Journal of Chromatography A 1744 (2025) 465731

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of deaminated impurities from the desired oligonucleotides using supercritical fluid chromatography

Momoka Hayashida [a,b,c], Risa Suzuki [c], Shinnosuke Horie [b,c,d], Junichi Masuda [c], Takao Yamaguchi [a,*], Satoshi Obika [a,*]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Shimadzu Analytical Innovation Research Laboratories, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Shimadzu Corporation, 1 Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan
[d] Shimadzu Europa GmbH, Albert-Hahn-Strasse 6-10, 47269 Duisburg, Germany

ARTICLE INFO

Keywords:
Supercritical fluid chromatography
Oligonucleotide
Deamination

ABSTRACT

With recent advancements concerning the optimization of the analytical conditions, it is feasible to analyze polar molecules using supercritical fluid chromatography (SFC). In this study, the applicability of SFC is evaluated for analyzing 5-, 10-, 15-, and 18-mer oligonucleotides, and SFC is then applied to analyze deaminated products, which are side products generated during oligonucleotide synthesis. These side products are difficult to separate from the target oligonucleotide, with the difficulty varying depending on the deamination position and sequences, even when using ion-pair reversed-phase liquid chromatography (IP-RPLC), a common method for oligonucleotide analysis. Our results demonstrate that SFC, with octylamine as a modifier additive, can achieve sharp chromatographic peaks for 5-, 10-, 15-, and 18-mer oligonucleotides modified with 2′-*O*-methoxyethyl RNA (2′-MOE), regardless of the presence of the hydrophobic 4,4′-dimethoxytrityl (DMTr) group on the sequence. After optimization of the column oven temperature, modifier additive, and stationary phase, SFC successfully separated oligonucleotides with various numbers and positions of deamination from the target oligonucleotide. SFC exhibited different selectivities for DMTr-on and DMTr-off oligonucleotides compared with those for IP-RPLC, which indicates that SFC can serve as a valuable alternative tool for the purification and analysis of oligonucleotides.

1. Introduction

Oligonucleotide therapeutics are being actively researched and developed as new modalities for drug discovery, with the number of products available in the market increasing annually. Antisense oligonucleotides (ASOs), a type of therapeutic oligonucleotide, consists of single-stranded oligonucleotides approximately 20-mer in length. To enhance their activity and safety, ASOs are chemically modified with sugars, nucleobases, and phosphodiester moieties. These oligonucleotides are primarily synthesized using a solid-phase phosphoramidite method, in which nucleotides are individually added to the growing chain through a coupling reaction with phosphoramidite on a solid support [1]. During the synthesis of oligonucleotides from the 3′ to the 5′ end, the 5′ end will have a 4,4′-dimethoxytrityl (DMTr, Fig. 1a) group once each coupling reaction is successfully completed. The DMTr group is then removed before coupling with the subsequent phosphoramidite. After the elongation is complete, the oligonucleotide is cleaved from the solid support, purified, and used as an active pharmaceutical ingredient (API). The crude sequence obtained is purified to achieve a purity appropriate for pharmaceuticals. There are two main purification methods: one in which the oligonucleotide is cleaved with the DMTr group still attached and purified using a reversed-phase column, followed by DMTr deprotection and further purification by liquid chromatography (LC), and the other in which the DMTr group is removed before the oligonucleotides are cleaved from the solid phase, with the oligonucleotide then cleaved from the solid support and purified directly by LC. Completely removing the many impurities generated during the synthesis is challenging. Therefore, quantification and characterization of impurities in API are important. For example, deamination products are generated when the amino group of a nucleobase is replaced with a hydroxyl group under the basic conditions used for cleavage and deprotection. This is mainly observed for cytosine, which is converted to

* Corresponding authors.
E-mail addresses: yamaguchi-ta@phs.osaka-u.ac.jp (T. Yamaguchi), obika@phs.osaka-u.ac.jp (S. Obika).

https://doi.org/10.1016/j.chroma.2025.465731
Received 4 December 2024; Received in revised form 24 January 2025; Accepted 27 January 2025
Available online 27 January 2025

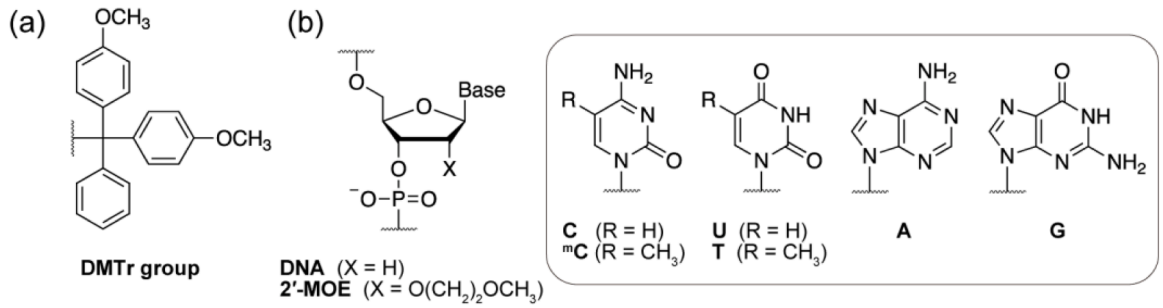


Fig. 1. (a) Structure of the DMTr group; (b) Structures of DNA and 2′-MOE used in this study, where ``Base'' represents a nucleobase, such as cytosine (C), 5-methylcytosine (^{m}C), uracil (U), thymine (T), adenine (A), or guanine (G).

uracil (Fig. 1b). Although adenine can also undergo deamination, its rate is 50 times slower than cytosine [2]. The impurities in which cytosine is converted to uracil in ASOs can cause adverse off-target effects on non-target RNA, necessitating appropriate analysis.

Chromatographic separation is crucial for the analysis of deamination because the mass difference between the parent sequence and the deaminated product is only 1 Da, making mass spectrometry (MS) detection challenging. Rentel et al. reported a quantification method for deamination products by evaluating isotopic distribution shifts [3]. However, chromatographic separation is recommended to detect small amounts of impurities and to avoid ion suppression. Roussis et al. reported separation using ion-pair reversed-phase liquid chromatography (IP-RPLC) with propylamine [4]. However, the separation can still be difficult, depending on the deamination position and sequence.

Supercritical fluid chromatography (SFC) uses pressurized carbon dioxide as mobile phase. Supercritical fluids have a lower viscosity and higher diffusivity than liquids, resulting in a high separation efficiency. SFC is also scalable for preparative purposes. Carbon dioxide is released upon fractionation for preparative separation, with the remaining solvent largely being organic, simplifying its removal, and thus SFC is also used for purification. Although applying SFC to highly polar compounds remains challenging because of the low polarity of carbon dioxide, recent reports have demonstrated that optimizing the cosolvent composition and increasing the cosolvent ratio can enable SFC to analyze highly polar compounds [5]. We previously analyzed 4-mer oligonucleotides using SFC and found that modifiers containing 2-aminoethanol allowed us to analyze polar short oligonucleotides [6]. However, 4-mer oligonucleotides containing more than two guanine or cytosine nucleobases, which are more polar than the other two nucleobases, showed peak tailing. Therefore, this method must be optimized to analyze longer and more polar oligonucleotides.

We investigated an approach for eluting polar compounds with well-defined peak shapes. This method involves the use of long-chain alkylamines as mobile phase additives, which can improve the peak shape by increasing the solubility of the target molecules in the mobile phase [7–10]. Sen et al. compared the SFC peaks using three modifiers containing isopropylamine, butylamine, or pentylamine for polar metabolite analysis, finding that the peak widths decreased as the alkyl chains became longer [11]. Other studies have shown that alkylamines improve the peak shapes of polar compounds [12]. Therefore, we applied this approach, involving the use of long-chain alkylamines as mobile phase additives, to oligonucleotide analysis. In this study, we evaluated the applicability of SFC to 5-,10-, 15-, and 18-mer modified oligonucleotides and used it to analyze deamination and assess its separation capability. We used sequences with and without DMTr groups, expecting that the high hydrophobicity of the DMTr group would make analysis easier for sequences containing it. We also investigated how the presence or absence of the DMTr group affected the retention behavior.

2. Materials and methods

2.1. Chemicals and solvents

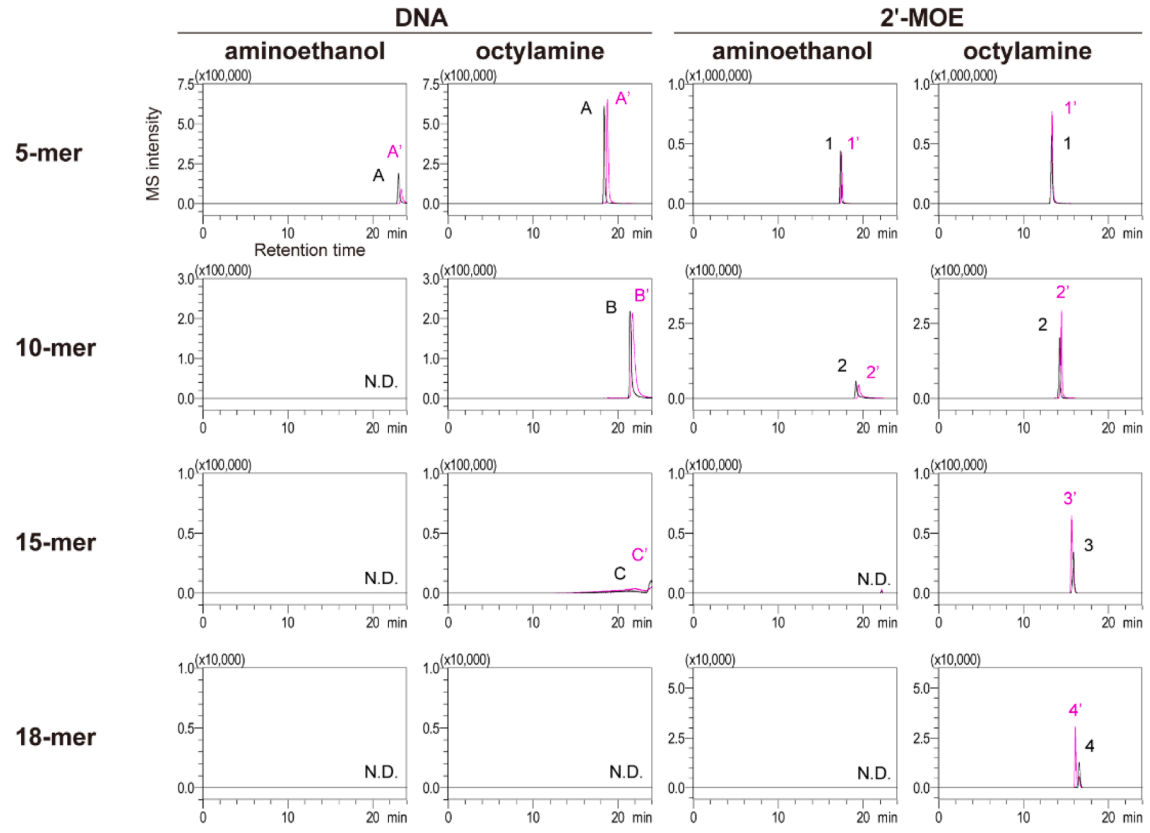
Synthetic oligonucleotides were purchased from Hokkaido System Science (Hokkaido, Japan) for reverse-phase cartridge purification. Methanol, aminoethanol, octylamine, dipropylamine, hexylamine, dibutylamine, and acetic acid were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). A Milli-Q water purification system, purchased from Merck Millipore (Massachusetts, United States), was used to prepare the mobile phases.

2.2. SFC instrumentation, detection, and analytical conditions

The SFC instrument used in this study was the Nexera UC system (Shimadzu Corporation, Kyoto, Japan). The instrument was equipped with carbon dioxide, modifier, and makeup pumps (LC-30ADSF, LC-30AD, and LC-30AD, respectively), an autosampler (SIL-30AC, partial loop injection, 5 μL loop), a column oven (CTO-20C), a photodiode array detector (SPD-M20A) with a high-pressure cell, and a back pressure regulator (SFC-30A). The SFC instrument was directly coupled to a quadrupole time-of-flight mass spectrometer (LCMS-9030). The LabSolutions LC-MS software was used for system control, data acquisition, and data processing. Shim-pack UC-Diol II (150 × 4.6 mm I.D., 3 μm, hereinafter called "Diol II column") and Shim-pack UC-Phenyl (150 × 4.6 mm I.D., 3 μm,), all purchased from Shimadzu Corporation (Kyoto, Japan), were the columns employed. The flow rate was set at 1.0 mL/min, and the back pressure regulator was set at 10 MPa and 50 °C. The injection volume was 1.0 μL of 100 μM sample solutions. Other detailed conditions for the modifier compositions, CO_2/modifier ratio, columns, flow rate, injection volume, column oven temperature, and back pressure are listed in Table S1. UV chromatograms were obtained at 260 nm with a resolution of 1.2 nm using a photodiode array detector. Extracted ion chromatograms (EICs) and total ion chromatograms (TICs) were obtained in negative ionization mode from 150 to 2000 *m/z* by electrospray ionization MS (ESI-MS). The interface temperature was set to 350 °C. The nebulizing gas flow rate was 3.0 L/min, whereas the heating and drying gas flow rates were both set to 10.0 L/min. The desolvation temperature was 250 °C, and the heat-block temperature was 400 °C. Capillary voltage was set to −3.5 kV (ESI-).

2.3. LC instrumentation, detection, and analytical conditions

The HPLC system used in this study was a Nexera XS system (Shimadzu Corporation, Kyoto, Japan). The system was equipped with two mobile-phase pumps (LC-40D XS), an autosampler (SIL-40C XS), a column oven (CTO-40C), and a photodiode array detector (SPD-M40) was directly coupled to a quadrupole time-of-flight mass spectrometer (LCMS-9030). The LabSolutions LC-MS software was used for system control, data acquisition, and data processing. The column oven temperature was set to 50 °C, and the injection volume was 1.0 μL of 100 μM



Peak	Sequence (DNA, 5'-3')		*m/z*	Rt (min)
A	DMTr-	TCACT	$[M-2H]^{2-}$, 868.70	18.4
A'		TCACT	$[M-2H]^{2-}$, 717.64	18.7
B	DMTr-	TCACTTTCAT	$[M-2H]^{2-}$, 1626.32	21.4
B'		TCACTTTCAT	$[M-2H]^{2-}$, 1475.26	21.7
C	DMTr-	TCACTTTCATAATGC	$[M-3H]^{3-}$, 1600.30	N.D.
C'		TCACTTTCATAATGC	$[M-3H]^{3-}$, 1499.59	N.D.
D	DMTr-	TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1440.51	N.D.
D'		TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1364.98	N.D.

Peak	Sequence (5'-3')		*m/z*	Rt (min)
1	DMTr-	TC<u>A</u>CT	$[M-2H]^{2-}$, 1068.31	13.3
1'		TC<u>A</u>CT	$[M-2H]^{2-}$, 916.74	13.3
2	DMTr-	TC<u>A</u>CTTTC<u>A</u>T	$[M-3H]^{3-}$, 1344.69	14.3
2'		TC<u>A</u>CTTTC<u>A</u>T	$[M-3H]^{3-}$, 1243.97	14.5
3	DMTr-	TC<u>A</u>CTTTC<u>A</u>TAATG<u>C</u>	$[M-4H]^{4-}$, 1491.63	15.9
3'		TC<u>A</u>CTTTC<u>A</u>TAATG<u>C</u>	$[M-4H]^{4-}$, 1416.09	15.7
4	DMTr-	TC<u>A</u>CTTTC<u>A</u>TAATG<u>C</u>TGG	$[M-5H]^{5-}$, 1430.15	16.5
4'		TC<u>A</u>CTTTC<u>A</u>TAATG<u>C</u>TGG	$[M-5H]^{5-}$, 1369.73	16.1

"<u>C</u>" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

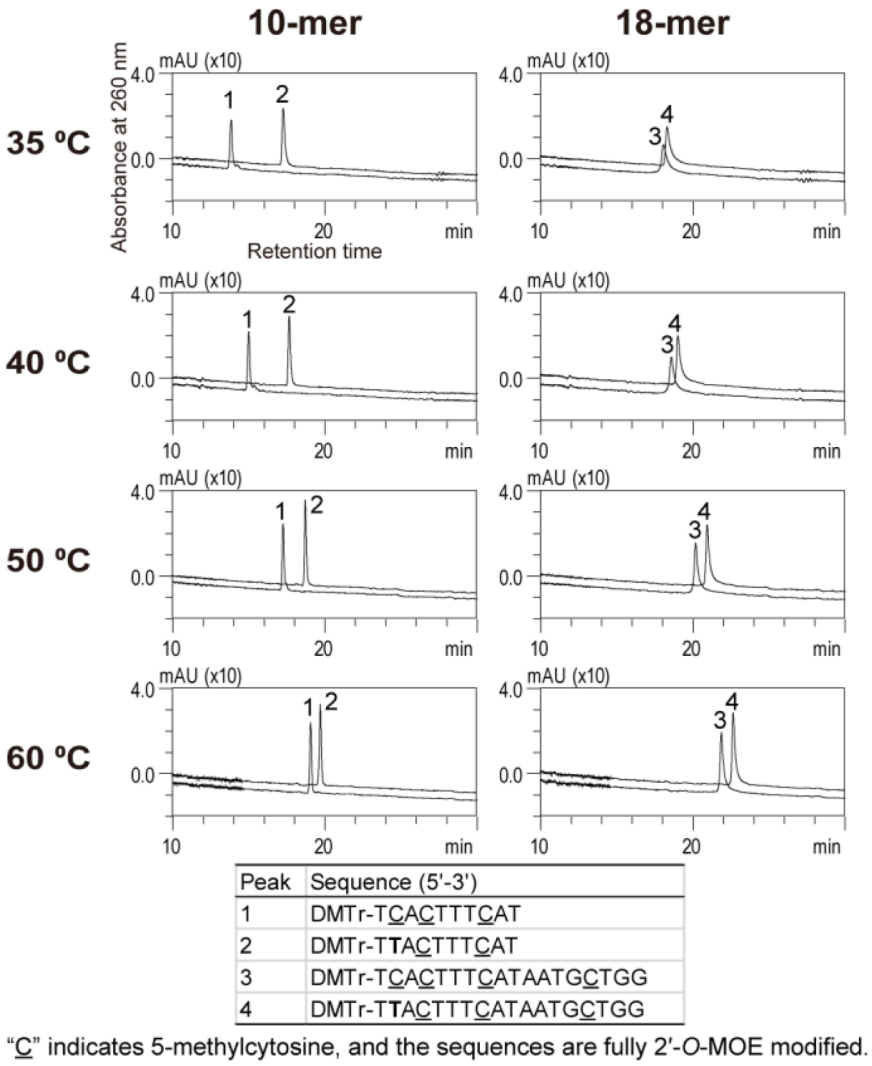
Fig. 2. Extracted ion chromatograms (EICs) of 5-, 10-, 15-, and 18-mer DNA and 2′-MOE-modified oligonucleotides. Black lines indicate oligonucleotides with DMTr groups, and pink lines indicate oligonucleotides without DMTr groups. Modifier: 50 mM 2-aminoethanol and 50 mM acetic acid in methanol and water (95:5, v/v), and 50 mM octylamine and 50 mM acetic acid in methanol and water (95:5, v/v). Column: Shim-pack UC-Diol II (150 × 4.6 mm I.D., 3 μm). The peaks labeled 1–4, 1′–4′, A–D, and A′–D′ on the chromatograms are shown in the tables below chromatograms. The retention times (Rt) represent averaged measurements from three replicates. For analytical conditions, see Table S1 (Entry 1).

sample solutions. The detailed conditions for the mobile phase compositions, columns, and flow rates are listed in Table S2.

3. Results and discussion

3.1. Evaluating the applicability of SFC to 5-, 10-, 15-, and 18-mer oligonucleotides

Initially, we investigated the peak shapes of 5- to 18-mer DNA using two modifiers: one containing 2-aminoethanol, as employed in our previous study [6], and the other containing octylamine, a relatively hydrophobic ion-pair reagent compatible with MS analysis. In our previous study, we examined 2-aminoethanol concentrations ranging from 10 mM to 100 mM and observed good peak shapes at concentrations of 40 mM or higher. Therefore, in the current study, we fixed the alkylamine concentration at 50 mM and performed oligonucleotide analysis. To neutralize the pH of the alkylamine, acetic acid was contained at the same concentration. A Diol II column packed with alkyldiol-modified silica, which was used in a previous study, was employed for this evaluation [6]. When a modifier containing 2-aminoethanol was used, 5-mer DNA required 60 % of the modifier for elution, whereas 10-, 15-, and 18-mer DNA were not eluted (Fig. 2, DNA-aminoethanol). In contrast, both 5-mer DNA and 10-mer DNA were eluted from the column when a modifier containing octylamine was used, indicating that octylamine enabled the analysis of longer DNA compared to 2-aminoethanol (Fig. 2, DNA-octylamine). However, peaks for 15- and 18-mer DNA were not detected. The retention times of the DMTr-off 5- and 10-mer DNA were slightly longer than those of the DMTr-on 5- and 10-mer DNA, respectively (Fig. 2). We also evaluated 5-, 10-, 15-, and 18-mer oligonucleotides, which were fully modified with 2′-*O*-methoxyethyl RNA (2′-MOE, Fig. 1b) and contained 5-methylcytosine instead of cytosine, both of which are commonly used in oligonucleotide therapeutics. Thus, we assessed the impact of these modifications on retention behavior and peak shape. The 5- and 10-mer oligonucleotides were eluted with the modifier containing 2-aminoethanol; however, the 15- and 18-mer oligonucleotides were not eluted (Fig. 2, 2′-MOE-aminoethanol). In contrast, all four oligonucleotides, including the 15- and 18-mers, were eluted with the octylamine modifier and the peaks were sharper (Fig. 2, 2′-MOE-octylamine). The retention times of DMTr-off 5- and 10-mer 2′-MOE-modified oligonucleotides were slightly longer than those of the corresponding DMTr-on oligonucleotides, as observed for the 5- and 10-mer DNAs. However, the opposite retention behavior was observed



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

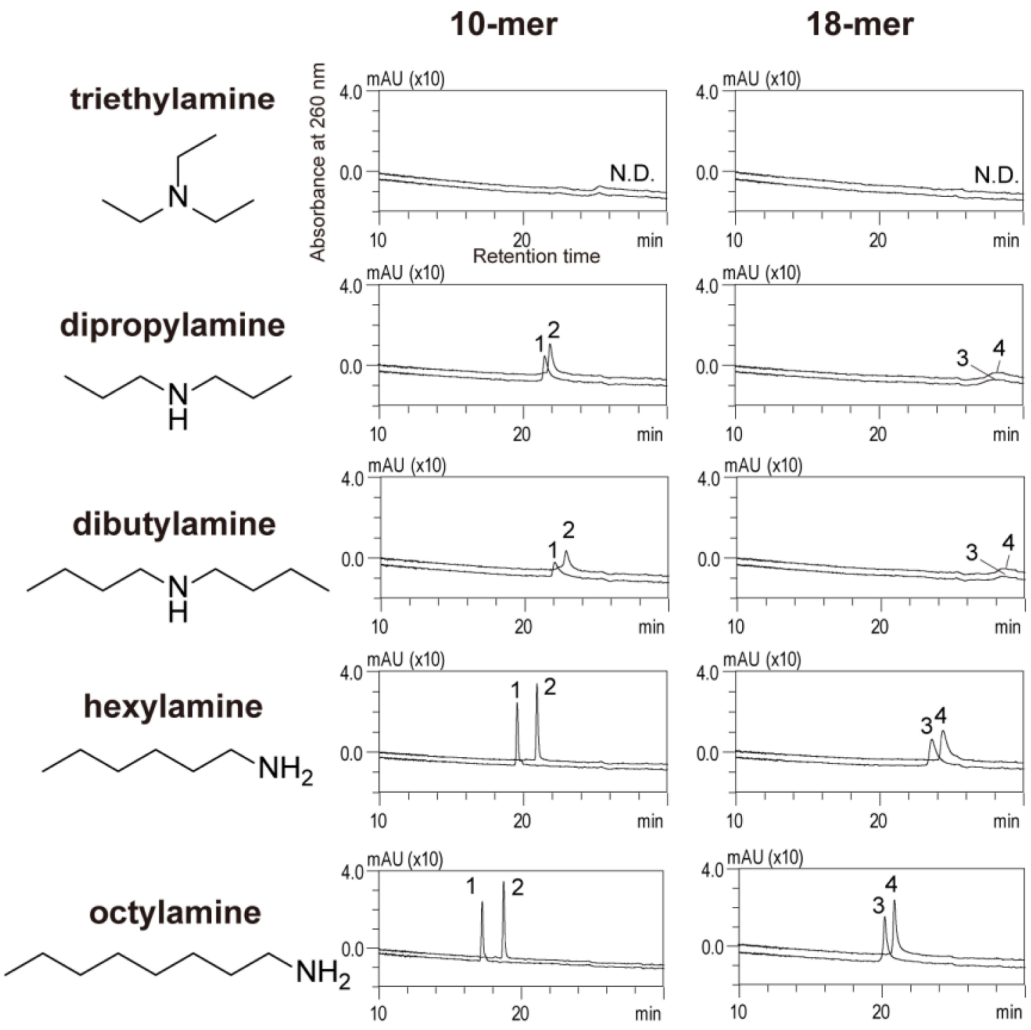
"C" indicates 5-methylcytosine, and the sequences are fully 2′-*O*-MOE modified.

Fig. 3. Overlayed UV chromatograms of the target sequences (peak 1 and 3) and the corresponding deaminated impurity (peak 2 and 4), employing column oven temperature of 35, 40, 50, and 60 °C. The peaks labeled 1–4 on the chromatograms are shown in the table below chromatograms. For analytical conditions, see Table S1 (Entry 3).

for the longer sequences (15- and 18-mer 2′-MOE-modified oligonucleotides), although the reason remains unclear. The 15- and 18-mer modified oligonucleotides could be analyzed, whereas their DNA counterparts could not be analyzed. This cannot be explained solely by hydrophobicity because the hydrophobicities of deoxyribose and MOE-modified ribose were similar (Fig. S1, Table S3). Although the cytosine nucleobases were methylated in the MOE-modified oligonucleotides, the overall difference in hydrophobicity was minimal (Fig. S1 and Table S3), and the number of 5-methylcytosines was limited. In our previous studies [6], the retention times of 4-mer DNA sequences were positively correlated with their polar surface areas. However, no such correlation was observed for the 5-mer oligonucleotides with various sugar modifications (Table S3, Fig. S2), suggesting that these oligonucleotides had different retention mechanisms. Among the modified oligonucleotides, MOE-modified sequences exhibited the shortest retention times. This is likely due to 2′-MOE modifications either enhancing the oligonucleotide's affinity for the mobile phase, which contains carbon dioxide, or these modifications reducing the interaction of the oligonucleotide with the stationary phase. As a result, we observed a unique retention behavior in these sugar-modified oligonucleotides, which are more commonly used in therapeutic applications than unmodified DNA. To the best of our knowledge, this is the first study to report 18-mer oligonucleotide analysis using SFC, enabled by the addition of

Table 1
The symmetry factors (*S*), the half-height widths ($W_{0.5}$), and the resolution factors (*Rs*) calculated from Fig. 3. More detailed chromatographic parameters were shown in Table S4.

(A) DMTr-10-mer					
	S		$W_{0.5}$ (min)		*Rs*
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1 and 2
35 °C	1.1	1.7	0.17	0.19	11.3
40 °C	1.1	1.5	0.15	0.16	10.1
50 °C	1.5	1.3	0.14	0.13	6.6
60 °C	1.0	1.0	0.14	0.14	3.0
(B) DMTr-18-mer					
	S		$W_{0.5}$ (min)		*Rs*
	Peak 3	Peak 4	Peak 3	Peak 4	Peak 3 and 4
35 °C	2.2	3.1	0.36	0.32	0.5
40 °C	1.8	3.6	0.29	0.26	1.0
50 °C	2.1	3.0	0.24	0.23	1.9
60 °C	2.1	2.1	0.22	0.21	2.2



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Fig. 4. Overlayed UV chromatograms of the target sequences (peak 1 and 3) and the corresponding deaminated impurity (peak 2 and 4), using the modifier compositions containing different alkylamine. Modifier: 50 mM alkylamine 50 mM acetic acid in methanol and water (95:5, v/v). The peaks labeled 1–4 on the chromatograms are shown in the table below chromatograms. For analytical conditions, see Table S1 (Entry 4).

long-chain alkylamines to the modifier.

3.2. *Optimization of conditions to separate deaminated products*

We optimized the column oven temperature, modifier additive, and column type, all of which may influence resolution, to achieve chromatographic separation with a resolution factor (*Rs*) of >1.5 between parent sequences with 2′-MOE modifications and their corresponding deaminated products, with thymidine replacing cytidine. First, we examined the effects of column oven temperature on the separation and peak shape using DMTr-on 10- and 18-mer sequences (Fig. 3, Table 1). These sequences were chosen because the DMTr-on and DMTr-off variants were nearly co-eluted when the Diol II column was used, and 10- and 18-mer oligonucleotides were considered appropriate as representative half-length and full-length models of therapeutic oligonucleotides. In IP-RPLC, higher column oven temperatures are known to improve peak shape by reducing solvent viscosity and enhancing the diffusion coefficient [13]. The effect of temperature on peak shape in hydrophilic interaction liquid chromatography (HILIC) is similar, although its significance varies depending on the conditions [14,15]. We evaluated whether similar effects could be observed with SFC. For the 10-mer sequences, sharp peaks with adequate peak shapes were achieved at 35 °C. The retention factors for the target sequence (peak 1) increased more significantly than those for the deaminated sequence (peak 2) as the temperature increased, resulting in decreasing resolution at higher column temperatures (Tables 1 and S4). Despite this, the baseline separation between the target and deaminated sequences (peak 1 vs. peak 2) was maintained, indicating that single base conversion from 5-methylcytosine to thymine in 10-mer oligonucleotide can be separated with SFC. For the 18-mer sequences, the symmetry factors (*S*) for peak 3 and peak 4 were 2.2 and 3.1 at 35 °C, indicating peak tailing (Tables 1 and S4), with partial co-elution of the target and the

Table 2
The symmetry factors (*S*), the half-height width ($W_{0.5}$), and the resolution factors (*Rs*) calculated from Fig. 4. More detailed chromatographic parameters were shown in Table S5.

(A) DMTr-10-mer

	S		$W_{0.5}$		*Rs*
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1 and 2
Triethylamine	N.D.	N.D.	N.D.	N.D.	N.D.
Dipropylamine	4.1	3.0	0.35	0.29	0.9
Dibutylamine	3.2	1.0	0.46	0.36	1.1
Hexylamine	1.1	1.5	0.13	0.14	5.9
Octylamine	1.5	1.3	0.14	0.13	6.6

(B)DMTr-18-mer

	S		$W_{0.5}$		*Rs*
	Peak 3	Peak 4	Peak 3	Peak 4	Peak 3 and 4
Triethylamine	N.D.	N.D.	N.D.	N.D.	N.D.
Dipropylamine	1.4	1.3	2.26	2.16	0.1
Dibutylamine	1.6	1.3	1.46	1.46	0.1
Hexylamine	2.4	2.6	0.51	0.48	1.0
Octylamine	1.8	2.5	0.24	0.22	1.9

deaminated sequences (*Rs* = 0.5 at 35 °C, Tables 1 and S4). The half-height widths ($W_{0.5}$) decreased and the resolutions improved as the temperature increased, achieving separation of the target 18-mer and its deaminated sequence at temperatures above 50 °C (*Rs* > 1.5; Tables 1 and S4). Based on these results, we selected 50 °C as the optimal column oven temperature.

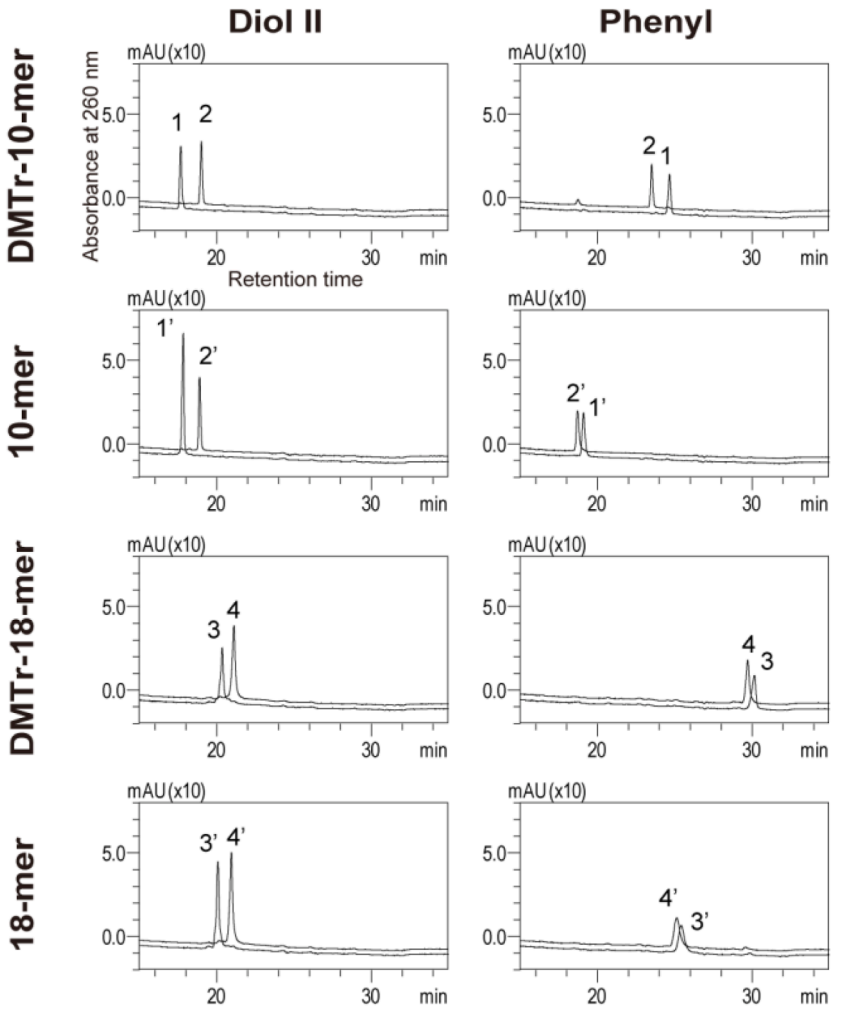
Subsequently, we compared the ion pair reagents in detail (Fig. 4 and Table 2). In IP-RPLC, the choice of alkylamine is known to influence retention. Improvements in peak shape using ion-pair reagents have also been reported for SFC [7–10]. We prepared methanol-water solutions (95:5, v/v) containing 50 mM of primary, secondary, and tertiary amines with the same carbon number (triethylamine, dipropylamine, and hexylamine) and analyzed the DMTr-on oligonucleotides. Triethylamine did not produce detectable peaks for either the 10- or 18-mer oligonucleotides. Dipropylamine produced broad peaks for the 10-mer and 18-mer sequences ($W_{0.5}$ = 0.35, 0.29, 2.26, and 2.16 for peak 1, 2, 3, and 4, Tables 2 and S5). Hexylamine produced sharp peaks for the 10-mer ($W_{0.5}$ = 0.13, 0.14 for peak 1 and 2, Tables 2 and S5) and slightly tailed peaks for the 18-mer (*S* = 2.4 and 2.6 for peak 3 and 4). We believe that the hydrophobicity of the alkylamines affects the peak shape, with hexylamine being the most hydrophobic and triethylamine being the most hydrophilic among the tested three amines. For further investigation, we analyzed the sequences using dibutylamine, a secondary amine that is more hydrophobic than hexylamine. However, dibutylamine caused significantly broad peaks for both 10- and 18-mer sequences ($W_{0.5}$ = 0.46, 0.36, 1.46, and 1.46 for peak 1, 2, 3, and 4, Tables 2 and S5). These results indicate that the primary amines were more effective in improving the peak shape. We hypothesized that primary amines interacted more efficiently with the phosphodiester backbone of oligonucleotides because of their lower steric hindrance, thereby forming ion pairs more effectively. Based on these results, we selected octylamine, which provided a better peak shape and higher resolution than the other tested alkylamines. 50 mM was selected for the octylamine concentration because the modifier containing 50 mM octylamine provided adequate resolution, particularly for DMTr-18-mer and its corresponding deaminated product (*Rs* > 1.5, Fig. S3 and Table S6).

Next, we investigated other columns in addition to the Diol II column. Although no peaks were observed for the columns containing C18 or 3-hydroxyphenyl (data not shown), peaks were detected for a column modified with phenyl groups (phenyl column) (Fig. 5). Notably, the deaminated product contained in the DMTr-on 10-mer was successfully separated using a phenyl column, with a resolution value (*Rs*) of 4.1 (Tables 3 and S7). Deaminated products contained in the 10-mer, DMTr-on 18-mer, and 18-mer were slightly separated, with *Rs* values of 1.1, 1.0, and 0.4, respectively. However, the separation of the deaminated products in the DMTr-off 10- and 18-mer was less efficient with the phenyl column than with the Diol II column. Therefore, the Diol II column was selected for further evaluations. A strong selectivity between the DMTr-on and DMTr-off sequences was observed with the phenyl column. This was probably due to the strong interactions between the phenyl groups in the stationary phase and the DMTr group on the DMTr-on oligonucleotides. As a result, the phenyl column exhibited different selectivity compared to the Diol II column. This separation behavior could be beneficial for isolating full-length DMTr-on oligonucleotides from DMTr-off impurities such as shortmers.

3.3. Evaluation of the separation behavior for deamination products

We compared the resolution of SFC with that of IP-RPLC for the analysis of the target sequence and its deamination products (Fig. 6). Both SFC and IP-RPLC employ isocratic conditions to avoid the influence of gradient elution and to simplify the understanding of the basic retention behavior. Propylamine was used as an ion-pair reagent in IP-RPLC because it has been reported to be suitable for separating deamination products [4]. The B ratio was optimized for each sequence to ensure elution within 15 min (Tables S1 and S2). Sequences with varying numbers of deaminations were analyzed, and complete separation of the target DMTr-on 10-mer (peak 1) and its deamination impurities (peaks 2–4) was achieved by SFC. Although IP-RPLC also separated the target DMTr-on 10-mer and its deamination impurities, SFC provided a higher resolution. For DMTr-on 18-mer, both SFC and IP-RPLC achieved separation of the target (peak 5) and its deamination products (peaks 6–8). However, the elution order differed between SFC and IP-RPLC. In SFC analysis, sequences with more deamination were eluted later. In contrast, IP-RPLC eluted sequences in the following order: peaks 1, 3, 4, and 2 for the DMTr-on 10-mer and peaks 5, 7, 8, and 6 for the DMTr-on 18-mer, indicating that SFC and IP-RPLC exhibited different retention behaviors. We also analyzed the DMTr-off oligonucleotides. In SFC analysis, DMTr-off and DMTr-on oligonucleotides were eluted at approximately the same time. SFC successfully separated the target DMTr-off 10-mer and its deamination products, as well as DMTr-off 18-mer. Peaks 7 and 7′ showed shoulders, with both the main and shoulder peaks exhibiting the same *m/z* in MS analysis. This could be attributed to the partial separation of sequences with different structural conformations. IP-RPLC also achieved separation of the target DMTr-off 10-mer and its deamination products, with better resolution for DMTr-off sequences than for DMTr-on sequences. In IP-RPLC, separation involves hydrophobic interactions between ion-paired oligonucleotides and C18 groups, as well as ion-exchange-like interactions between the amino groups of alkylamines adsorbed on C18 and the oligonucleotides. When using ion-pairing reagents with low hydrophobicity, such as propylamine, the ion-pairing effect with oligonucleotides becomes more dominant than adsorption on C18 [16]. Based on this, we hypothesized that hydrophobic interactions played a more significant role than ion-exchange-like interactions under the analytical conditions used in this study. In IP-RPLC, nucleobase differences in DMTr-on sequences were difficult to be detected as changes in retention because the highly hydrophobic DMTr group provided substantial hydrophobic retention and the contribution of nucleobase differences to overall retention was relatively small. Conversely, DMTr-off oligonucleotides lack a highly hydrophobic region, which results in stronger interactions with the stationary phase and nucleobases, and better separation of the deamination products. In the SFC, DMTr-on and DMTr-off oligonucleotides exhibited similar retention behaviors, indicating that the DMTr group had minimal influence on retention. This is likely because the diol column used in SFC interacts more effectively with highly polar nucleobases and phosphodiesters and not as effectively with the DMTr group, emphasizing hydrophilic interactions over hydrophobic ones.

To evaluate the separation capability of SFC for deaminated positions, we analyzed sequences with deamination at different positions



Peak	Sequence (5'-3')	
1	DMTr-	TCACTTTCAT
2	DMTr-	TTACTTTCAT
1'		TCACTTTCAT
2'		TTACTTTCAT
3	DMTr-	TCACTTTCATAATGCTGG
4	DMTr-	TTACTTTCATAATGCTGG
3'		TCACTTTCATAATGCTGG
4'		TTACTTTCATAATGCTGG

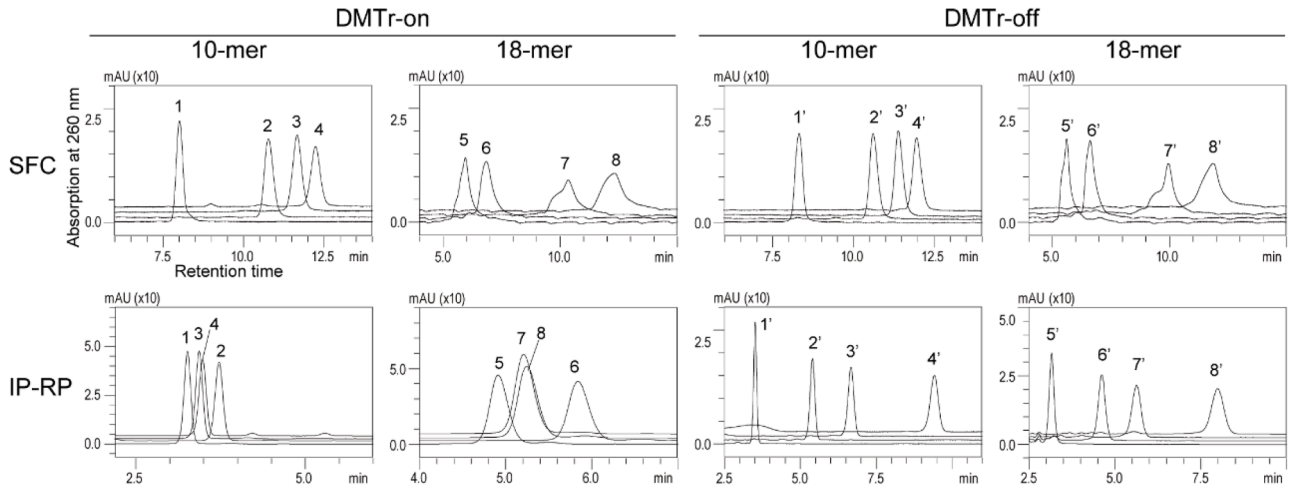
"C" indicates 5-methylcytosine, and the sequences are fully 2′-*O*-MOE modified.

Fig. 5. Overlayed UV chromatograms of the target sequences (peak 1, 1′, 3, and 3′) and the corresponding deaminated impurities (peak 2, 2′, 4 and 4′), using the Diol II and Phenyl columns. The peaks labeled 1–4 and 1′−4′ on the chromatograms are shown in the table below chromatograms. For analytical conditions, see Table S1 (Entry 6).

Table 3
The resolution factors (*Rs*) calculated from Fig. 5. More detailed chromatographic parameters were shown in Table S7.

	Peak 1 and 2	Peak 1′ and 2′	Peak 3 and 4	Peak 3′ and 4′
Diol II	5.4	4.7	2.4	3.0
Phenyl	4.1	1.1	1.0	0.4

(Fig. 7). Using SFC, we successfully separated the target DMTr-on 10-mer (peak a) from its deamination impurities (peaks b–d). Additionally, positional isomers (peaks b–d) were separated, demonstrating their ability to distinguish between sequence changes. For DMTr-on 18-mer, peak h was co-eluted with the target (peak e), whereas the other three (peaks f, g, and i) were successfully separated. SFC showed similar retention behavior for both DMTr-on and DMTr-off oligonucleotides, indicating its capability to handle both types of oligonucleotides. In contrast, IP-RPLC showed different selectivities for DMTr-on and DMTr-off oligonucleotides. The IP-RPLC analysis revealed that the target DMTr-on 10-mer (peak a) were co-eluted with one of the deaminated sequences (peak d), whereas the other two peaks (peaks b and c) were separated. For DMTr-on 18-mer, peaks g and f were separated from the target (peak e), but peaks h and i were co-eluted with the target. IP-RPLCs showed good separation of DMTr-off oligonucleotides, which is consistent with the results shown in Fig. 6. Overall, our results indicate that SFC and IP-RPLC exhibit different retention behaviors in



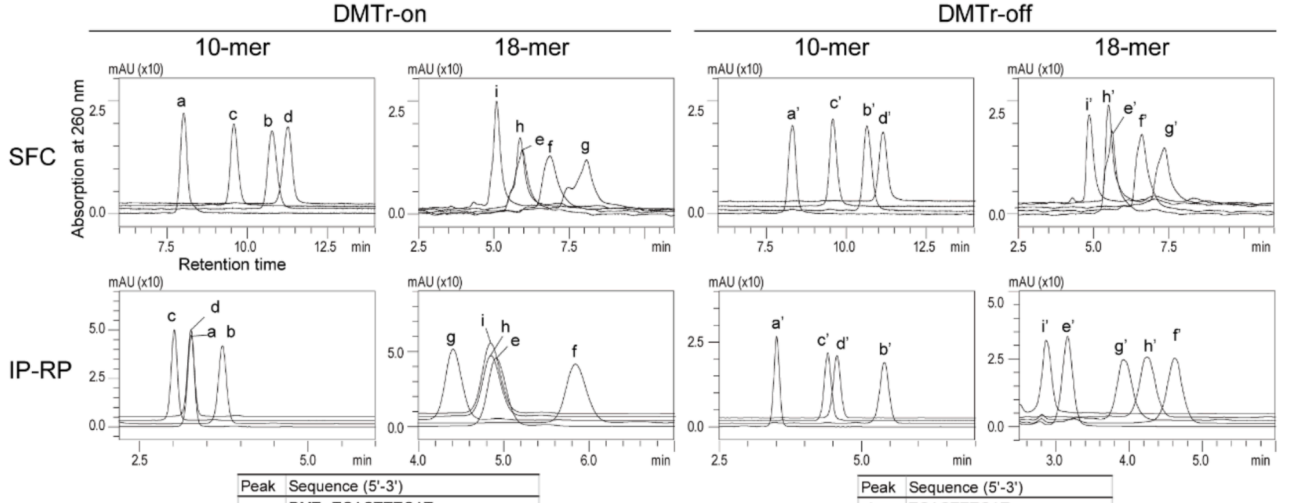
Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TTATTTTCAT
4	DMTr-TTATTTTTAT
5	DMTr-TCACTTTCATAATGCTGG
6	DMTr-TTACTTTCATAATGCTGG
7	DMTr-TTATTTTCATAATGCTGG
8	DMTr-TTATTTTTATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Peak	Sequence (5'-3')
1'	TCACTTTCAT
2'	TTACTTTCAT
3'	TTATTTTCAT
4'	TTATTTTTAT
5'	TCACTTTCATAATGCTGG
6'	TTACTTTCATAATGCTGG
7'	TTATTTTCATAATGCTGG
8'	TTATTTTTATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Fig. 6. Overlayed UV chromatograms of the target sequences (10 and 18-mer with and without DMTr group) and impurities with different numbers of deamination using SFC and IP-RPLC. The peaks labeled 1–8 and 1′–8′ on the chromatograms are shown in the table below chromatograms. For analytical conditions for SFC, see Table S1 (Entry 7), and for analytical conditions for IP-RPLC, see Table S2 (Entry 1).



Peak	Sequence (5'-3')
a	DMTr-TCACTTTCAT
b	DMTr-TTACTTTCAT
c	DMTr-TCATTTTCAT
d	DMTr-TCACTTTTAT
e	DMTr-TCACTTTCATAATGCTGG
f	DMTr-TTACTTTCATAATGCTGG
g	DMTr-TCATTTTCATAATGCTGG
h	DMTr-TCACTTTTATAATGCTGG
i	DMTr-TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Peak	Sequence (5'-3')
a'	TCACTTTCAT
b'	TTACTTTCAT
c'	TCATTTTCAT
d'	TCACTTTTAT
e'	TCACTTTCATAATGCTGG
f'	TTACTTTCATAATGCTGG
g'	TCATTTTCATAATGCTGG
h'	TCACTTTTATAATGCTGG
i	TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Fig. 7. Overlayed UV chromatograms of the target sequences (10 and 18-mer with and without DMTr group) and impurities deaminated at different positions using SFC and IP-RPLC. The peaks labeled a–i and a′–i′ on the chromatograms are shown in the table below chromatograms. For analytical conditions, see Table S1 (Entry 7), and for analytical conditions for IP-RPLC, see Table S2 (Entry 1).

oligonucleotide analyses. The retention behavior of SFC was influenced by the interactions between the stationary phase and nucleobases, whereas the hydrophobic DMTr group did not have a significant impact on this behavior.

4. Conclusions and perspectives

In this study, we optimized the analytical conditions for applying SFC to 5-, 10-, 15-, and 18-mer oligonucleotides. We also evaluated the applicability of SFC for separating deaminated impurities in both the DMTr-on and DMTr-off forms, thereby assessing SFC for application in the purification process after oligonucleotide synthesis. We first analyzed 5-, 10-, 15-, and 18-mer DNA and 2′-MOE modified oligonucleotides using modifiers containing either aminoethanol or octylamine. Our results showed that octylamine provided sharp peaks for 5- to 18-mer 2′-MOE modified oligonucleotides regardless of the presence of the DMTr group. Further optimization of the column oven temperature, modifier additive, and stationary phase enabled successful separation of the deaminated products. SFC demonstrated the ability to separate sequences with different numbers and positions of deamination from the target oligonucleotides. To the best of our knowledge, this is the first study reporting the use of SFC to analyze long-chain oligonucleotides, such as 10-, 15-, and 18-mers, and to separate structurally similar sequences. Notably, for DMTr-on oligonucleotides, SFC separated the sequences that were co-eluted in IP-RPLC. This trend was consistent for sequences with different numbers and positions of deamination, likely because of the distinct interactions between the stationary phase and the nucleobase moieties of the analytes. These results suggest that SFC has a significant potential for impurity analysis in oligonucleotide therapeutics, especially those conjugated with hydrophobic organ-targeting ligands, beyond just DMTr groups.

CRediT authorship contribution statement

Momoka Hayashida: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Risa Suzuki:** Methodology. **Shinnosuke Horie:** Supervision, Methodology, Conceptualization. **Junichi Masuda:** Supervision, Conceptualization. **Takao Yamaguchi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Satoshi Obika:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported in part by AMED under Grant numbers JP21ae0121022, JP21ae0121023, JP21ae0121024.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.465731.

Data availability

Data will be made available on request.

References

[1] M.H. Caruthers, A.D. Barone, S.L. Beaucage, D.R. Dodds, E.F. Fisher, L.J. McBride, M. Matteucci, Z. Stabinsky, J.Y. Tang, Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. Methods Enzymol, Academic Press, 1987, pp. 287–313, https://doi.org/10.1016/0076-6879(87)54081-2.
[2] T. Lindahl, B. Nyberg, Heat-induced deamination of cytosine residues in deoxyribonucleic acid, Biochemistry 13 (1974) 3405–3410, https://doi.org/10.1021/bi00713a035.
[3] C. Rentel, J. DaCosta, S. Roussis, J. Chan, D. Capaldi, B. Mai, Determination of oligonucleotide deamination by high resolution mass spectrometry, J. Pharm. Biomed. Anal. 173 (2019) 56–61, https://doi.org/10.1016/j.jpba.2019.05.012.
[4] S.G. Roussis, M. Pearce, C. Rentel, Small alkyl amines as ion-pair reagents for the separation of positional isomers of impurities in phosphate diester oligonucleotides, J. Chromatogr. A 1594 (2019) 105–111, https://doi.org/10.1016/j.chroma.2019.02.026.
[5] J. Molineau, M. Hideux, C. West, Chromatographic analysis of biomolecules with pressurized carbon dioxide mobile phases—A review, J. Pharm. Biomed. Anal. 193 (2021) 113736, https://doi.org/10.1016/j.jpba.2020.113736.
[6] M. Hayashida, R. Suzuki, S. Horie, J. Masuda, T. Yamaguchi, S. Obika, Applicability of supercritical fluid chromatography for oligonucleotide analysis: a proof-of-concept study, J. Chromatogr. A 1708 (2023) 464333, https://doi.org/10.1016/j.chroma.2023.464333.
[7] J.A. Blackwell, R.W. Stringham, J.D. Weckwerth, Effect of mobile phase additives in packed-column subcritical and supercritical fluid chromatography, Anal. Chem. 69 (1997) 409–415, https://doi.org/10.1021/ac9608883.
[8] C. West, J. Melin, H. Ansouri, M.M. Metogo, Unravelling the effects of mobile phase additives in supercritical fluid chromatography. Part I: polarity and acidity of the mobile phase, J. Chromatogr. A 1492 (2017) 136–143, https://doi.org/10.1016/j.chroma.2017.02.066.
[9] M. Doué, G. Dervilly-Pinel, K. Pouponneau, F. Monteau, B. Le Bizec, Analysis of glucuronide and sulfate steroids in urine by ultra-high-performance supercritical-fluid chromatography hyphenated tandem mass spectrometry, Anal. Bioanal. Chem. 407 (2015) 4473–4484, https://doi.org/10.1007/s00216-015-8573-x.
[10] S.J. Kumari, A. Ubhayasekera, S.R. Acharya, J. Bergquist, A novel, fast and sensitive supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) method for analysis of arachidonic acid metabolites, Analyst 143 (2018) 3661–3669, https://doi.org/10.1039/c8an00788h.
[11] A. Sen, C. Knappy, M.R. Lewis, R.S. Plumb, I.D. Wilson, J.K. Nicholson, N. W. Smith, Analysis of polar urinary metabolites for metabolic phenotyping using supercritical fluid chromatography and mass spectrometry, J. Chromatogr. A 1449 (2016) 141–155, https://doi.org/10.1016/J.CHROMA.2016.04.040.
[12] O. Gyllenhaal, L. Edström, B.A. Persson, Ion-pair supercritical fluid chromatography of metoprolol and related amino alcohols on diol silica, J. Chromatogr. A 1134 (2006) 305–310, https://doi.org/10.1016/j.chroma.2006.08.051.
[13] M. Gilar, K.J. Fountain, Y. Budman, U.D. Neue, K.R. Yardley, P.D. Rainville, R. J. Russell Ii, J.C. Gebler, Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides: retention prediction, J. Chromatogr. A 958 (2002) 167–182, https://doi.org/10.1016/s0021-9673(02)00306-0.
[14] H. Lardeux, V. D'Atri, D. Guillarme, Recent advances and current challenges in hydrophilic interaction chromatography for the analysis of therapeutic oligonucleotides, TrAC Trends Anal. Chem. 176 (2024) 117758, https://doi.org/10.1016/j.trac.2024.117758.
[15] Z. Vosáhlová, K. Kalíková, M. Gilar, J. Szymarek, M. Mazurkiewicz-Bełdzińska, S. Studzińska, Hydrophilic interaction liquid chromatography with mass spectrometry for the separation and identification of antisense oligonucleotides impurities and nusinersen metabolites, J. Chromatogr. A 1713 (2024) 464535, https://doi.org/10.1016/j.chroma.2023.464535.
[16] M. Donegan, J.M. Nguyen, M. Gilar, Effect of ion-pairing reagent hydrophobicity on liquid chromatography and mass spectrometry analysis of oligonucleotides, J. Chromatogr. A 1666 (2022) 462860, https://doi.org/10.1016/j.chroma.2022.462860.