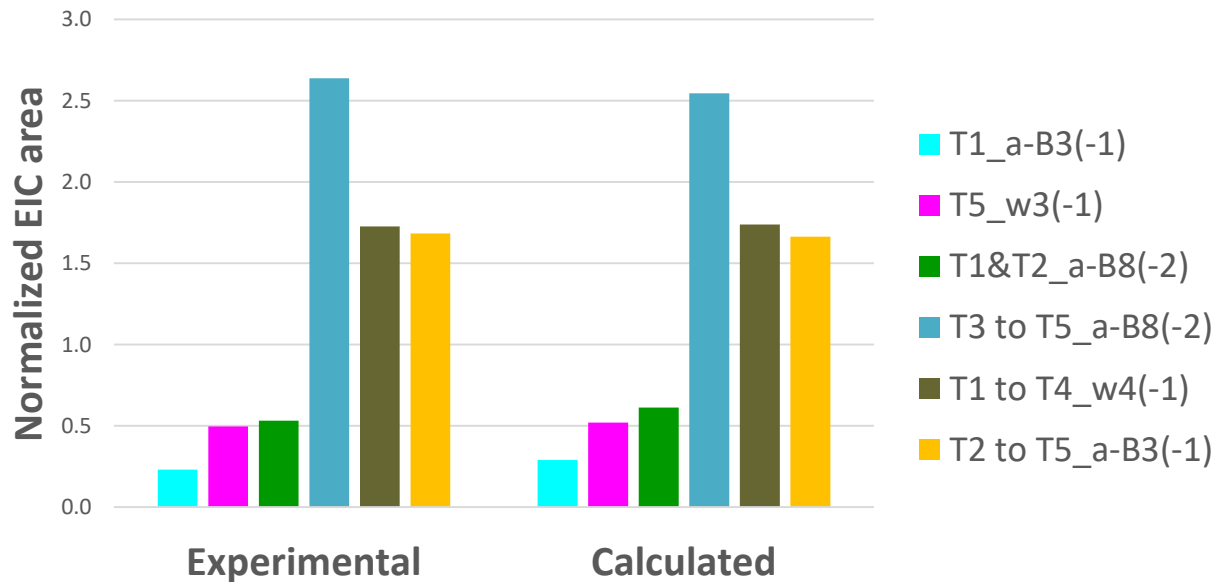
Holds true for more complex isomeric mixtures

# Mixture composition prediction: using "Solver" (Excel)



SOLVER → Resolving the mixture composition

Iterative combinations of individuals to reach the best fit for mixture
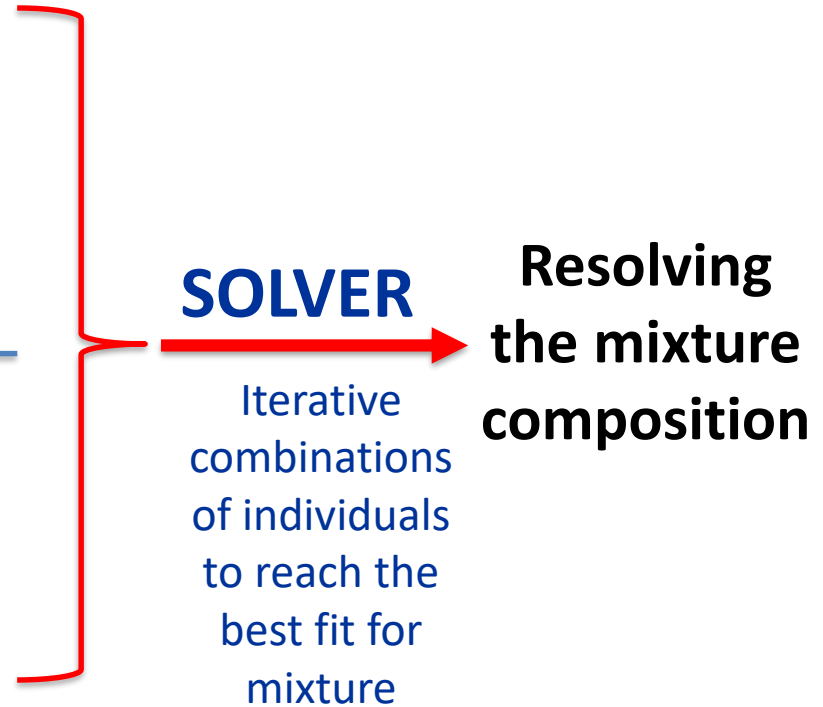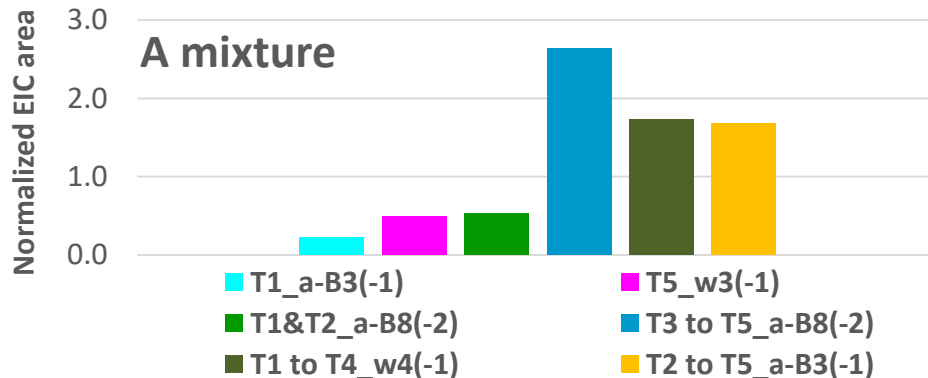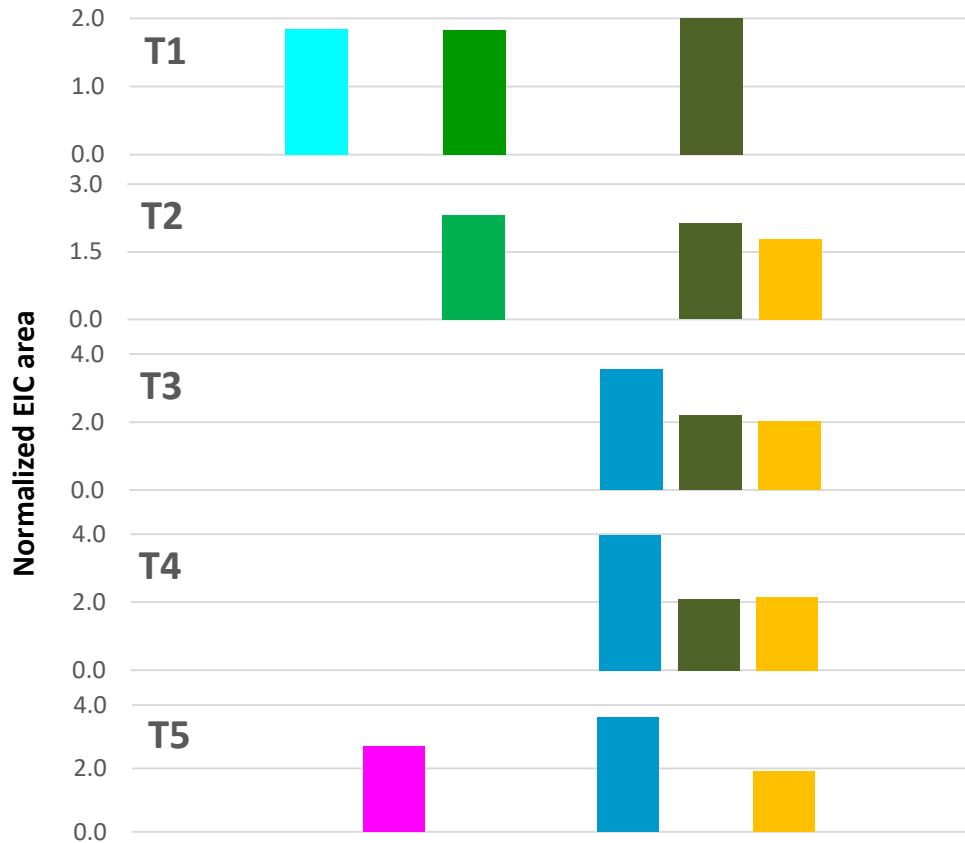
# Mixture composition prediction: using "Solver" (Excel)
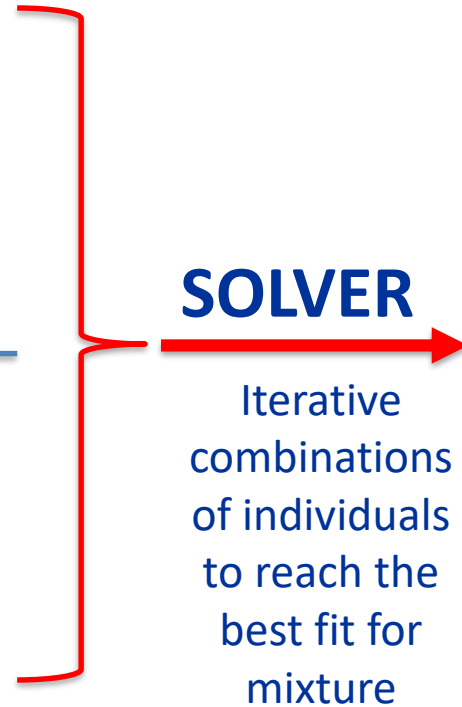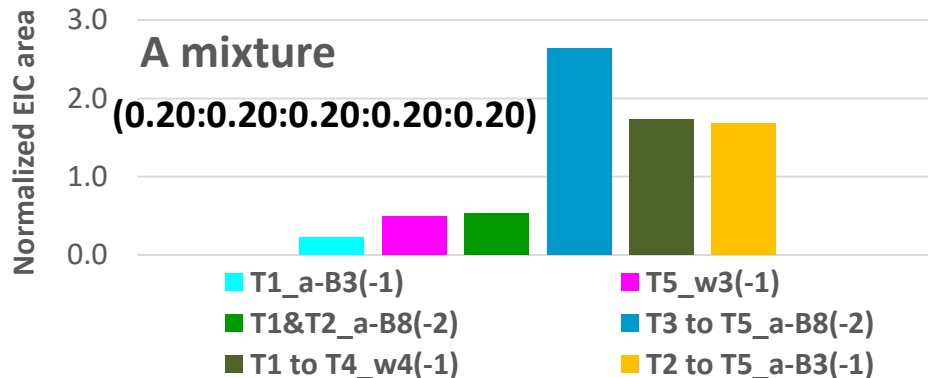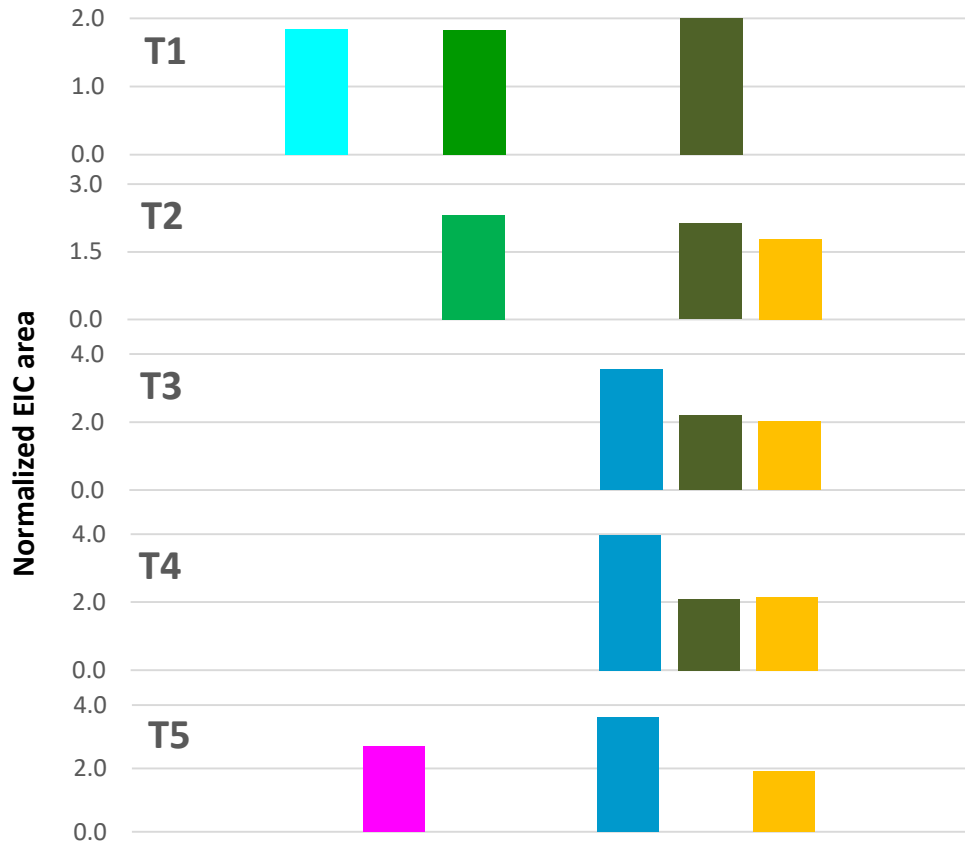
**Predicted**
**T1:T2:T3:T4:T5**
**=**
**0.18:**
**0.20:**
**0.23:**
**0.20:**
**0.20**

**SOLVER**

Iterative combinations of individuals to reach the best fit for mixture

A mixture (0.20:0.20:0.20:0.20:0.20)

- ■ T1_a-B3(-1)
- ■ T1&T2_a-B8(-2)
- ■ T1 to T4_w4(-1)
- ■ T5_w3(-1)
- ■ T3 to T5_a-B8(-2)
- ■ T2 to T5_a-B3(-1)

# Mixture compositions: True *vs* Predicted



Predicted by SOLVER
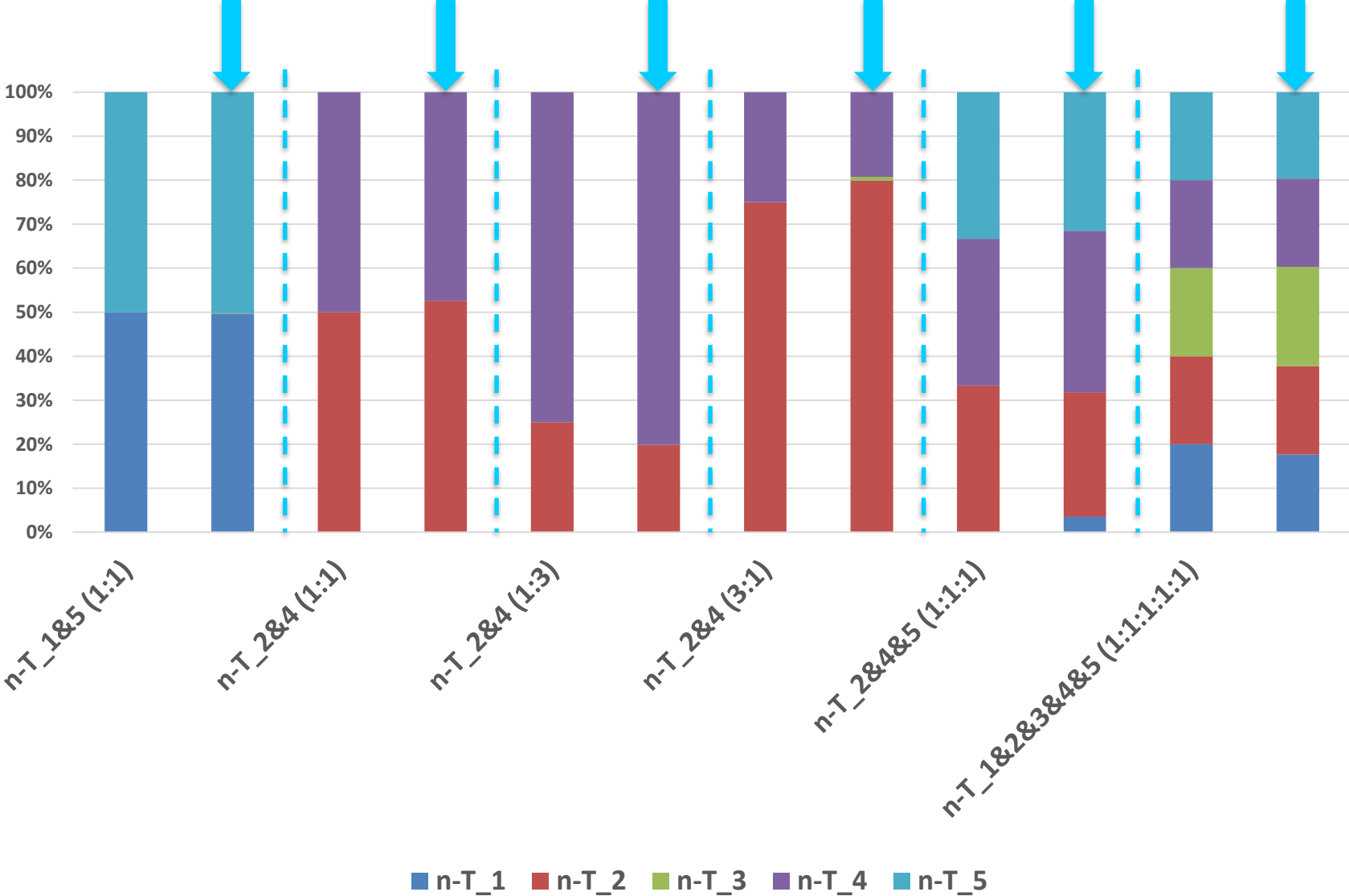
Legend: n-T_1, n-T_2, n-T_3, n-T_4, n-T_5

Categories: n-T_1&5 (1:1), n-T_2&4 (1:1), n-T_2&4 (1:3), n-T_2&4 (3:1), n-T_2&4&5 (1:1:1), n-T_1&2&3&4&5 (1:1:1:1:1)

# What to conclude?

o Combination of normalized EIC areas of <span style="color:red">characteristic fragments (fingerprints)</span> of individual isomers reflects the composition in their composite mixture.

o May be used to compare isomer distribution profiles during manufacturing process or from batch to batch (quality control), or in generic equivalents.

# What next?

o Other types of positional isomers in addition to deletion/addition sequences: $(PO)_1$ impurities, deaminated, abasic …

# **Outline**

# To address isomer challenge
o **Approach II: IM MS**
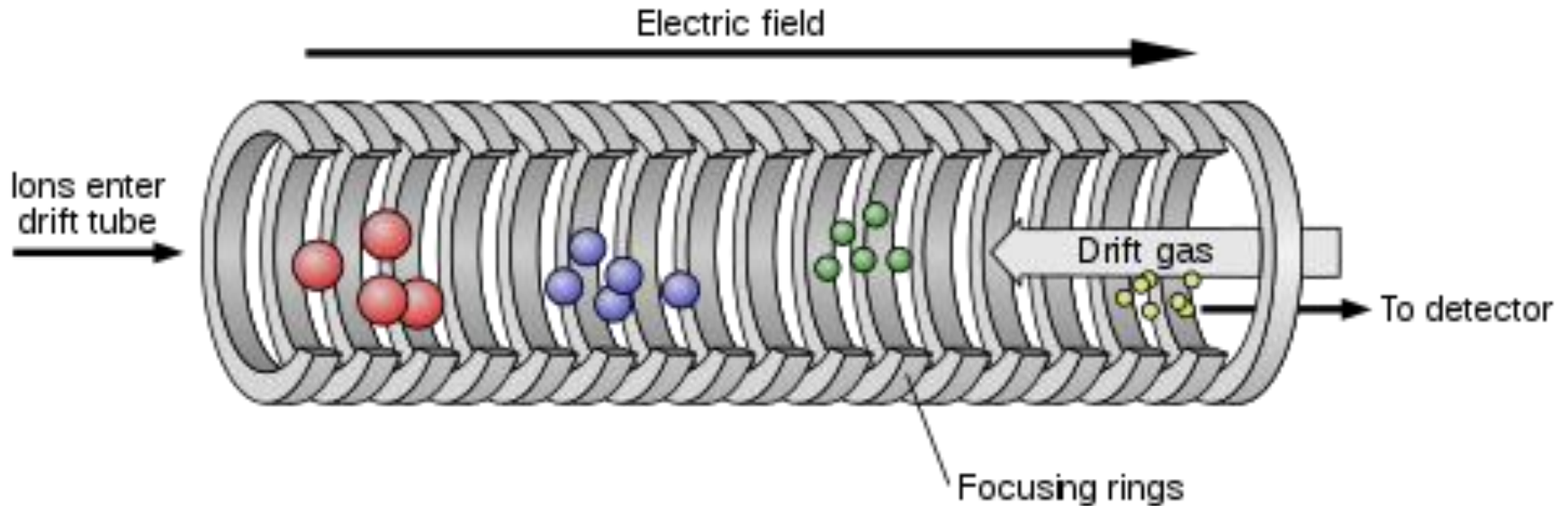


Distinct Drift Time (DT)

# IM MS of synthetic sequence isomers

Isomer 1
**ACGTACGTACGT**

**DT=29.24ms**
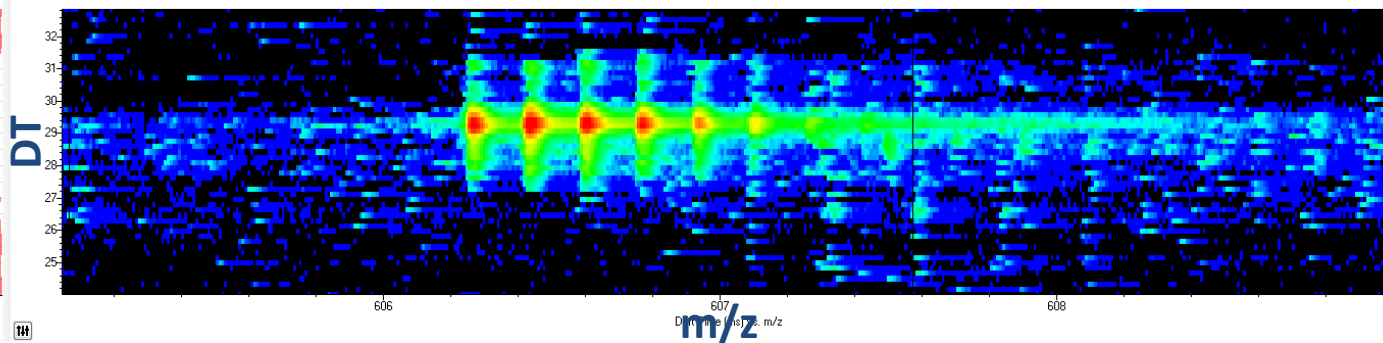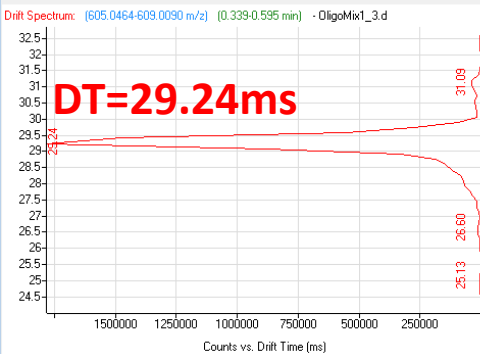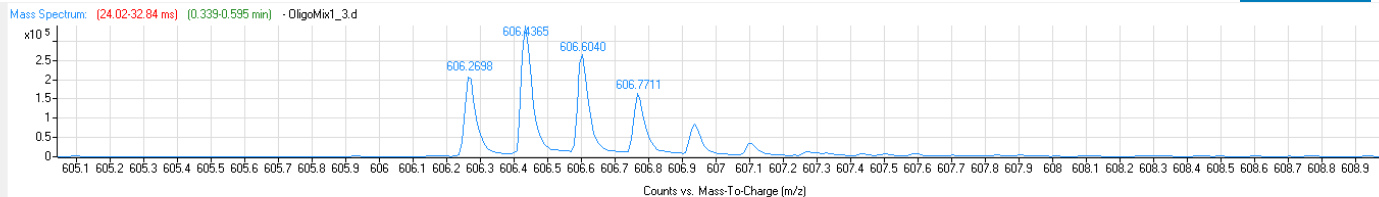
Isomer 2
**TGCATGCATGCA**

**DT=28.59ms**
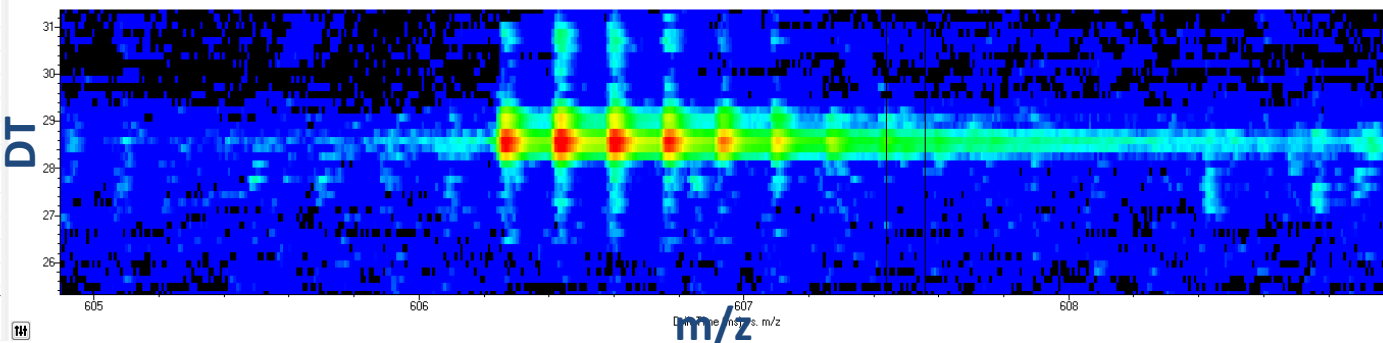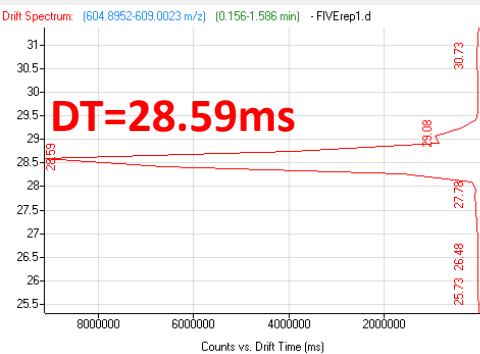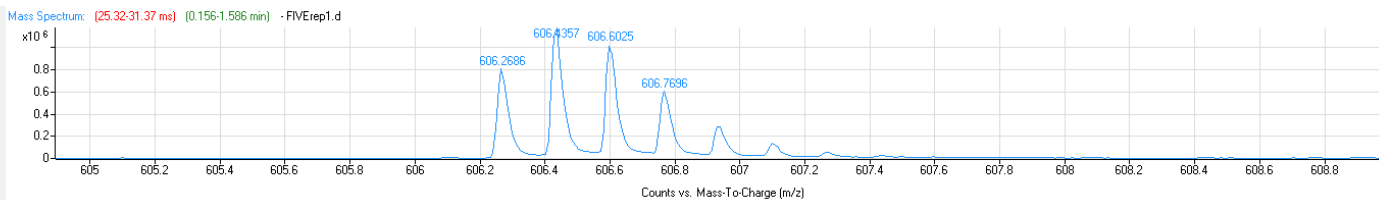
# IM MS and MS/MS of mixture of isomers



**Mixture**

**MS**
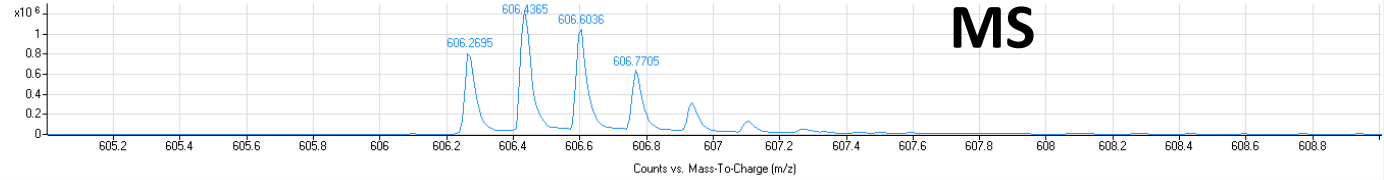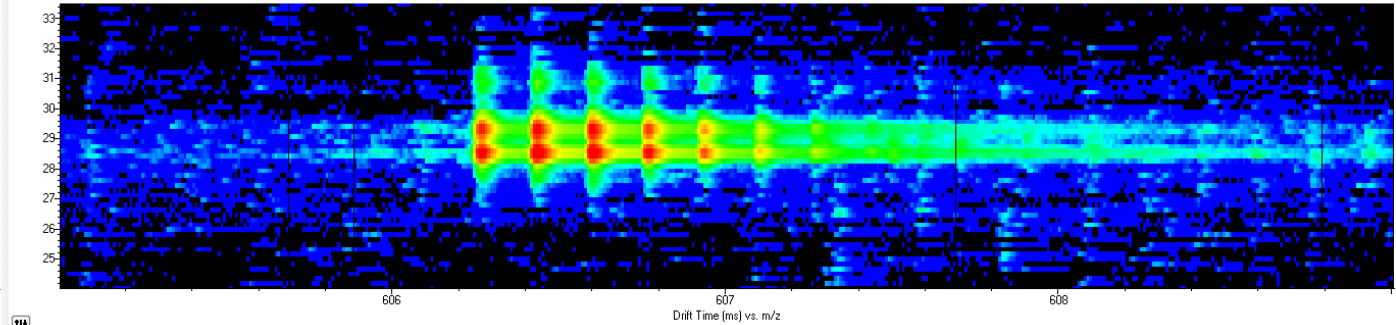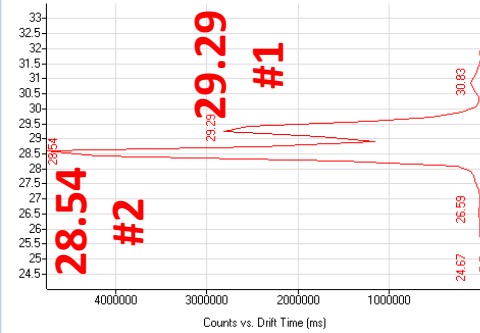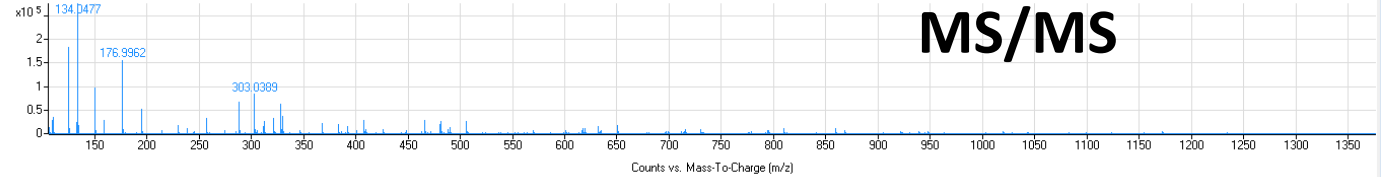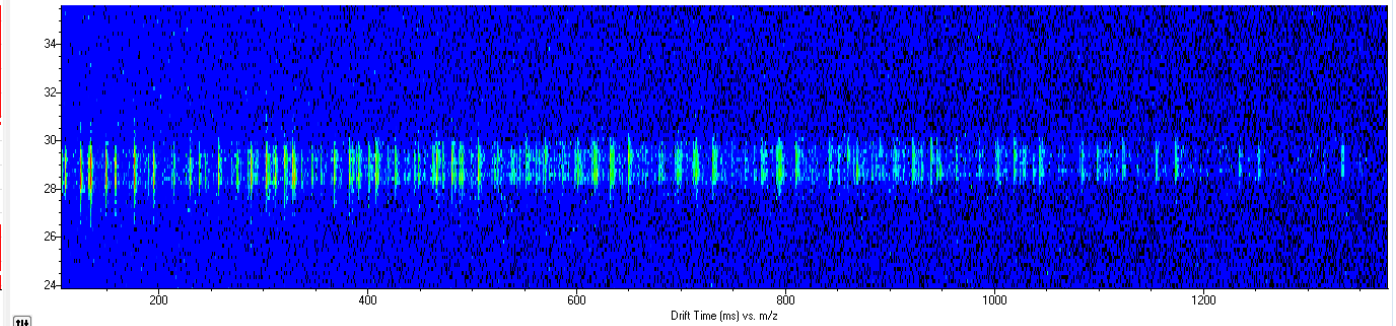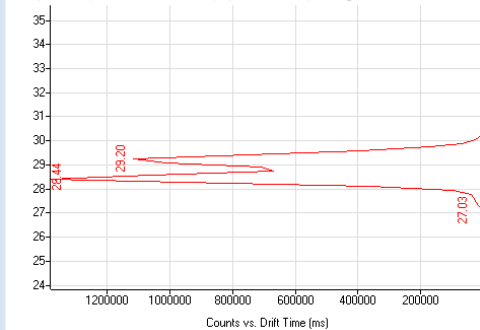
**MS/MS**

Alignment of fragments with precursor ions by drift time

# Zoomed IM MS/MS of mixture of isomers
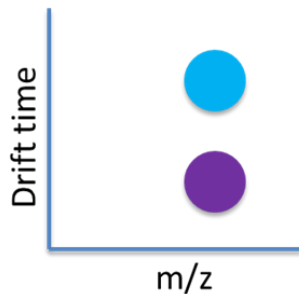## - clarifying fragments for each isomer
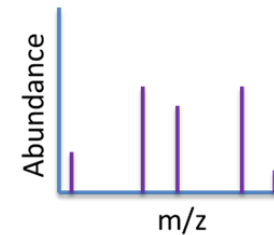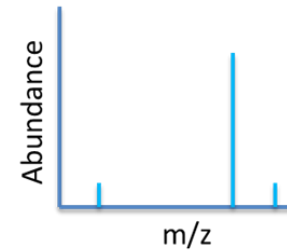
# IM on *vs* off

Isomers
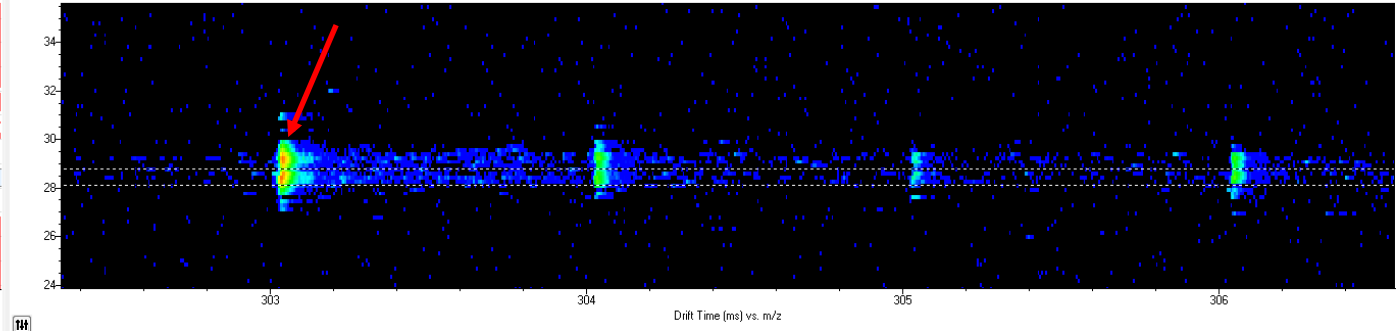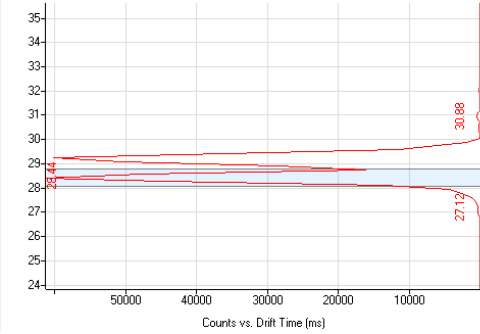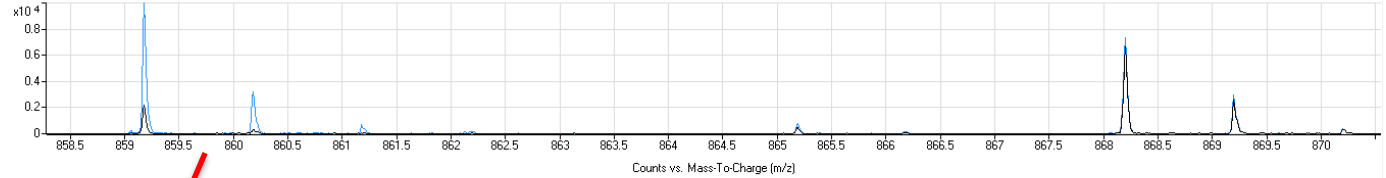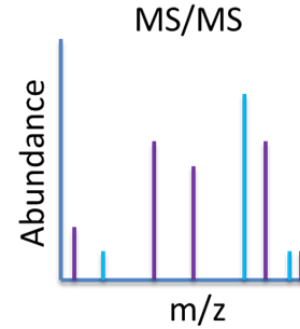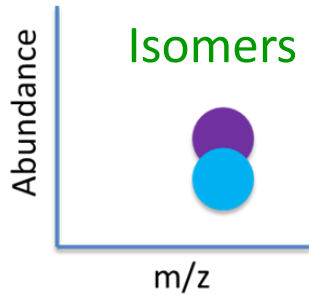
Without IM

MS/MS
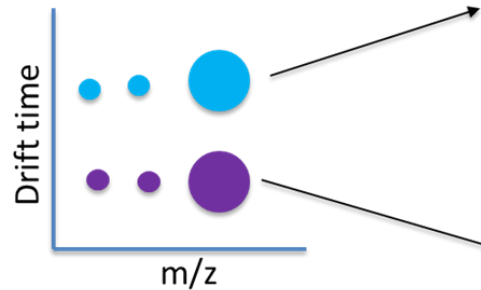
With IM

Precursor ions separated by drift time
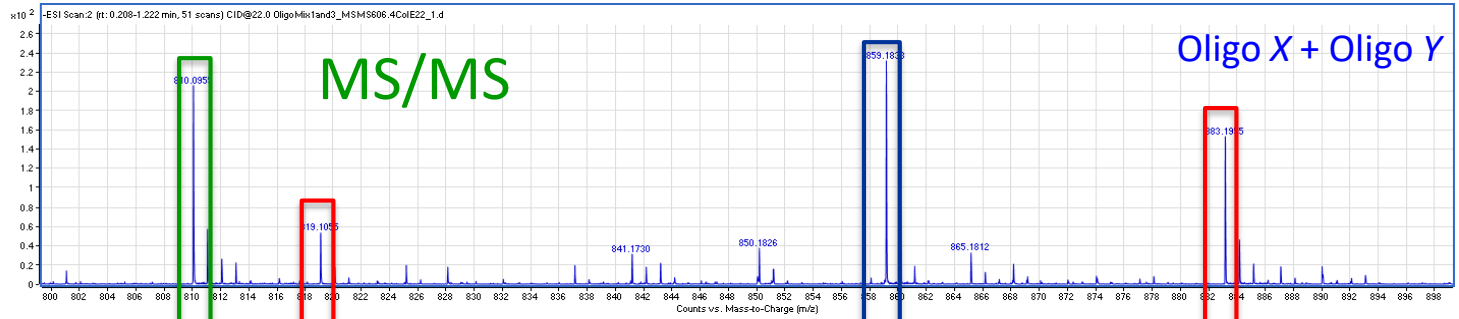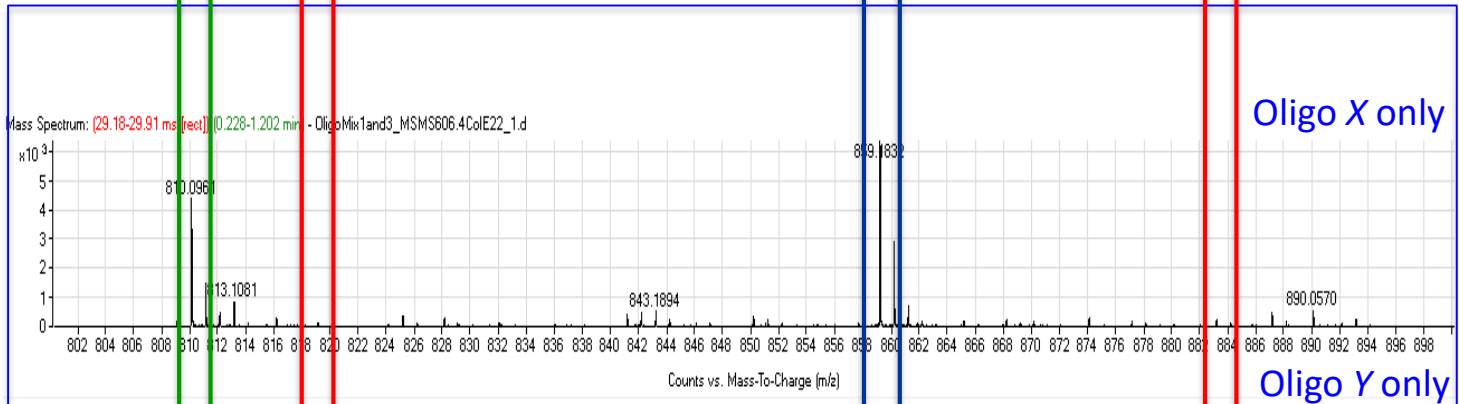
Fragment ions aligned by drift time

✓ **IM (drift-time)** ➡ **Additional dimension of separation**

✓ **Fragments assigned to drift-time separated isomeric precursors**

⬇

**MS/MS spectral clarity = Better sequence ID**

# IM on *vs* off
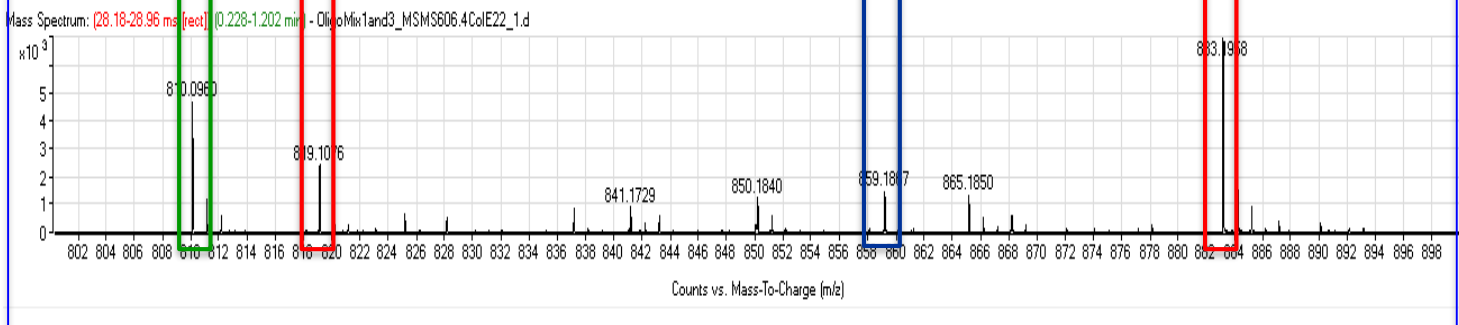


MS/MS

Oligo *X* + Oligo *Y*

Without IM

Oligo *X* only

With IM

Oligo *Y* only

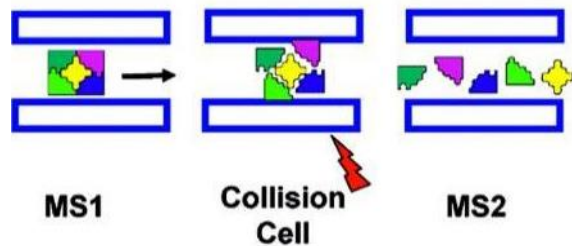# **Outline**

❑ Challenge in impurity analysis of synthetic oligonucleotides – Composite impurity mixtures

❑ **High resolution mass spectrometry in differentiating isomeric impurities**

➢ High resolution tandem mass spectrometry (HR MS/MS)

➢ **Ion mobility mass spectrometry (IMMS)**

❑ Summary and Q&A

# Challenge in impurity analysis: composite nature



MS1   Collision Cell   MS2

## High Resolution MS/MS:

- ✓ Characteristic fragments (**fingerprints**)-based differentiation of isomers by tandem MS analysis

- ✓ Combination of normalized EIC areas of characteristic fragments (**fingerprints**) of individual isomers reflecting the isomeric ratio in the mixture



## Ion Mobility MS and MS/MS:

- ✓ Shape-based separation of isomers by drift time

- ✓ Fragments aligned by drift time of isomeric precursors enabling MS/MS spectral clarity
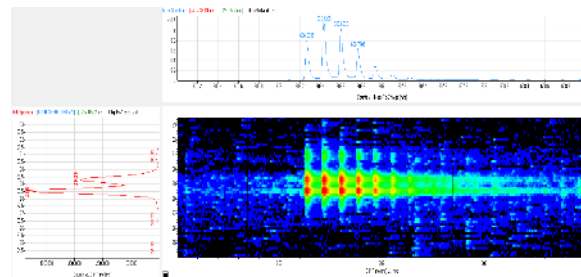
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