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Short-Chain Oligonucleotide Analysis Using Supercritical Fluid Chromatography

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■ Abstract

The applicability of supercritical fluid chromatography (SFC) to the analysis of impurities in oligonucleotide was evaluated. In this study, we demonstrated that 4-mer oligonucleotides can be analyzed by optimizing the analytical conditions. As a result of comparative studies, 4-mer oligonucleotides with different amounts of phosphorothioate modification could be separated using a mixture of methanol and water containing 2-aminoethanolacetic acid as a modifier (co-solvent) and Shim-pack™ UC-Diol II as a column. These results demonstrate the applicability of this method to oligonucleotide analysis.

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1. Introduction

Oligonucleotide therapeutics are drugs in which oligonucleotide is the active ingredient. Oligonucleotide therapeutics are manufactured through chemical synthesis, but impurities are produced during the process, so measurement and characterization of the impurities are required. Currently, reversed-phase ion-pair chromatography is often used for this analysis, but it is difficult to separate some impurities that are structurally similar to the target product. Therefore, a separation method with selectivity different from existing methods is required.

SFC is an analytical method that employs carbon dioxide as the mobile phase at temperatures and pressures above the respective critical points. Compared to liquids, carbon dioxide is reported to have high resolution and special selectivity as a component of SFC mobile phase because of its low viscosity and high diffusivity.

On the other hand, since the mobile phase containing carbon dioxide has low polarity, SFC was considered unsuitable for the analysis of highly polar compounds. However, in recent years, the composition of the modifier mixed with carbon dioxide and the choice of stationary phase have made it possible to analyze highly polar compounds, such as peptides and nucleotides[2].

In this study, we evaluated the applicability of SFC to oligonucleotide analysis. Single-strand oligonucleotides of around 20-mer are often used as oligonucleotide therapeutics, but due to the extremely high polarity of the molecules and the difficulty of analysis, we first used 4-mer oligonucleotides for evaluation and investigated their basic retention behaviors.

2. Experiments

In this study, we focused on the separation of oligonucleotides with different phosphorothioate (PS) contents. PS modification means one oxygen atom in the phosphoric acid portion of an oligonucleotide is replaced with a sulfur atom, which is used in many commercial drugs (Fig. 1). Separation of oligonucleotide sequences with different PS contents is important for quality control, as the synthetic process of oligonucleotide containing PS modifications produces impurities in which the Sulfur atom is converted to an oxygen atom. To separate 4-mer oligonucleotides with different PS content using SFC, we developed an analytical method in three steps: (1) verification of elution conditions, (2) evaluation of separation performance of stationary phase, and (3) optimization of modifier additives in mobile phase. Finally, we investigated the oligonucleotide sequences and basic retention characteristics to which the analytical method could be applied.

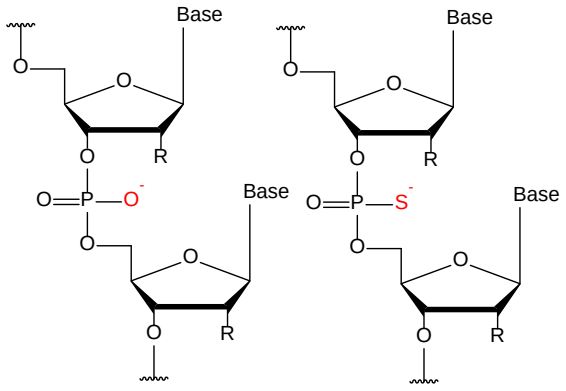


Fig. 1 Phosphate of oligonucleotide

Detection conditions for mass spectrometer (MS) are shown in Table 1; SFC analytical conditions are described in the text.

Table 1 MS conditions

System	: LCMS-9030
Polarity	: Negative
Interface temp	: 350 °C
Nebulizer gas	: 3.0 L/min
Heating gas	: 10.0 L/min
Drying gas	: 10.0 L/min
DL temp	: 250 °C
Heat block temp	: 400 °C
Interface Voltage	: -3.5 kV
MS scan range	: *m/z* 150-2000

3. Results and Discussion

3-1. Investigation of Elution Conditions

Considering the possibility that oligonucleotides are not eluted from the column due to their high polarity of oligonucleotides, we first analyzed a 4-mer oligothymidine T4 using three different modifiers to confirm elution (Fig. 2). We used 80% modifiers, similar to LC conditions (Table 2). Diverse stationary phases, ranging from low to high polarity functional groups, were selected. Methanol, which has high solubility for oligonucleotides, was chosen as the base solvent for the modifiers. No peaks were detected with methanol or methanol-water mixtures (95:5) (Fig 2-A, B), but sharp peaks were detected on five out of six columns when using a methanol-water mixture (95:5) containing 50 mmol/L ammonium formate (Fig. 2-C). This suggests that ammonium ions were important for eluting oligonucleotides from the column. Columns modified with amide groups showed strong interactions with oligonucleotides, preventing their elution, and thus no peaks were detected. Consequently, we compared the separation efficiency of each stationary phase using a methanol-water mixture (95:5) containing 50 mmol/L ammonium formate, excluding the amide column.

Table 2 Analytical conditions for Fig. 2

System	: Nexera^{TM} UC
Column	: Shim-pack UC-GIS II/RP/Phenyl/CN/Diol II/ Amide (150 mm x 4.6 mm I.D., 3 µm)
Temperature	: 35 °C
Injection volume	: 5 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2, B) modifier, A/B=20:80
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: MS (Table 1)

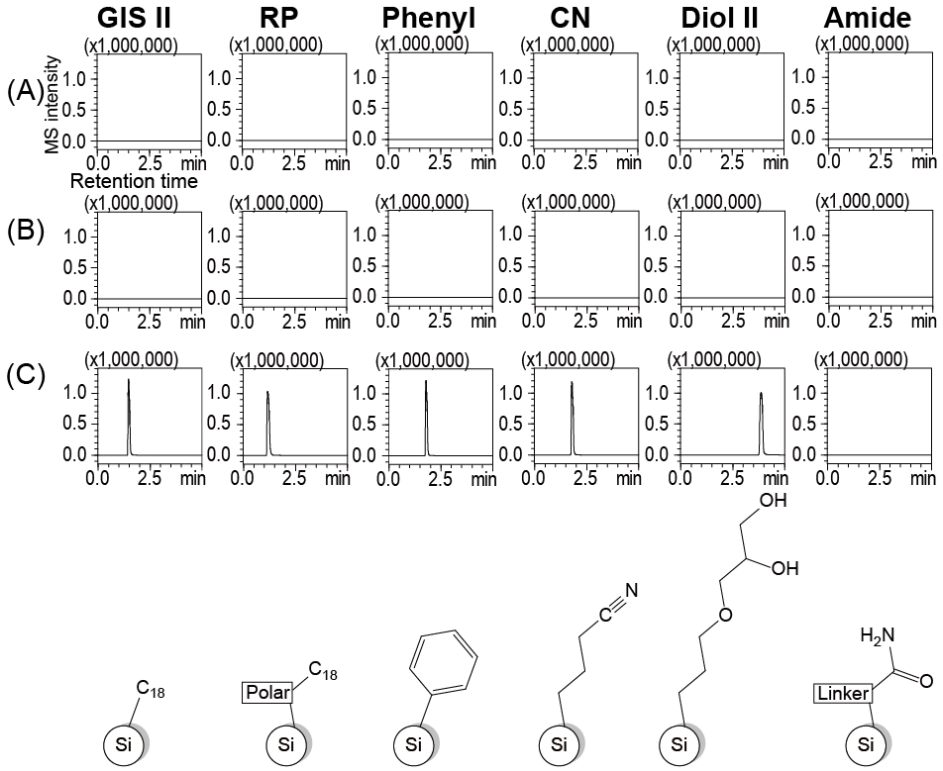


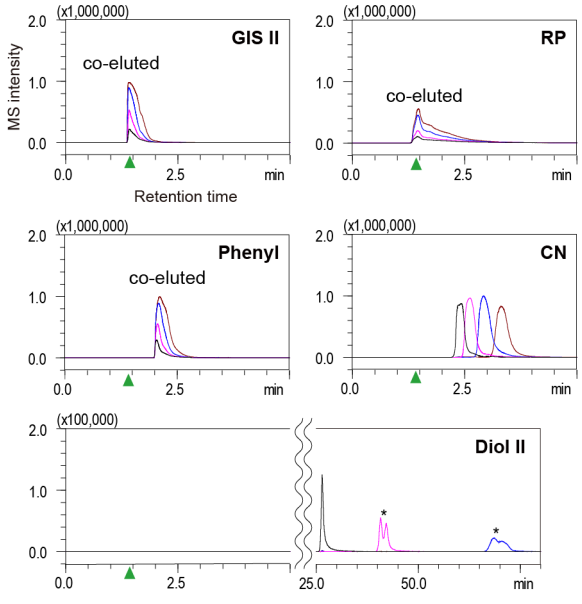
Fig. 2 Extracted ion chromatograms of T4s using three different modifiers (*m/z* 1153.22),
(A)methanol, (B)methanol and water (95:5, *v/v*) , and (C)50 mmol/L ammonium formate in methanol and water (95:5, *v/v*)

3-2. Evaluation of Separation Ability of Stationary Phases

T4s with different PS contents were analyzed employing decreased modifier ration of 50% to increase retention (Fig. 3, Table 3). GIS II, RP, and Phenyl columns having low-polarity functional groups as stationary phases all T4s were co-eluted. Retention times were short, and tailing was present when the modifier ratio was further reduced to increase retention, strongly suggesting that a complete separation was not achievable with these columns. Peaks were separated on the CN and Diol II columns, and in particular, complete separation of all the sequences was accomplished on the Diol II column. T4+3PS (sequence described in Fig. 3) eluted after 80 minutes (data not shown). Based on these results, we prepared a column[3)] with similar retention characteristics to the Diol II column and investigated the functional groups effective for the separation.

Table 3 Analytical conditions for Fig. 3

System	: Nexera UC
Column	: Shim-pack UC-GIS II, RP, Phenyl, CN, Diol II (150 mm×4.6 mm I.D., 3 µm)
Temperature	: 35 °C
Injection volume	: 5 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2, B) 50 mmol/L ammonium formate in methanol and water (95:5, *v/v*), A/B=50:50
Flow rate	: 1.0 ml/min
Back pressure	: 10 MPa, 50 °C
Detection	: MS (Table 1)



Name	Sequence (5′-3′)	*m/z*, [M-H]⁻
■ T4	$T_oT_oT_oT$	1153.22
■ T4+1PS	$T_sT_oT_oT$	1169.20
■ T4+2PS	$T_sT_oT_sT$	1185.18
■ T4+3PS	$T_sT_sT_sT$	1201.15

Thymidine (T), PO linkage ($_o$), PS linkage ($_s$), PS diastereomers (*), hold-up time (▲)

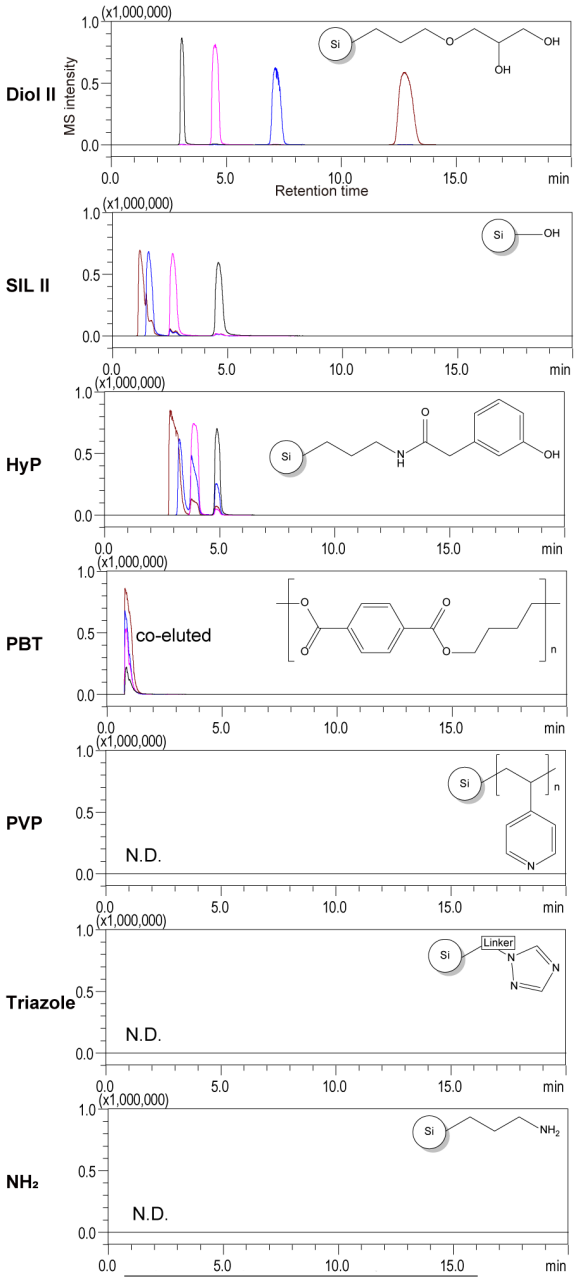
Fig. 3 Overlayed EICs of T4s with different PS contents

Seven columns having retention characteristics similar to Diol II column were used to analyze T4s with different PS contents (Fig. 4, Table 4), which were separated or partially separated on Diol II, SIL II, and HyP columns; PBT columns did not retainT4s, while PVP, Triazole, and NH2 columns did not elute all T4s within a given time. All the columns that separated T4s with varying PS contents had hydroxy groups, indicating the importance of hydroxy groups. The best separation was achieved on Diol II column, where retention times were increased with the increase of PS contents (Fig. 4-Diol II).

In general, diol column shows retention behavior based on hydrophilic interactions; since PO bonds are more polar than PS bonds, all PO-bonded sequences should be strongly retained, but as a result, opposite elution order was observed. This indicates that Diol II has a special selectivity for PS-bonds. Based on these results, we selected Diol II column as the separation column.

Table 4 Analytical conditions for Fig. 4

System	: Nexera UC
Column	: Shim-pack UC-Diol II, SIL II, HyP, Triazole and NH_2 (150 mm x 2.1 mm I.D., 3 µm) Shim-pack UC-PBT and PolyVP (150 mm x 3.0 mm I.D., 3 µm)
Temperature	: 35 °C
Injection volume	: 5 µL
Mobile phases	: A) CO_2, B) 50 mmol/L ammonium formate in methanol and water (95:5, *v/v*) A/B=50:50
Flow rate	: 1.0 ml/min
Detection	: MS (Table 1)



Name	Sequence (5′-3′)	*m/z*, [M-H]⁻
■ T4	$T_oT_oT_oT$	1153.22
■ T4+1PS	$T_sT_oT_oT$	1169.20
■ T4+2PS	$T_sT_oT_sT$	1185.18
■ T4+3PS	$T_sT_sT_sT$	1201.15

Thymidine (T), PO linkage ($_o$), PS linkage ($_s$)

Fig. 4 Overlayed EICs of T4s with different PS contents

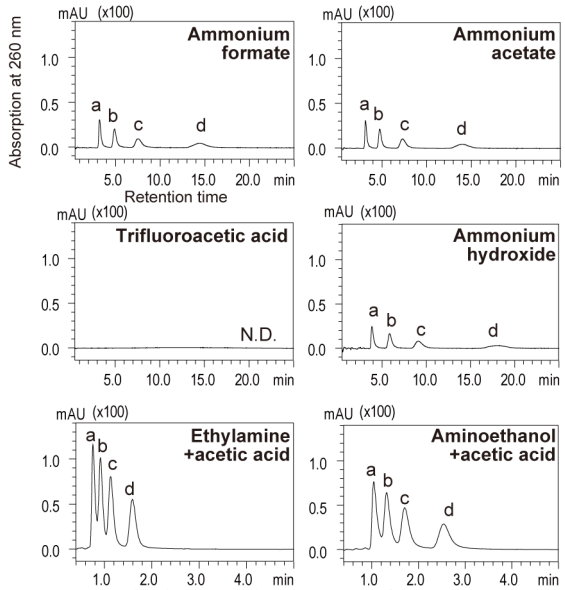
3-3. Investigation of Modifier Conditions Applicable to Highly Polar Sequences

Thymine has low polarity among nucleobases and is easily analyzed by SFC. Therefore, when applied the SFC method to a sequence with higher polarity and complexity than T4 (5′-TAGC-3′), significant peak tailing was observed, so modifier additives were investigated (Fig. 5, Table 5). Ammonium acetate and ammonium hydroxide were employed, but no improvement in peak tailing was observed, and when trifluoroacetic acid was added, oligonucleotides were not eluted, and no peak was detected. We then turned our attention to ion-pair agents used in reversed-phase HPLC, and when ethylamine and aminoethanol were used, the peak shape was improved.

This is thought to be owing to the formation of ion pairs in the phosphate portions of the oligonucleotides, which reduced the polarity of the molecule and improved its affinity for the mobile phase. This result indicates that ion-pair agents are effective when applying SFC to highly polar oligonucleotide sequences.

Table 5 Analytical conditions for Fig. 5

System	: Nexera UC
Column	: Shim-pack UC-Diol II (100 mm x 2.0 mm I.D., 3 µm)
Temperature	: 35 °C
Injection volume	: 1 µL
Mobile phases	: A) CO_2, B) 50 mmol/L or 0.1% (*v/v*) additive in methanol and water (95:5, *v/v*), A/B=50:50
Flow rate	: 1.5 ml/min
Detection	: 260 nm (PDA coupled to a high-pressure cell)



Peak	Name	Sequence (5′-3′)
a	TAGC	$T_oA_oG_oC$
b	TAGC+1PS	$T_sA_oG_oC$
c	TAGC+2PS	$T_sA_oG_sC$
d	TAGC+3PS	$T_sA_sG_sC$

Thymidine (T), deoxyadenosine (A), deoxyguanosine (G), deoxycytidine (C), PO linkage ($_o$), PS linkage ($_s$)

Fig. 5 Overlayed EICs of T4s with different PS contents

3-4. Application to Different Sequences

Since the peak shape changed significantly between T4 and TAGC, a total of 14 oligonucleotide sequences (all PO-bound) with different base sequences were analyzed and their peak shapes were compared (Fig. 6, Table 6). Based on the results of the comparison of modifier salt concentrations, 40 mM was selected, which provided the best separation and peak shape[1)].

The peak shape was found to be good for sequences with G and C less than two bases (Fig. 6-A, B). Since G and C are more polar than A and T, the polarity of the sequence was considered to affect the peak shape. The octanol/water partition coefficient (log*P*) calculated from the molecular structure of the sequence is plotted against the theoretical plate number for the peak. The results showed a positive correlation, i.e., less polar sequence provided better peak shape (Fig. 6-C).

Table 6 Analytical conditions for Fig. 6

System	: Nexera UC
Column	: Shim-pack UC-Diol II (100 mm x 2.0 mm I.D., 3 µm)
Temperature	: 35 °C
Injection volume	: 5 µL
Mobile phases	: A) CO_2, B) 40 mmol/L aminoethanol + 40 mmol/L acetic acid in methanol and water (95:5, *v/v*), A/B=50:50
Flow rate	: 1.5 ml/min
Detection	: 260 nm (PDA coupled to a high-pressure cell)

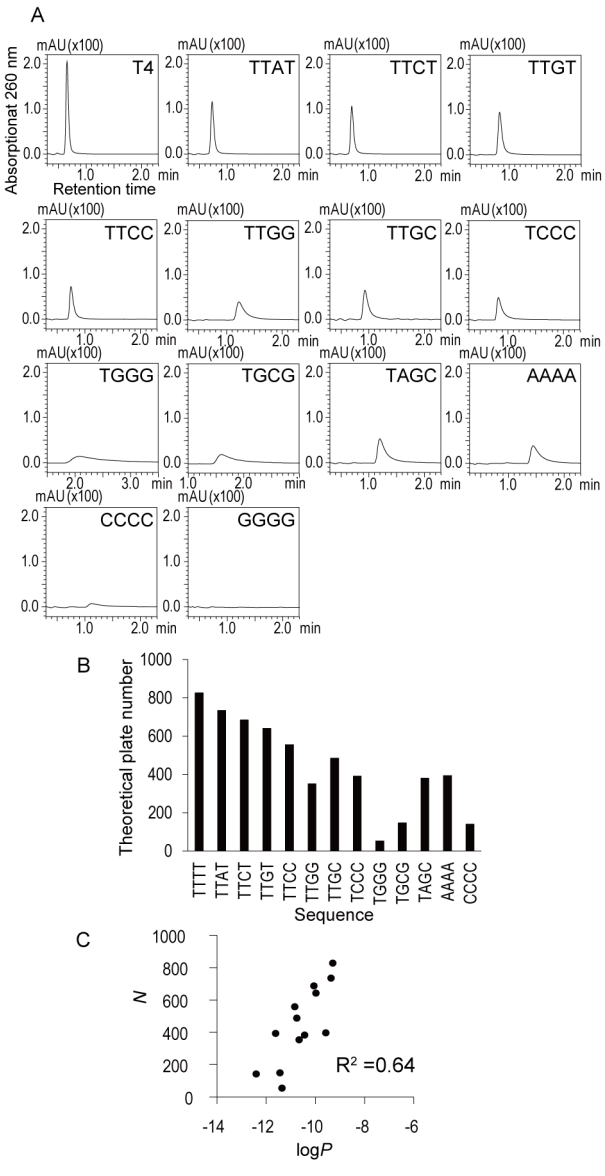


Fig. 6 (A) UV chromatograms, (B) theoretical plate numbers (*N*), and (C) Relation between the log*P* and *N* for 14 sequences

Fig. 4 also shows that Diol II column provided a special retention behavior for PS modifications. We plotted the polar surface area*1 against the retention time of fourteen sequences (Fig. 7-A), and found a high positive correlation, with a correlation coefficient higher than that of log*P* versus molecular weight plot. This can be attributed to the hydrophilic interaction between the hydroxy groups of diol column and the polar groups of oligonucleotides. On the other hand, a similar plot of retention time versus polar surface area for T4s with different PS contents (Fig. 7-B) showed a negative correlation. The fact that the retention behavior of T4s was opposite to that of the bases also indicates that T4 exhibits a special retention behavior with respect to the phosphate portion.

*1 PSA means the polarized molecular surface area.

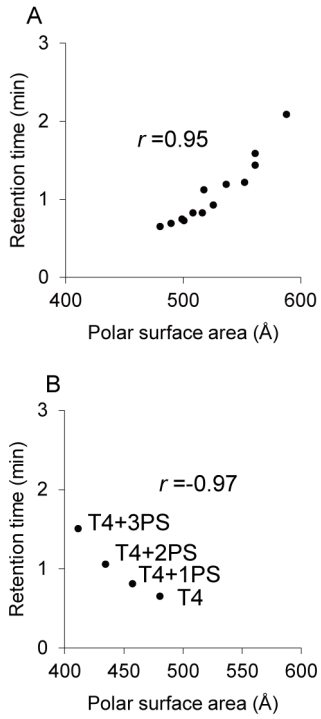


Fig. 7 Relation between the retention times and PSA for (A) 14 sequences and (B) T4 with varying PS contents

4. Conclusions

To evaluate the applicability of SFC for oligonucleotide analysis, we developed a separation method using 4-mer oligonucleotides with varying PS contents as model compounds and investigated their basic retention characteristics. First, we identified conditions that allowed oligonucleotides to elute, finding that a methanol-water mixture containing 50 mmol/L ammonium formate enabled elution and peak detection. Using these conditions, we analyzed T4 with varying PS contents on 12 columns, finding good separation with the Diol II column. To apply this method to more polar sequences, we re-evaluated modifier conditions and found that adding aminoethanol improved peak shape. Applying the optimized method to 14 oligonucleotide sequences showed that sequences with two or fewer G or C bases were suitable for this method. Additionally, higher-polarity sequences showed stronger retention, while oligonucleotides with varying PS contents showed weaker retention with higher polarity, indicating unique retention behavior for PS modifications. The high selectivity for sequences with different PS contents suggests that SFC can be applied to separating impurities with a PS linkage replaced by a PO linkage that can be included in PS-modified oligonucleotides in the synthesis process.

In the future study, the applicability of SFC to longer oligonucleotides than 4-mer will be evaluated.

5. Acknowledgments

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<References>

1) M. Hayashida *et al.*, *J. Chromatogr. A*, **2023**, 464333

2) J. Molineau *et al.*, *J. Pharm. Biomed. Anal.*, **2021**, 193, 113736

3) Q. Gros *et al.*, *J. Chromatogr. A*, **2021**, 1639, 461923

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