



# Factors Influencing Diastereomer Separations in Oligonucleotide Analysis

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This is an Application Brief and does not contain a detailed Experimental section.

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## Abstract

This work demonstrates that the hydrophobicity and concentration of ion pairing agents influence the separation of diastereomer containing oligonucleotide samples.

### Benefits

Separation of diastereomers can be influenced through the type ("weak" vs. "strong") and concentration of IP agent used in oligonucleotide analyses.

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## Introduction

Chemical modifications are commonly incorporated into synthetic oligonucleotides to increase stability and safety *in vivo* against endo- and exonucleases as well as for improving efficacy through increased cellular uptake and binding properties. Modification to the phosphodiester backbone during synthesis, such as replacement of an oxygen molecule with a sulfur, creates a phosphorothioate (PS) linkage. The PS modification introduces a chiral center into the phosphate group leading to the formation of  $2^n$  diastereomers, where  $n$  is the number of phosphorothioate modifications, i.e. two diastereomers for single PS linkage, four for two PS linkages, etc. It has been shown that the oligonucleotide diastereomers can be resolved on reversed-phase columns.<sup>1</sup>

The partial separation of numerous diastereomers is the cause of band broadening of full PS oligonucleotides and is a reason why their analysis is more difficult than phosphodiester (PO) oligonucleotides in Ion Pairing Reversed Phase Liquid Chromatography (IP-RPLC).<sup>2</sup> IP-RPLC is one of the prevalent techniques in the analysis of synthetic oligonucleotides due to its enhanced separation performance and its ability to incorporate MS compatible mobile phase buffers. Understanding factors that influence the separation of diastereomers in oligonucleotide IP-RPLC separations is necessary for streamlining method development and improving productivity in the characterization and analysis of oligonucleotides.

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## Results and Discussion

When adsorbed onto hydrophobic bonded phases, IP reagents such as n-alkyl amines, provide a means to separate oligonucleotides based on charge interaction with their phosphate backbone. To a lesser degree, the hydrophobicity of the nucleobases plays a role in the oligonucleotides retention. When selecting more hydrophobic ion-pairing agents (more adsorbed on stationary phase) or using elevated concentrations of IP agents, the retention mechanism of oligonucleotides is dominated by ionic interaction and the hydrophobic interaction responsible for diastereomeric separation is suppressed. To demonstrate this effect, three oligonucleotides of the same primary sequence were purchased containing zero, one, and two chiral PS linkages (noted by the asterisks).

The sequences are as follows: 5'-rArGrCrUrGrArCrCrCrUrGrArArGrUrUrCrA rUrCT-3', 5'-rArGrCrUrGrArCrCrCrUrGrArArG\*rUrUrCrArUrCT-3', and 5'-rArGrCrUrGrArCrCrCrUrGrArArGrUrUrCrArU\*rC\*T-3'.

Evaluation of a "weak" triethylammonium acetate (TEAA) and "strong" hexylammonium acetate (HAA) ion-pairing system is shown in Figures 1 and 2. Mobile phases were prepared at 15mM and adjusted to pH 7.0. The Waters ACQUITY UPLC OST BEH C<sub>18</sub> Column (130Å, 1.7 µm, 2.1 mm x 50 mm) was selected for this work based on the superior column life time and performance for oligonucleotide separations. As shown in Figure 1A, separation of the diastereomers were observed for the oligonucleotides containing additional chiral PS centers when using the "weak" IP agent TEAA. Separation of the diastereomers was not observed with the "strong" IP agent HAA as shown in Figure 1B.

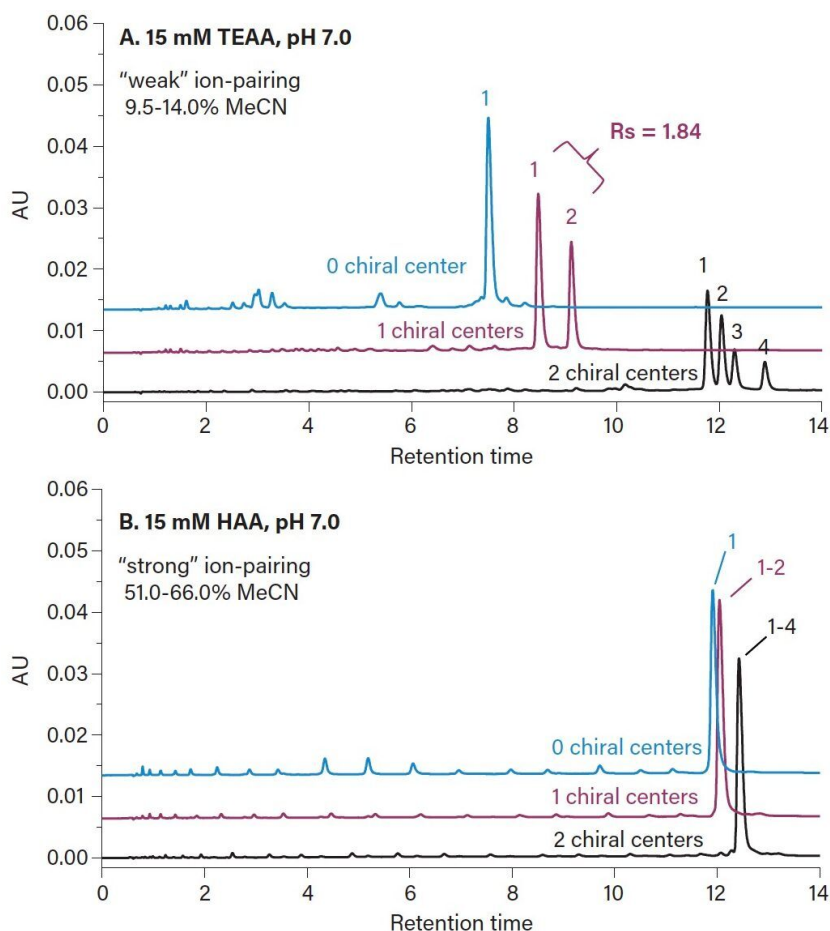
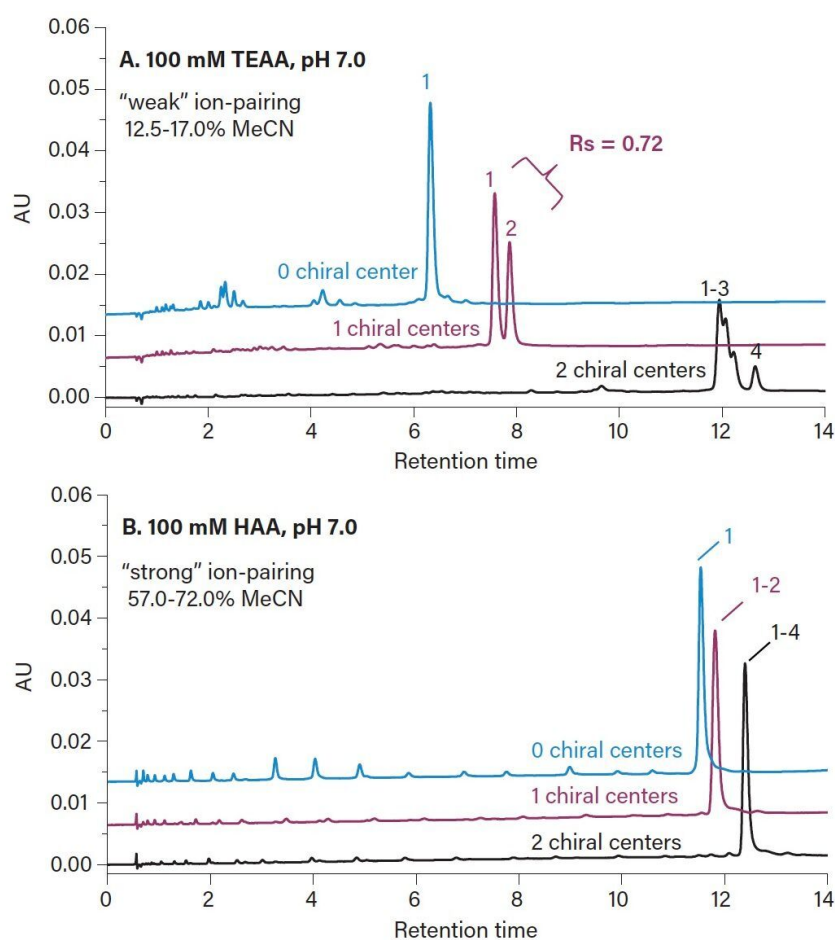


Figure 1. Evaluation of TEAA and HAA IP Agent. Analysis of three ssRNA strands containing zero, one, and two chiral centers was conducted on an ACQUITY H-Class Bio System with a Waters OST BEH C<sub>18</sub> Column (130Å, 1.7 µm, 2.1 mm x 50 mm). Mobile phase A (H<sub>2</sub>O) and B (50:50 H<sub>2</sub>O:MeCN) were prepared at 15 mM IP agent concentration using A) TEAA and B) HAA adjusted to pH 7. Using a 15 minute separation gradient, the diastereomers, if present in the sample, were resolved using the "weak" TEAA IP agent compared to the "strong" HAA IP agent.

The much higher concentration of organic modifier needed to elute the oligonucleotides when using "strong" IP agent presumably suppressed the hydrophobic interactions needed for resolution of diastereomers. This phenomenon was further investigated using a higher concentration of IP agent. As shown in Figure 2A, higher concentration of TEAA (100 mM) suppresses diastereomeric resolution (2.5-fold), however partial separation of the diastereomers was still observed. Figure 2B illustrates that both 15 mM and 100 mM

"strong" IP HAA buffer system offer comparable separation performance with mostly charge-based or length based retention of N and N-x truncated synthetic products.



*Figure 2. IP-Reagent Concentration Evaluation. Using the system configuration described in Figure 1, the impact of high IP-reagent concentration on the separation of oligonucleotides containing zero, one, and two chiral centers was evaluated for the A) TEAA and B) HAA IP agents. A) The higher concentration of TEAA suppressed diastereomeric resolution compared to Figure 1A (2.5-fold); however, partial separation of the diastereomers was still observed. B) No diastereomeric resolution was observed in HAA IP system regardless of HAA concentration.*

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## Conclusion

Understanding factors that influence the separation of diastereomers in oligonucleotide IP-RPLC separations is important for streamlining method development and improving productivity in the characterization and analysis of oligonucleotides. This work demonstrates how separation of diastereomers can be controlled through the type ("weak" vs. "strong") and to a lesser degree the concentration of IP agent used in oligonucleotide analyses.

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## References

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