Oligonucleotide impurity analysis using an innovative SFC approach

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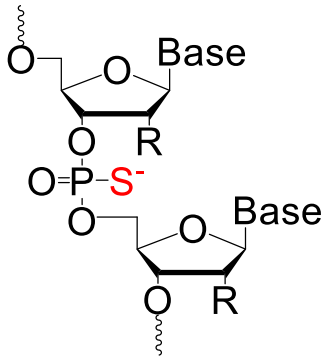
in place of Momoka Hayashida, Shimadzu Corporation

1. Introduction | Oligonucleotide therapeutics

Oligonucleotide therapeutics **draw** attention as one of the new drug modalities.

- Target at mRNA and miRNA that cannot be approached by existing drugs.
  ➞Expected to be a treatment for diseases that have been difficult to treat.
- 20 products were approved worldwide.
  (except for mRNA therapeutics, as of June 2024).
  (National Institute of Health Sciences, Division of Molecular Target and Gene Therapy Products, "Approved ON therapeutics," 2024-10-15)
- Composed of chemically modified oligonucleotides to improve biostability.
  e.g. phosphorothioate (PS) modification was used in 12 approved oligonucleotide therapeutics.

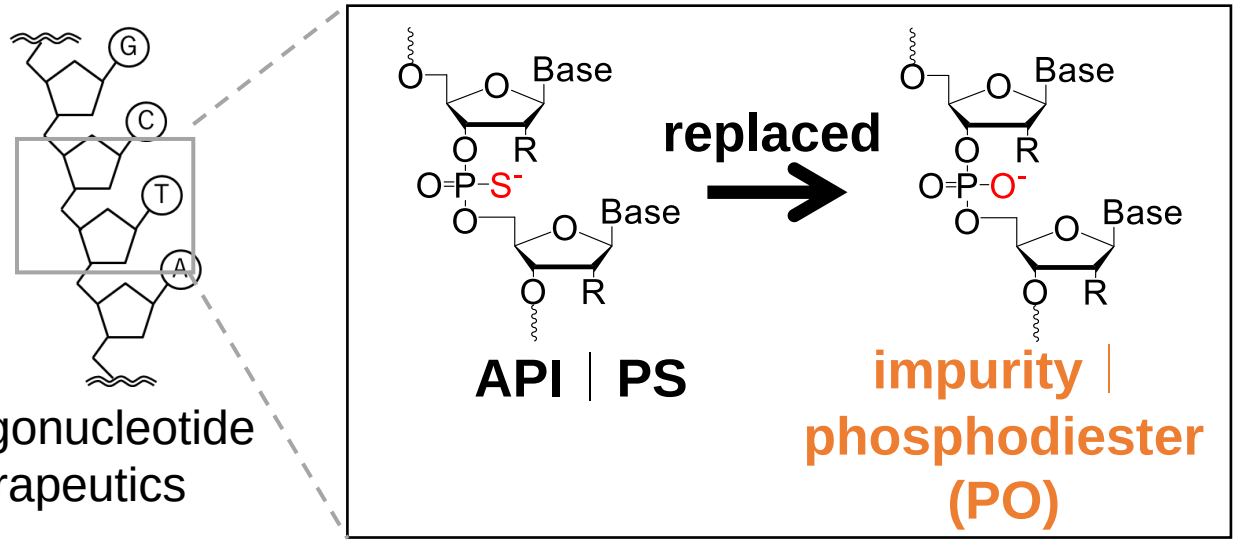


PS modification

Quality control methods have been discussed

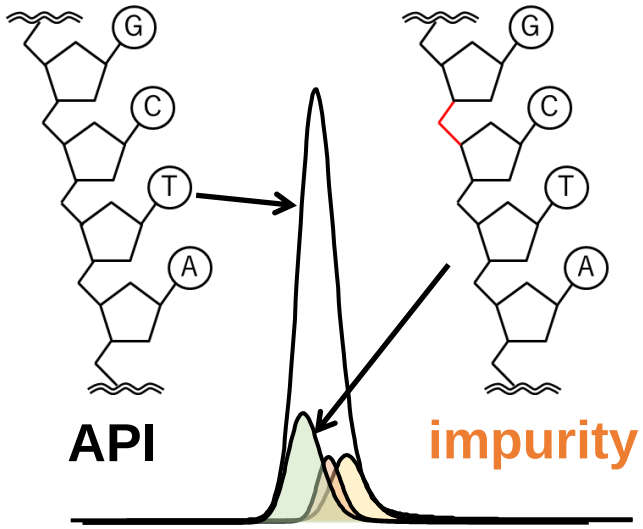
1. Introduction | Quality control issues

Co-elution of structurally related impurities



PS-modified oligonucleotide therapeutics and its impurities

A. Krotz *et al.*, *Bioorganic Med. Chem. Lett.* **1997**, 7, 73–78.



Chromatogram of synthesized oligonucleotide therapeutics (ion pair reversed-phased liquid chromatography; IPRP-LC)

J.M. Sutton. *et al.* , *J. Am. Soc. Mass Spectrom*. **2020**, 31(9), 1775-1782.

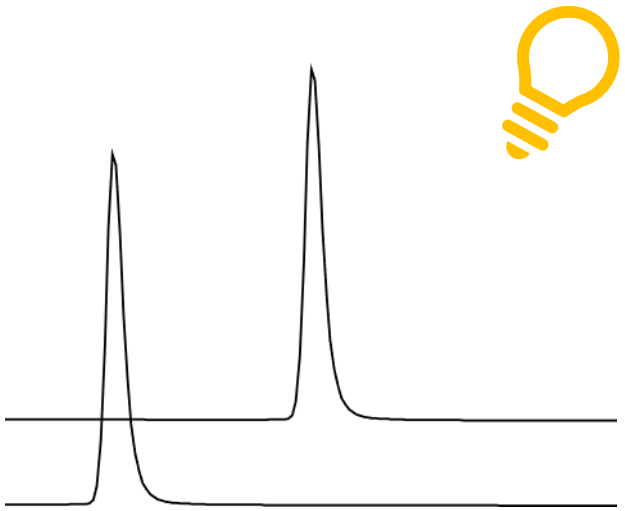
The method with different selectivity from IPRP-LC has been desired

➡ We focus on SFC

1. Introduction | Features of SFC with CO_2

Feature **1**

High resolution



Nucleotide analysis: M.C. Beike *et al*, *J. Chromatogr. A* **2016**, 1436 84-90

Feature **2**

Superior structural recognition

Ac-**R_3** -R_1 -R_2 -NH_2

Ac-R_1 -R_2 -**R_3** -NH_2

Peptide analysis: J. Molineau *et al.* , *J. Chromatogr. A* **2021**, 1658 462631

Feature **3**

Low-polarity of CO_2

To the best of our knowledge, oligonucleotide analysis with SFC remains to be reported.

→controlling co-solvent compositions allows for the SFC applications to oligonucleotide.

1. Introduction | Goal of this work

Evaluate the applicability of SFC separation to oligonucleotide analysis

①

Evaluation the separation performance for a 4-mer oligonucleotide, a model compound

- Optimization of column and modifier composition
- Separation performance for 4-mer with varying PS contents
- Retention behavior

(M. Hayashida *et el.*, *J of Chromatogr. A*, **2023**, 1708, 464333)

②

Apply to longer sequences

- Optimization of modifier composition for longer sequences
- Separation performance for deaminated products

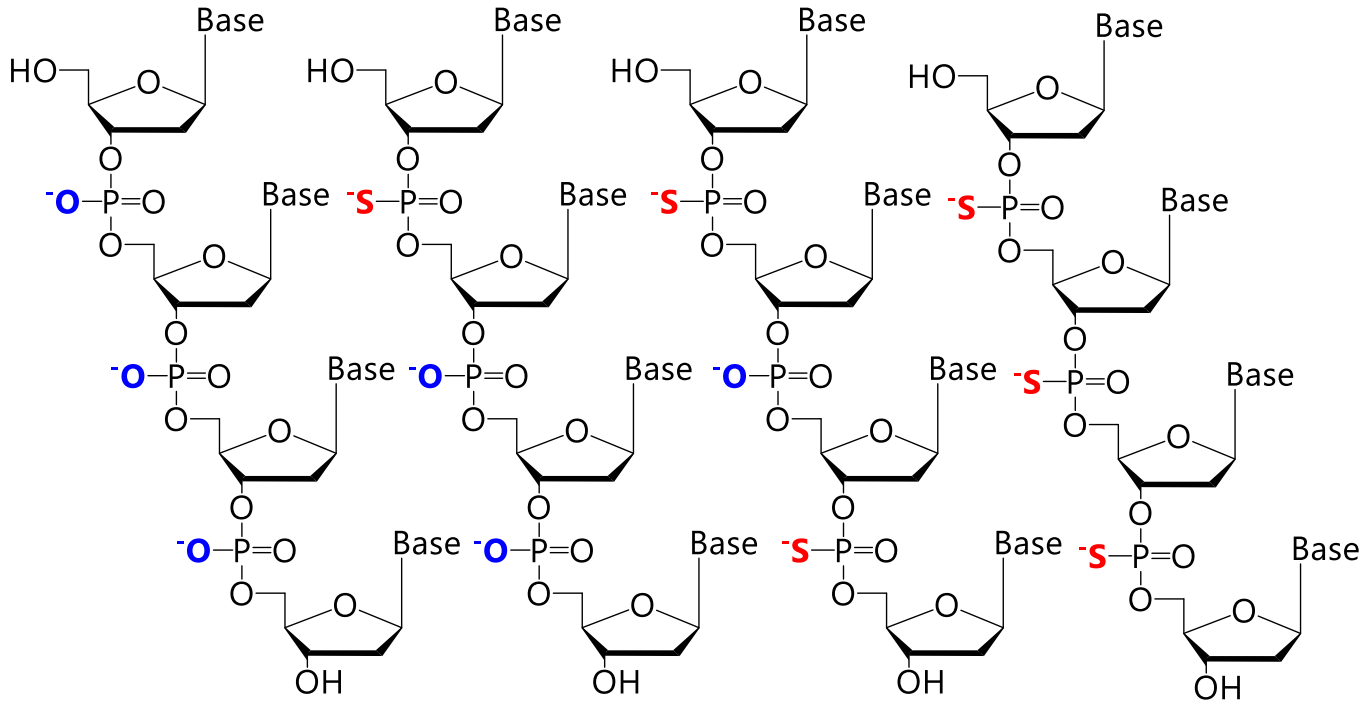
(M. Hayashida *et el.*, *J of Chromatogr. A*, **2025**, 1744, 465731)

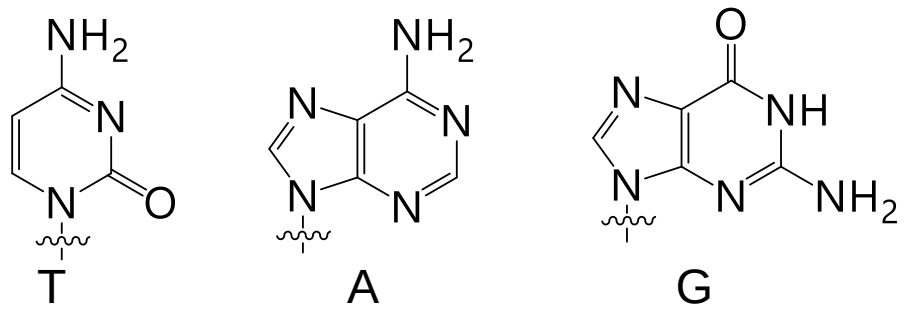
1. Materials | Analytes

Analytes

Target	Sequence	[M-H]$^-$
a. T4	5′-$T_oT_oT_oT$-3′	1153.22
b. T4+1PS	5′-$T_sT_oT_oT$-3′	1169.20
c. T4+2PS	5′-$T_sT_oT_sT$-3′	1185.18
d. T4+3PS	5′-$T_sT_sT_sT$-3′	1201.15
e. TAGC	5′-$T_oA_oG_oC$-3′	1172.24
f. TAGC+1PS	5′-$T_sA_oG_oC$-3′	1188.22
g. TAGC+2PS	5′-$T_sA_oG_sC$-3′	1204.19
h. TAGC+3PS	5′-$T_sA_sG_sC$-3′	1220.17

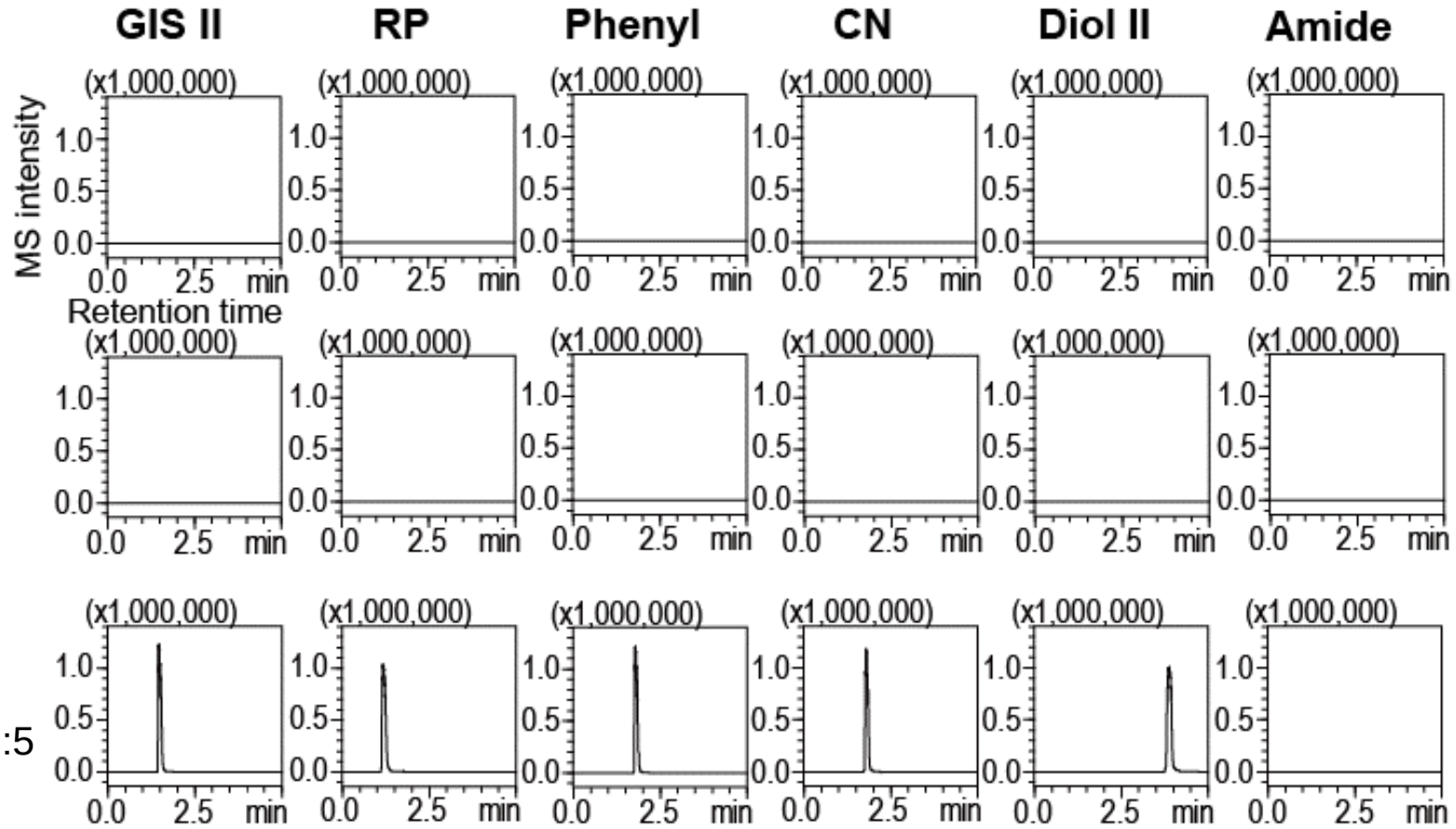
T: thymidine, A: adenosine, G: guanosine, C: cytidine,
s :PS linkage, o:PO linkage





1-1. Results | Eluting condition

T4 were eluted from five types of column using the modifier containing ammonium formate



Analytical conditions

Flow rate : 1 mL/min

MP : CO_2/modifier = **20:80**

Make-up : 0.1 mL/min, MeOH

Column : Shim-pack UC
(4.6 X 150 mm; 3 µm)

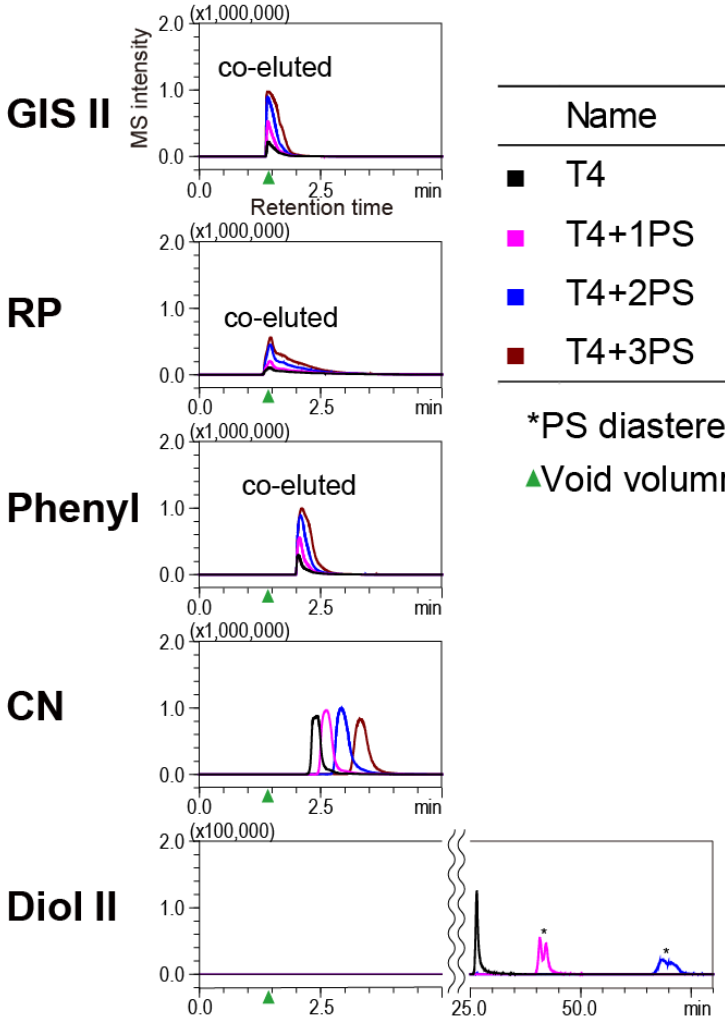
BPR : 10.0 MPa, 50 °C

Oven temp. : 35 °C

Injection vol. : 1 µL, 100 µM

1-2. Results | 1st Column screening

We evaluate different types of column.



	Name	*m/z*, [M-H]⁻
■	T4	1153.22
■	T4+1PS	1169.20
■	T4+2PS	1185.18
■	T4+3PS	1201.15

*PS diastereomers

▲Void volumn (t_0)

Analytical conditions

Flow rate : 1 mL/min

MP : CO_2/MeOH/1 M AmF solution = **50:47.5:2.5**

Make-up : 0.1 mL/min, MeOH

Column : Shim-pack UC (4.6 X 150 mm; 3 µm)

BPR : 10.0 MPa, 50 °C

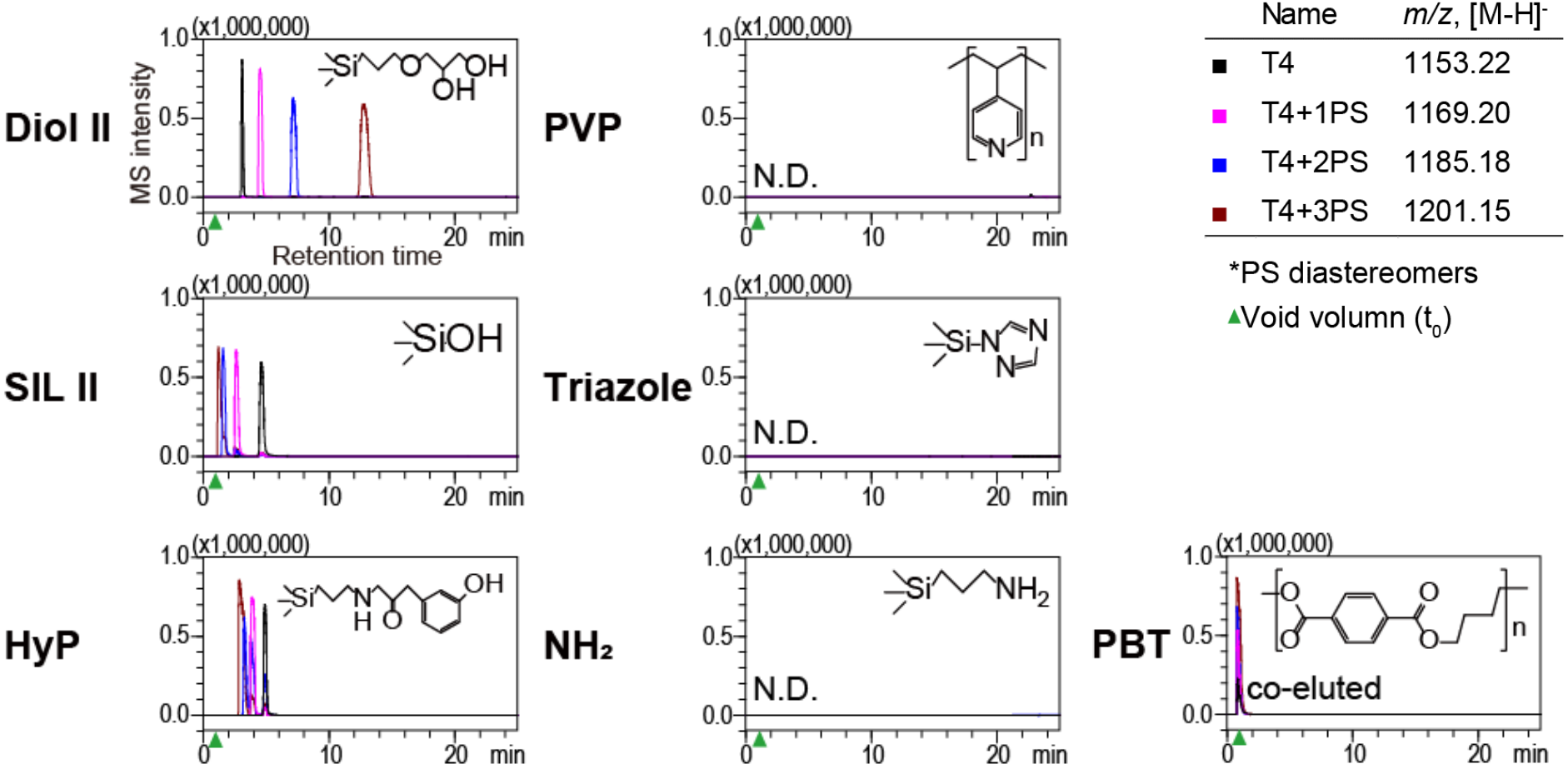
Oven temp. : 35 °C

Injection vol. : 1 µL, 100 µM

➡ **Diol II column** provides better separations.

1-3. Results | 2nd Column screening

We evaluate columns modified with polar stationary phases.



	Name	*m/z*, [M-H]⁻
■	T4	1153.22
■	T4+1PS	1169.20
■	T4+2PS	1185.18
■	T4+3PS	1201.15

*PS diastereomers

▲Void volumn (t_0)

Analytical conditions

Flow rate : 1 mL/min

MP : CO_2/MeOH/1 M AmF solution = **50:47.5:2.5**

Make-up : 0.1 mL/min, MeOH

Column : Shim-pack UC (**2.1** X 150 mm; 3 µm)

BPR : 10.0 MPa, 50 °C

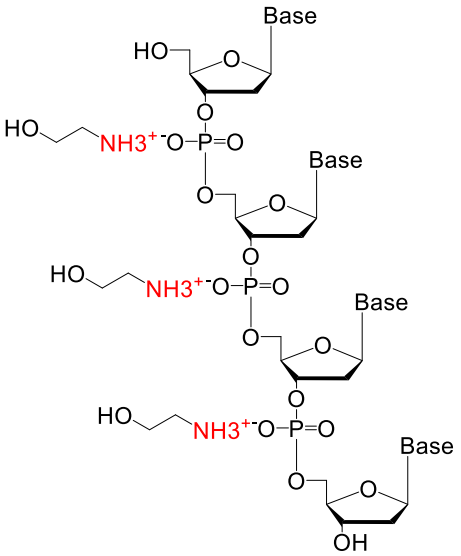
Oven temp. : 35 °C

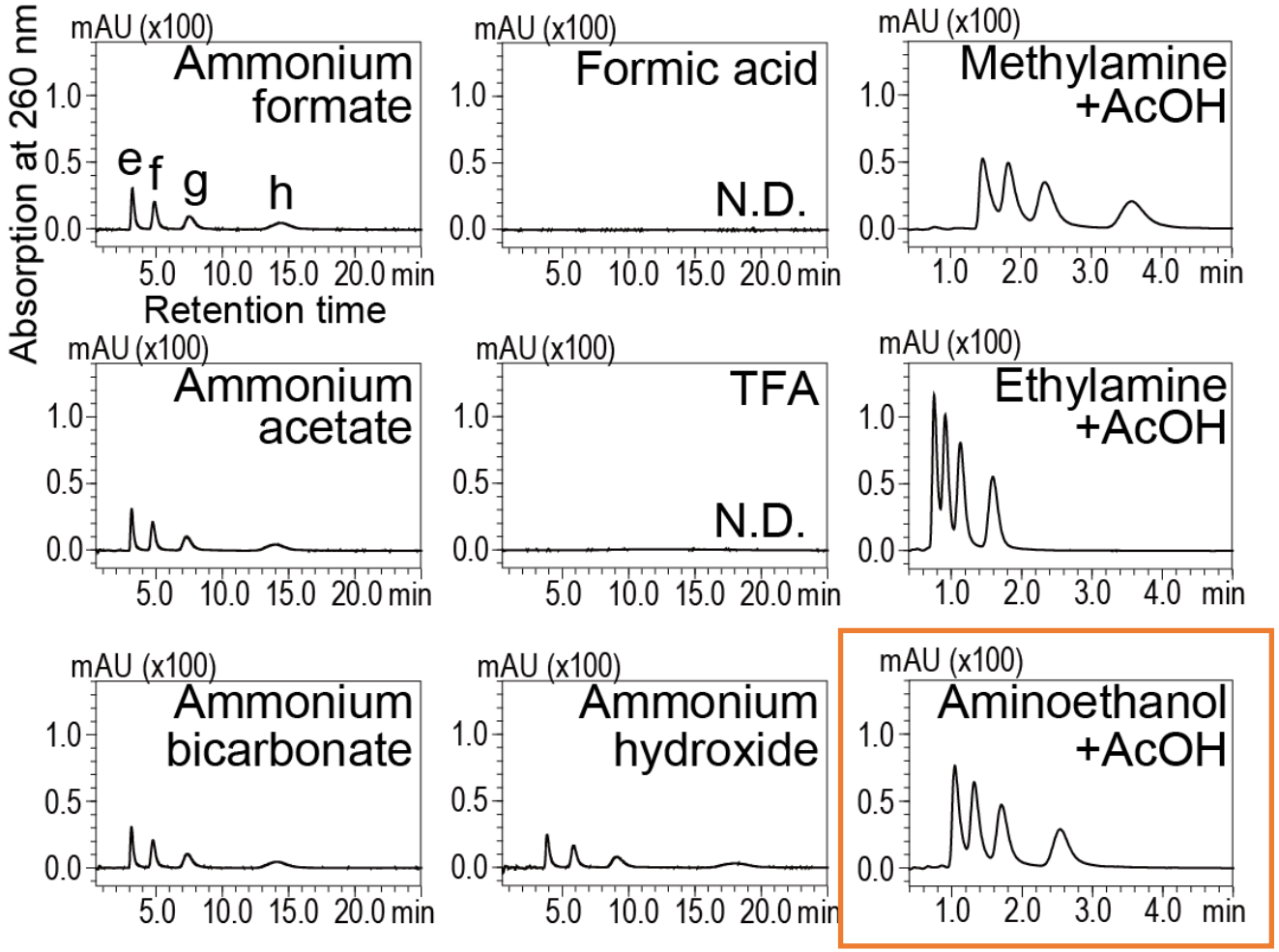
Injection vol. : 1 µL, 100 µM

➡ **Diol II column** provides better separations.

1-4. Results | Optimize additives for hydrophilic sequnces

(e) TAGC
(f) TAGC+1PS
(g) TAGC+2PS
(h) TAGC+3PS





Analytical conditions

Flow rate : **1.5** mL/min

MP : CO_2/additive in methanol buffer (95:5) = 50:50

Make-up : 0.1 mL/min, MeOH

Column : Shim-pack UC (**2.1** X 150 mm; 3 µm)

BPR : 10.0 MPa, 50 °C

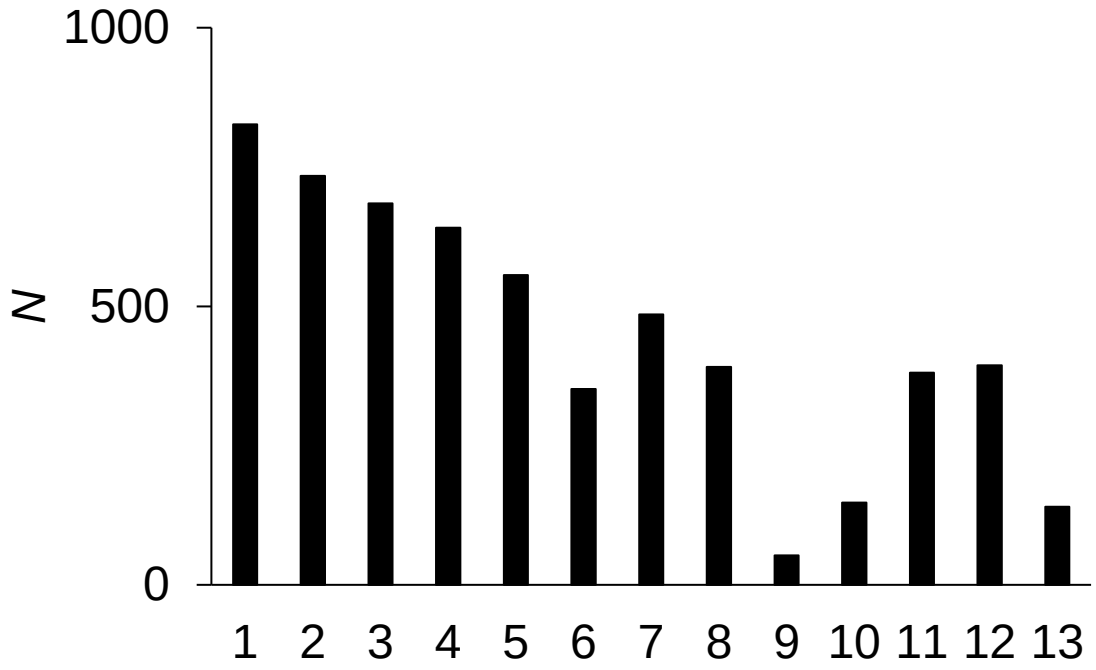
Oven temp. : 35 °C

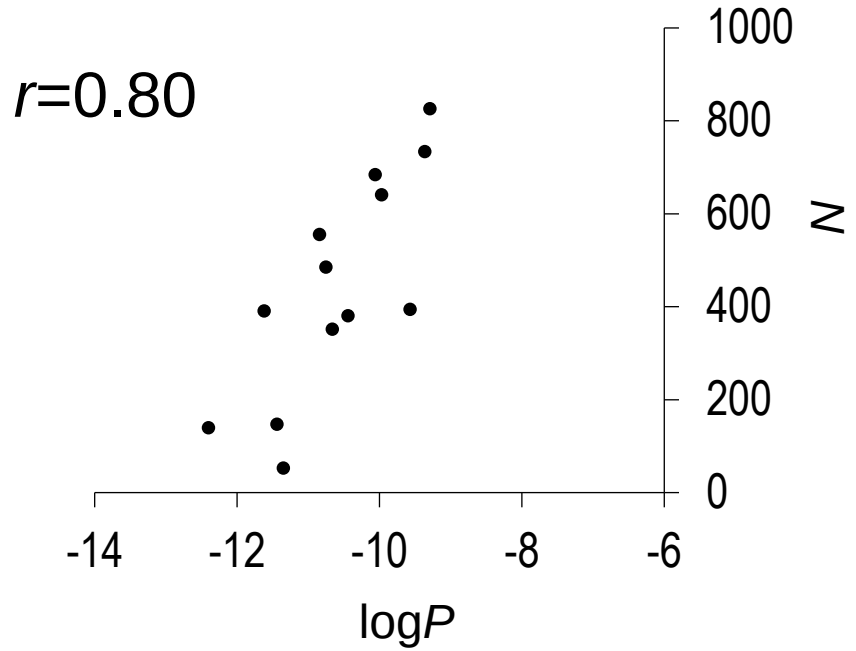
Injection vol. : 1 µL, 100 µM

➡ **Aminoethanol acetate** provides better peak shape and separation performance

1-5. Results | Sequence-dependency for peak shape

- Good peak shapes were obtained in the sequences whose **GC content was less than ½**

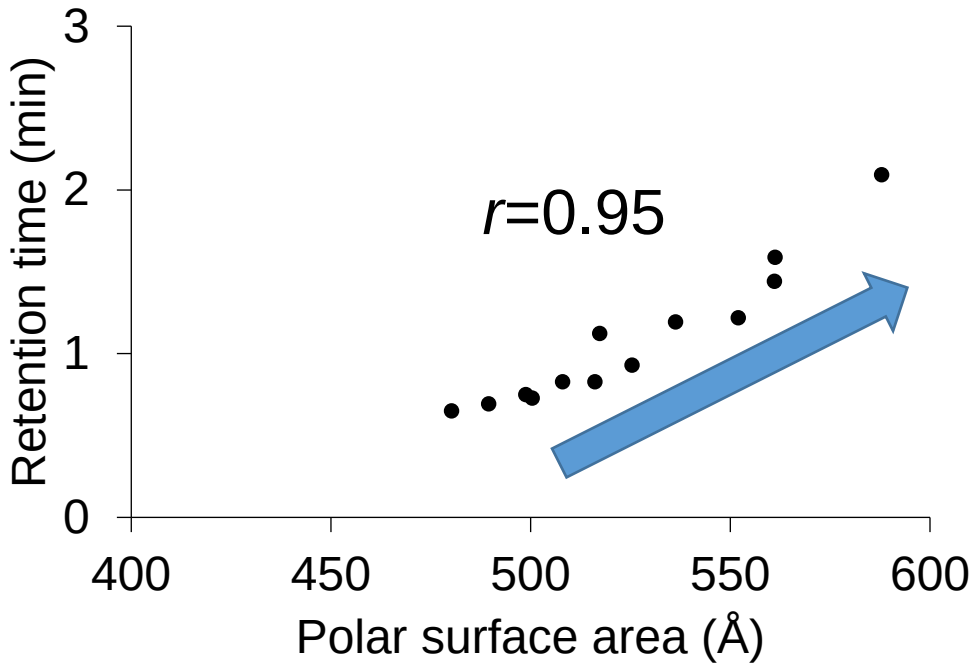


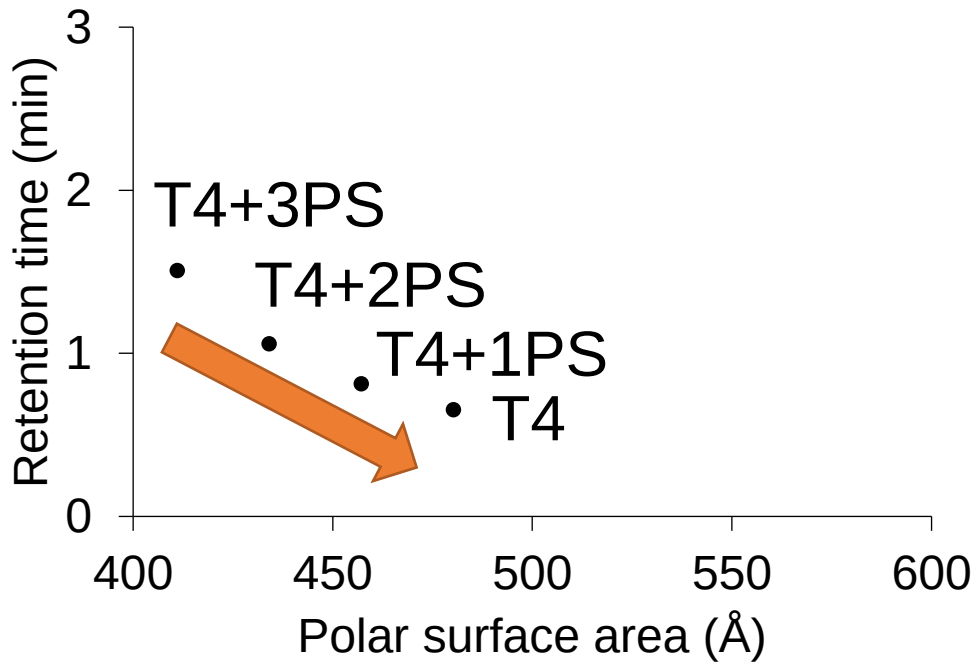


N: theoretical plate numbers
r: correlation coefficient

1-6. Results | Retention behavior

- Retention times of the selected sequences were **positively correlated with polar surface areas**





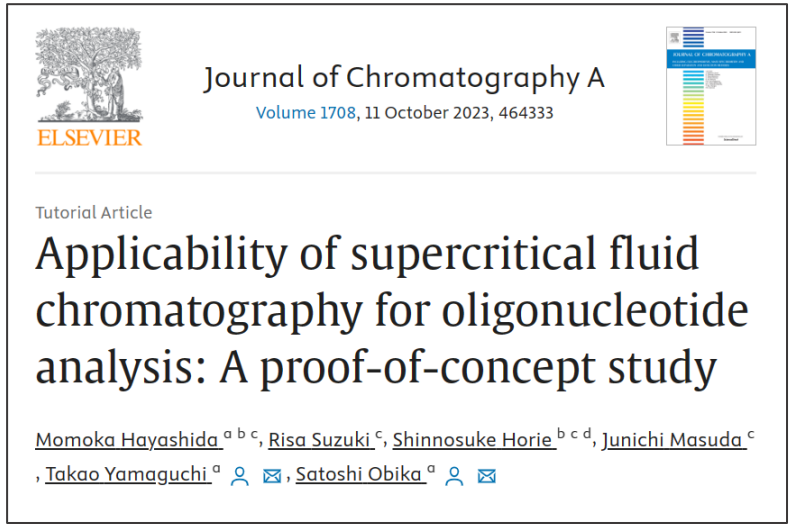
Hydrophilic interaction

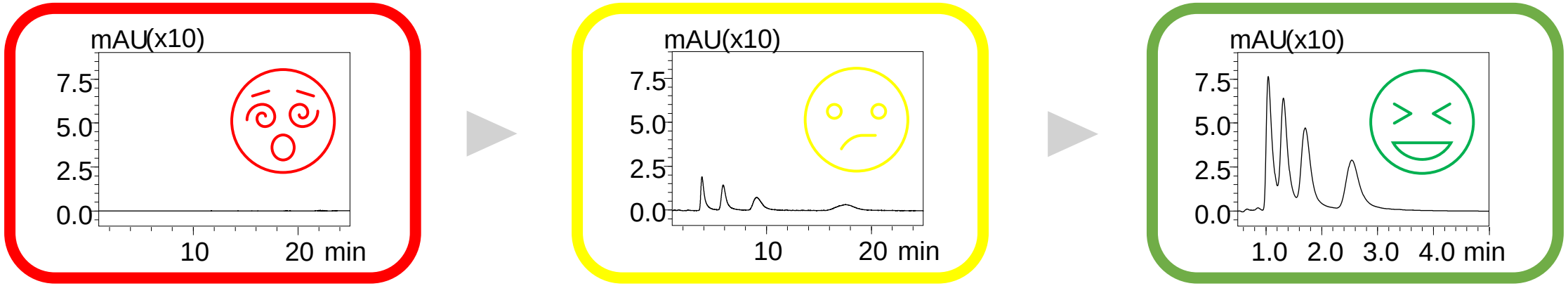
Unique selectivity of Diol II to PS contents

1. Summary

We evaluated the applicability of SFC to oligonucleotide analysis, using 4-mer oligonucleotides with different PS contents.

- Column screening revealed that Diol II provided better selectivity to PO and PS linkages.
- After examining several additives, we obtained good peak shapes and resolutions with aminoethanol.
- When the GC contents were less than 1/2, good peak shapes were obtained in the optimized method.





2. Introduction | Next topics

Evaluate the applicability of SFC separation to oligonucleotide analysis

①

Evaluation the separation performance for a 4-mer oligonucleotide, a model compound

- Optimization of column and modifier composition
- Separation performance for 4-mer with varying PS contents
- Retention behavior

(M. Hayashida *et el.*, *J of Chromatogr. A*, **2023**, 1708, 464333)

②

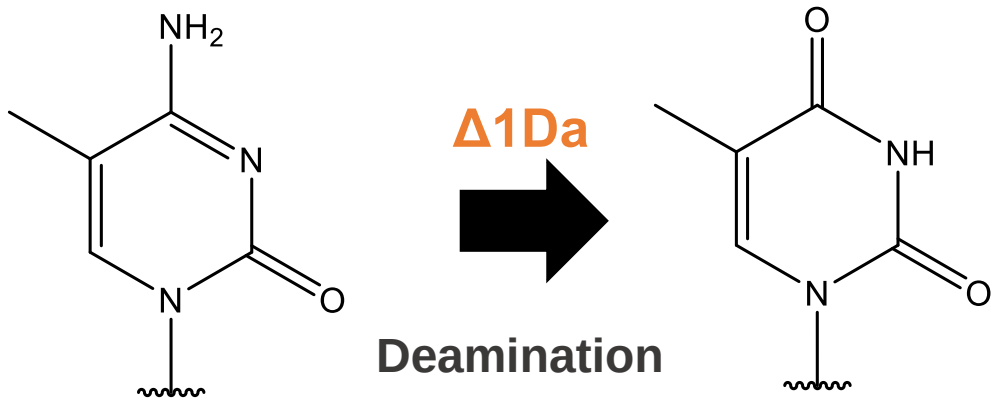
Apply to longer sequences

- Optimization of modifier composition for longer sequences
- Separation performance for deaminated products

(M. Hayashida *et el.*, *J of Chromatogr. A*, **2025**, 1744, 465731)

2. Introduction | Deamination analysis

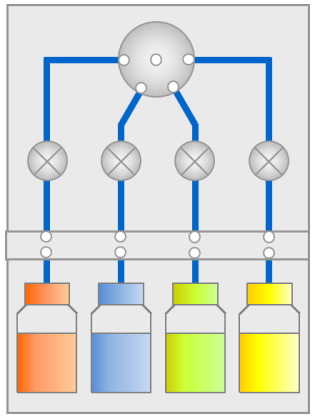
- The **4-amino group** of a cytidine, protected with acyl group, can be converted into a **hydroxyl group** due to the basic conditions of deprotection.
- Deamination analysis using MS is often challenging, and thus chromatographic separation is important.



5-methylcytosine (mC) 5-methyluracil (mU)

A method with **different selectivity from ion pair reverse phase (IPRP) chromatography** has been desired.

2. Materials | Purification for chemically synthesized oligonucleotides

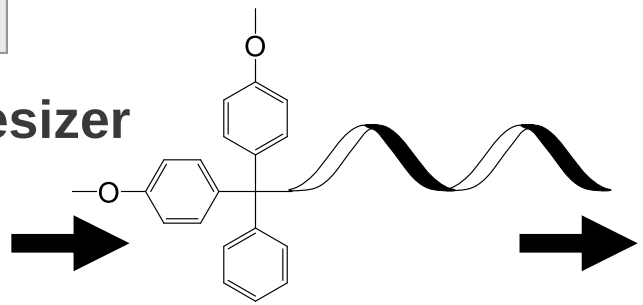


Oligo synthesizer

DMTr-off oligonucleotide

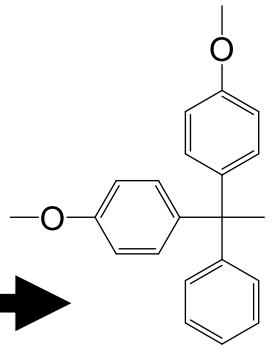
Purification

*DMTr (4,4'-Dimethoxytrityl):
a protecting group



DMTr-on oligonucleotide

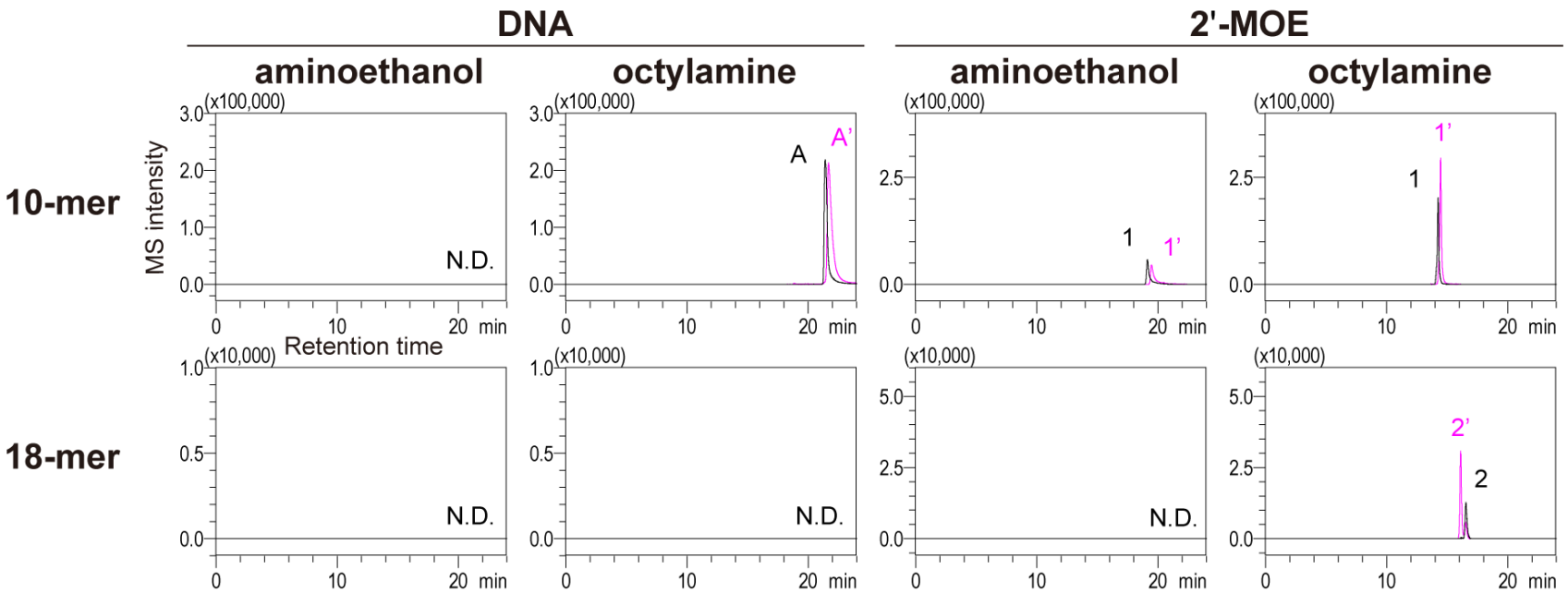
Purification



Deprotection

Purification

2-1. Results | Additives for the modifier



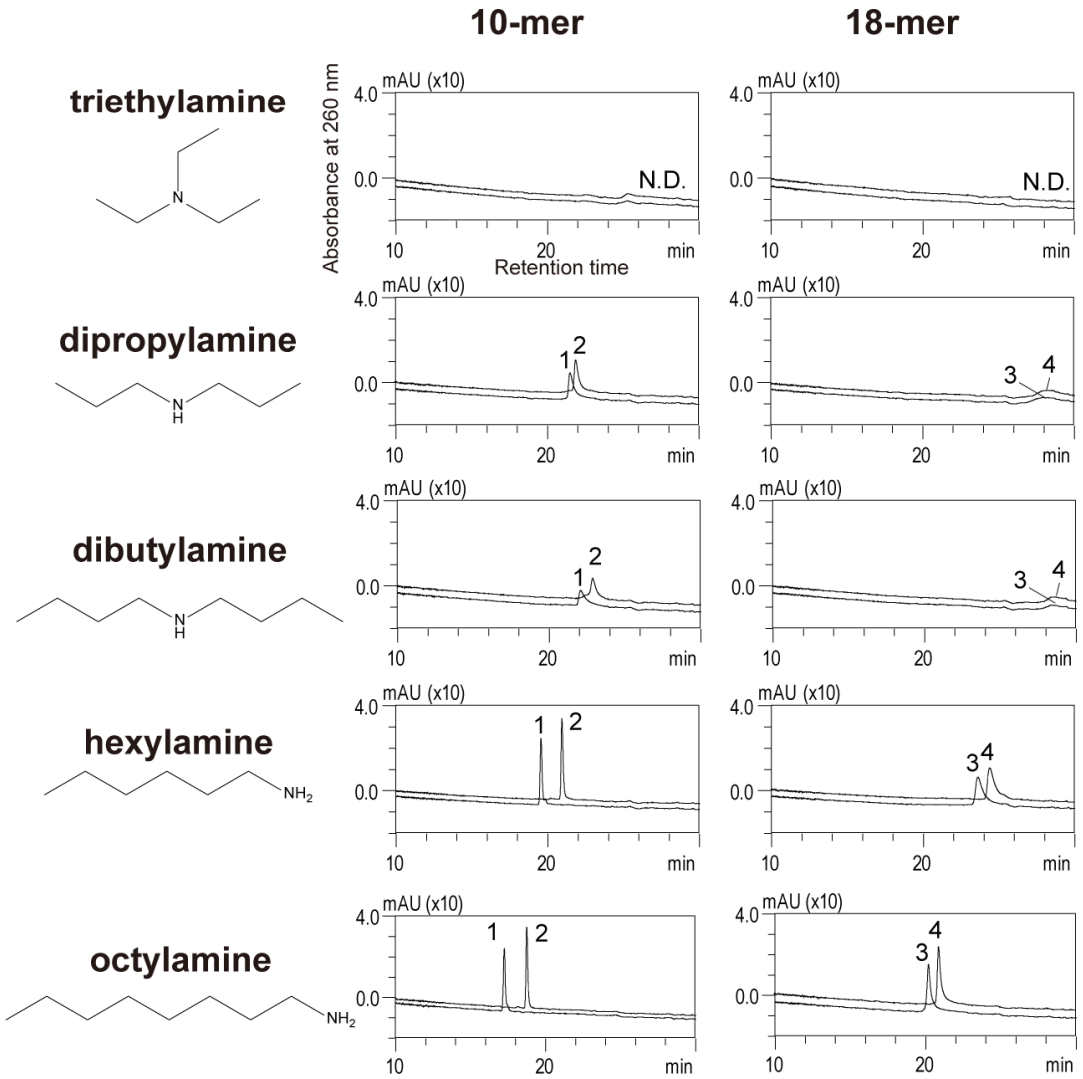
Analytical conditions
Flow rate : 1 mL/min
Modifier: methanol/water=95:5 containing 50 mM 2-aminoethanol or octylamine acetate
B conc.: 20% (0–2 min), 20–60% (2–20 min), 60% (20–24 min), 60–20% (24–25 min), 20% (25–30 min)
Make-up : 0.1 mL/min, methanol
Column : Shim-pack UC-Diol II (4.6x150 mm; 3 µm)
BPR : 10.0 MPa, 50 °C
Oven temp. : 35 °C
Injection vol. : 1 µL, 100 µM

Peak	Sequence (DNA, 5'-3')	*m/z*
A	DMTr- TCACTTTCAT	$[M-2H]^{2-}$, 1626.32
A'	TCACTTTCAT	$[M-2H]^{2-}$, 1475.26
B	DMTr- TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1440.51
B'	TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1364.98

Peak	Sequence (5'-3', full 2'-MOE, C: 5-methyl cytosine)	*m/z*
1	DMTr- TCACTTTCAT	$[M-3H]^{3-}$, 1344.69
1'	TCACTTTCAT	$[M-3H]^{3-}$, 1243.97
2	DMTr- TCACTTTCATAATGCTGG	$[M-5H]^{5-}$, 1430.15
2'	TCACTTTCATAATGCTGG	$[M-5H]^{5-}$, 1369.73

- **Octylamine acetate** provided better peak shape for 10- and 18-mer
- Sharp peak for **2′-MOE-modified 18-mer** were detected

2-2. Results | Optimize additives



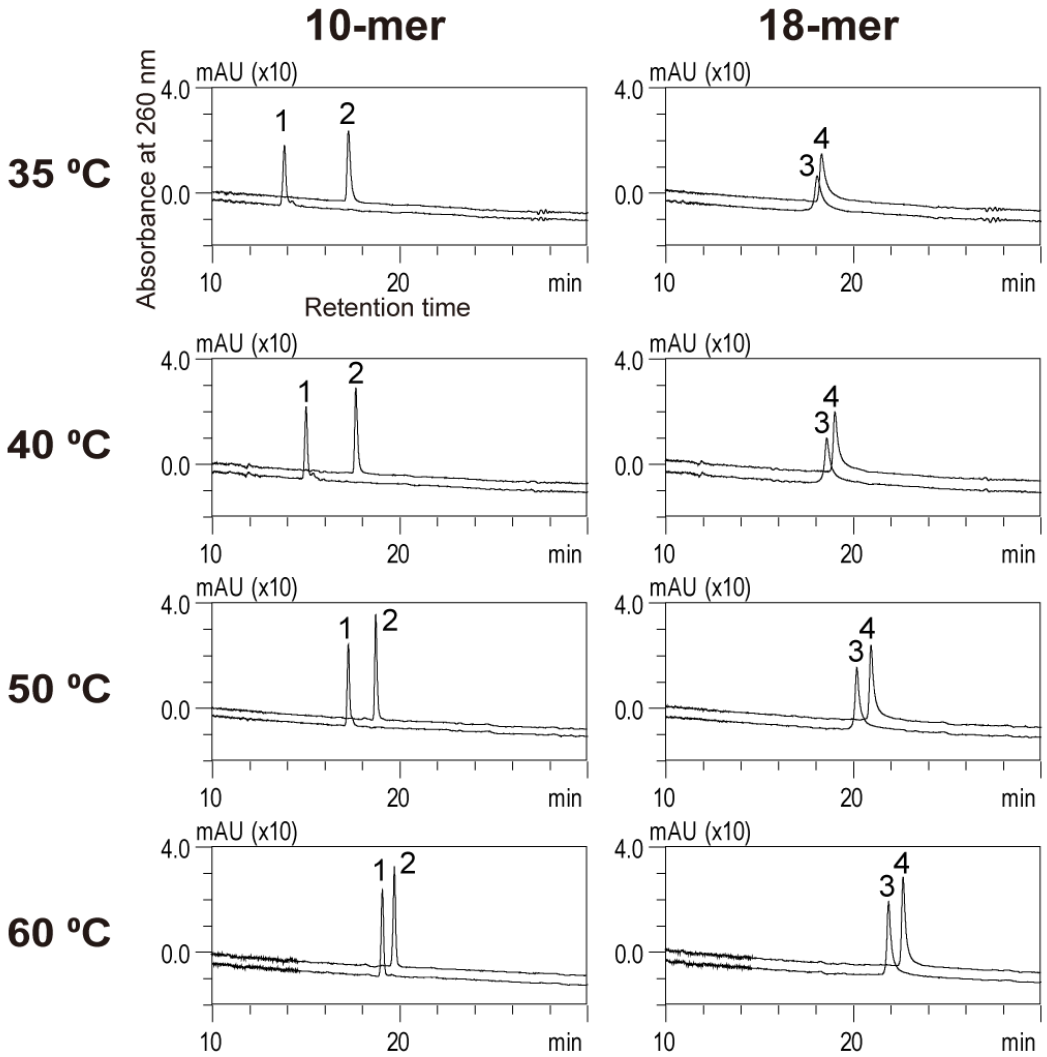
Peak	Sequence (5'-3', full 2'-MOE, C: 5-methyl cytosine)
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

Analytical conditions
Flow rate : 1 mL/min
Modifier: methanol/water=95:5 containing 50 mM additive and 50 mM acetic acid
B conc.: 30% (0–5 min), 30–60% (5–30 min), 60% (30–35 min), 60–30% (36–40 min)
Make-up : 0.1 mL/min, methanol
Column : Shim-pack UC-Diol II (4.6x150 mm; 3 µm)
BPR : 10.0 MPa, 50 °C
Oven temp. : 35 °C
Injection vol. : 1 µL, 100 µM

- **Primary amine** provided better peak shapes than secondary and tertiary amines

➡ **Octylamine acetate** provided shaper peaks for 18-mer

2-3. Results | Optimize column oven temp.

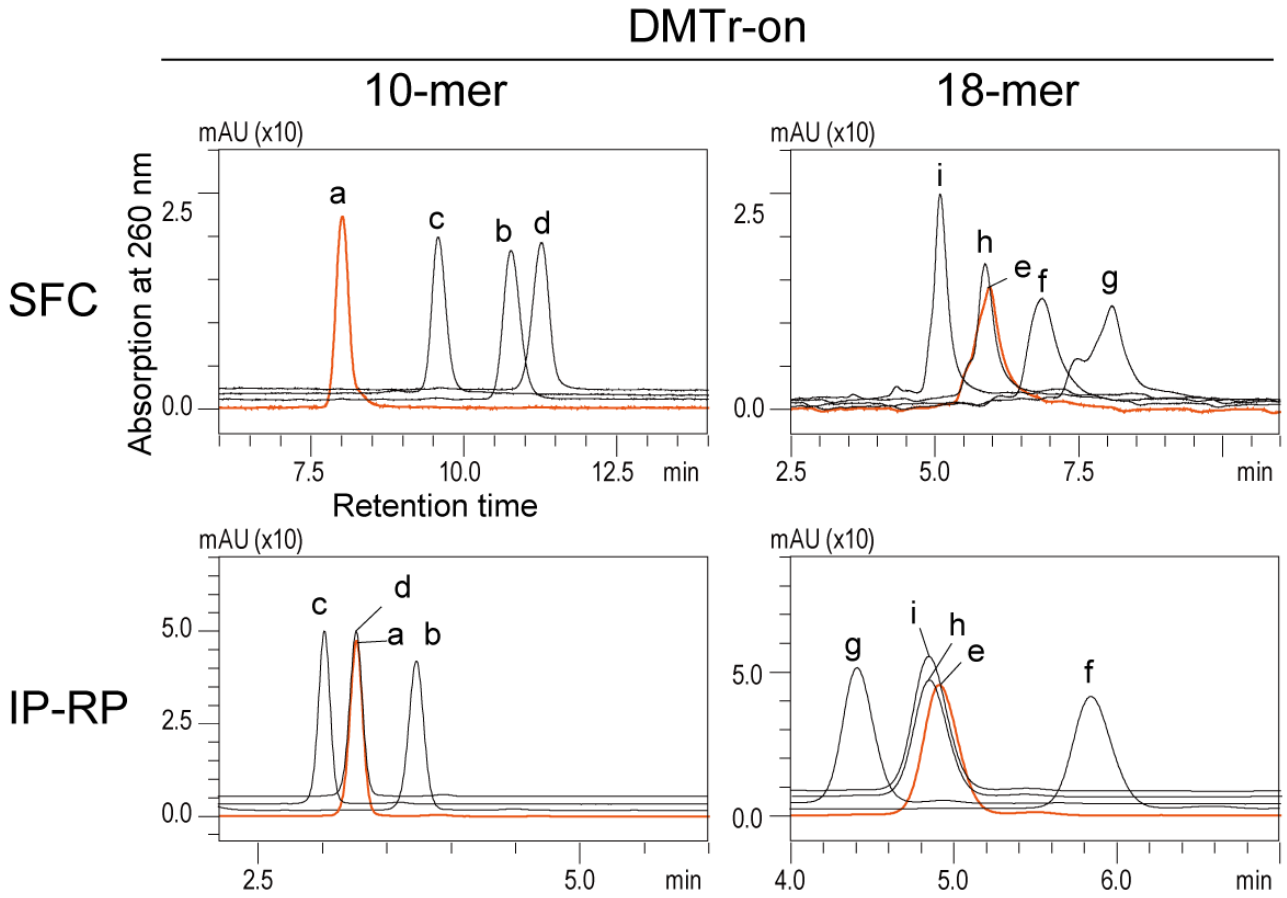


Peak	Sequence (5'-3', full 2'-MOE, C: 5-methyl cytosine)
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

Analytical conditions
Flow rate : 1 mL/min
Modifier: methanol/water=95:5 containing 50 mM octylamine acetate
B conc.: 30% (0–5 min), 30–60% (5–30 min), 60% (30–35 min), 60–30% (36–40 min)
Make-up : 0.1 mL/min, methanol
Column : Shim-pack UC-Diol II (4.6x150 mm; 3 µm)
BPR : 10.0 MPa, 50 °C
Oven temp. : 35-50 °C
Injection vol. : 1 µL, 100 µM

50 and 60 °C provided better separation performance
→ 50 °C was selected

2-4. Results | Separate deaminated impurities contained in DMTr-on oligos

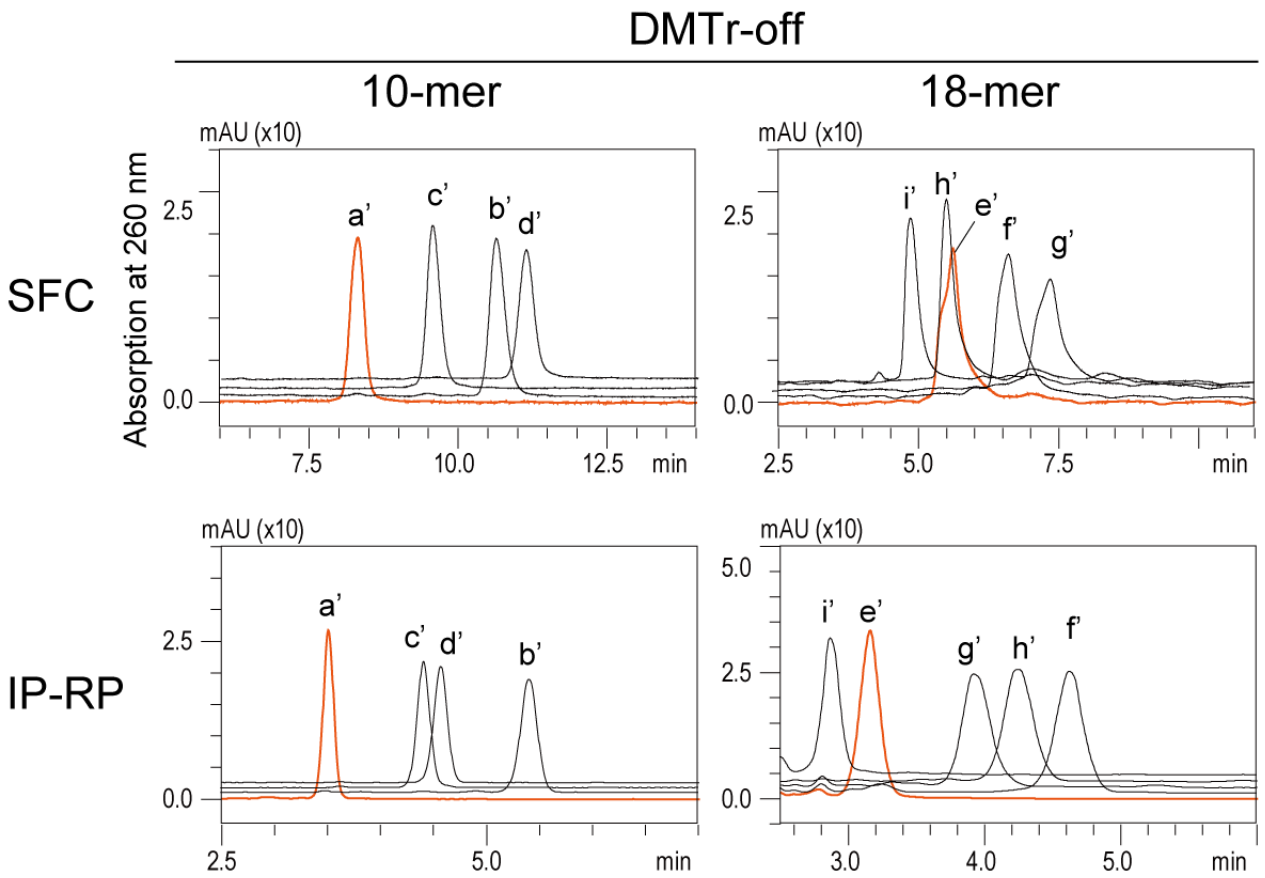


Peak	Sequence(5'-3', full 2'-MOE, C: 5-methyl cytosine)
a	DMTr-TCACTTTCAT
b	DMTr-T**T**ACTTTCAT
c	DMTr-TCA**T**TTTCAT
d	DMTr-TCACTTT**T**AT
e	DMTr-TCACTTTCATAATGCTGG
f	DMTr-T**T**ACTTTCATAATGCTGG
g	DMTr-TCA**T**TTTCATAATGCTGG
h	DMTr-TCACTTT**T**ATAATGCTGG

Analytical conditions for SFC
Flow rate：1.0 mL/min, MP：A)CO_2, B)50 mM octylamine acetate in methanol buffer (95:5) B conc.: 40% (10-mer), 45% (18-mer), Column：Shim-pack UC-Diol II (4.6×150 mm; 3 µm) BPR：10.0 MPa, 50 ºC, Oven temp.：50 ºC, Injection vol.：1 µL, 100 µM
Analytical conditions for IPRP
Flow rate：1.0 mL/min, MP：A)10 mM propylamine in water, B)ACN B conc.: 25% (10-mer), 23% (18-mer), Column：Shim-pack GIST C18 (4.6×150 mm; 3 µm), Oven temp.：50 ºC, Injection vol.：1 µL, 100 µM

- **SFC** successfully separated some deaminated products from the 10- and 18-mer main products.
- **Sequence d** were coeluted with the main product using IPRP-LC.

2-5. Results | Separate deaminated impurities contained in DMTr-off oligos



Peak	Sequence(5'-3', full 2'-MOE, C: 5-methyl cytosine)
a'	TCACTTTCAT
b'	T**T**ACTTTCAT
c'	TCA**T**TTTCAT
d'	TCACTTT**T**AT
e'	TCACTTTCATAATGCTGG
f'	T**T**ACTTTCATAATGCTGG
g'	TCA**T**TTTCATAATGCTGG
h'	TCACTTT**T**ATAATGCTGG
i'	TCACTTTCATAATG**T**TGG

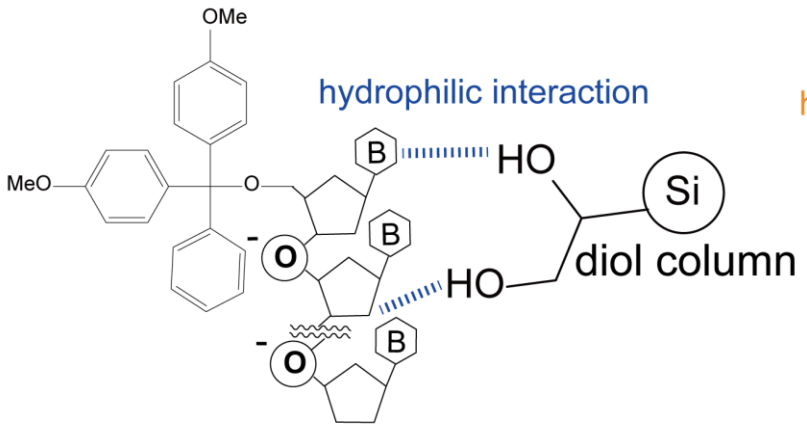
Analytical conditions for SFC
Flow rate：1.0 mL/min, MP：A)CO_2, B)50 mM octylamine acetate in methanol buffer (95:5) B conc.: 40% (10-mer), 45% (18-mer), Column：Shim-pack UC-Diol II (4.6×150 mm; 3 µm) BPR：10.0 MPa, 50 ºC, Oven temp.：50 ºC, Injection vol.：1 µL, 100 µM
Analytical conditions for IPRP
Flow rate：1.0 mL/min, MP：A)10 mM propylamine in water, B)ACN B conc.: 12% (10-mer), 13% (18-mer), Column：Shim-pack GIST C18 (4.6×150 mm; 3 µm), Oven temp.：50 ºC, Injection vol.：1 µL, 100 µM

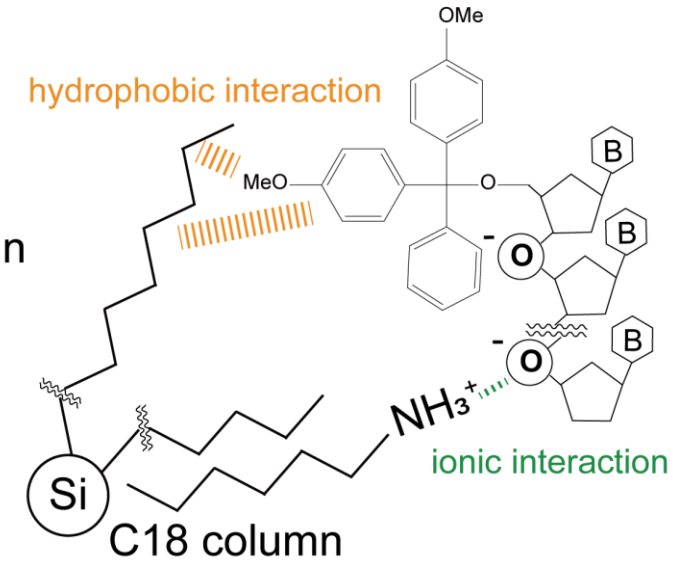
- DMTr-on and off sequences were almost **coeluted using SFC**.
- The separation capability **for DMTr-off sequences was higher** than that for DMTr-on sequences using IPRP-LC

2. Discussions | Different retention behavior

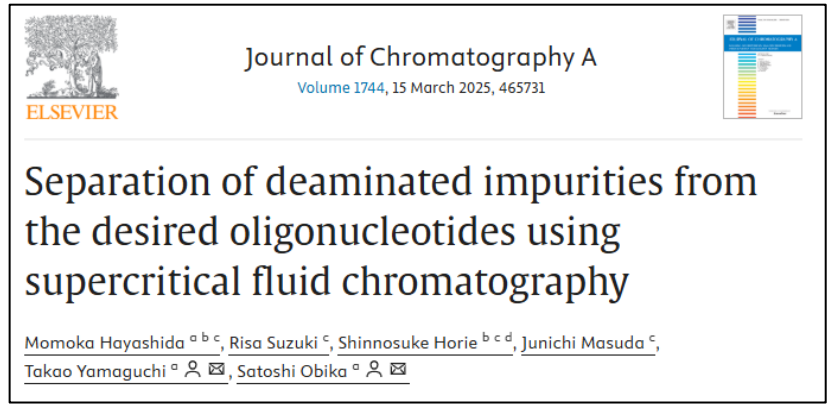
- The retention behavior of DMTr-on oligonucleotides using SFC was **dominated by interaction with oligonucleotides.**
- That using IPRP-LC was **strongly influenced by DMTr group** and thus the target sequences and their deaminated impurities were difficult to separate because of the similar hydrophobicity

→ SFC could be a suitable method for analyzing oligonucleotides having **hydrophobic organ-targeting ligands** as well as the DMTr group





For more details…



2. Summary

- Octylamine acetate provided a sharp peak for 10- and 18-mer 2-MOE-modified oligonucleotides.
- 50 ºC was better for column oven temp. to separate deaminated products.
- DMTr-on and –off oligos were co-eluted using SFC.
- Several deaminated impurities were separated from the main products using SFC.
- SFC and IPRP-LC exhibited **different** selectivity

1 & 2. Conclusion

This study demonstrated the applicability of SFC in oligonucleotide analysis

①

Evaluation the separation performance for a 4-mer oligonucleotide, a model compound

- 4-mer oligonucleotides with different PS contents were separated

②

Apply to longer sequences

- We obtained peaks for 10- and 18-mer oligonucleotides
- Deaminated products were separated from the main products

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