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Evaluation of Alternative Ion-pairing Reagents in the Analysis of Oligonucleotides with the ACQUITY QDa Detector

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Abstract

This application note evaluates the applicability of alternative IP reagents in the analysis of oligonucleotides.

As new therapeutic oligonucleotides come to market with novel physicochemical properties, IP-RPLC/MS-based techniques that incorporate alternative IP buffers to TEA:HFIP may be required to achieve optimal chromatography conditions. The Waters OST columns exhibit a high degree of ruggedness when using alternative methods that require high pH and elevated temperatures over extended time periods, and can be incorporated into existing ACQUITY QDa workflows. Collectively, this work demonstrates that Waters offers a cost-effective solution to the challenges facing today's analysts in oligonucleotide analyses that is flexible, robust, and readily deployable.

Benefits

· Method compatibility with alternative ion-pairing reagents

· Minimizing costs through low volume use of IP-RPLC reagents

· Increased productivity through the use of on-line orthogonal detection techniques

Introduction

Electrospray ionization- (ESI) based analyses of oligonucleotides are often performed with buffers comprised of triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). This is in part due to the increased mass spectrometry (MS) sensitivity afforded by the HFIP buffering reagent as demonstrated by Apffel and colleagues.[1,2] The benefit of mass information in identifying challenging base modifications is well established.[3] However, as oligonucleotides with novel base modifications come to market with varied physicochemical properties, achieving optimal chromatography conditions may require alternative ion-pairing reagents other than TEA, which can be buffered with HFIP. Recently, it was demonstrated that amines other than TEA could be used with HFIP and produce adequate MS response using an IP-RPLC/MS-based technique.[4] As shown in previous work using the ACQUITY QDa Mass Detector, mass information afforded by MS detection offers improved productivity in synthetic therapeutic oligonucleotide workflows.[5]

A natural extension of this work is to evaluate the applicability of alternative IP reagents in the analysis of oligonucleotides using the ACQUITY QDa. A set of polyT standards ranging from 15 nt to 35 nt in length as

well as a ssRNA sequence (5'-UCGUCAAGCGAUUACAAGGTT-3') were used in this study to assess method repeatability, column longevity, and assay comparability. Triethylamine (TEA), butylamine (BA), and dibutylamine (DBA) were evaluated in this study as alternative IP reagents.

Experimental

Sample Description

Triethylamine (99.5% purity), butylamine (99.5% purity), dibutylamine (99.5% purity), and 1,1,1,3,3,3-hexafluoro 2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. Optima series solvents were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were purchased from Waters (P/N 186004135) and prepared at a concentration of 10 pmol/µL. A ssRNA upper strand (5'-UCGUCAAGCGAUUACAAGGTT-3') was purchased from Integrated DNA Technologies. Mass loads on column were kept constant at 50 pmol.

LC conditions

LC system:	ACQUITY UPLC H-Class
LC detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC Oligonucleotide BEH C_{18}, 1.7 µm, 2.1 mm x 50 mm
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL

Mobile phase

TEA:HFIP

Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

BA:HFIP

Mobile phase A:	15 mM BA, 50 mM HFIP prepared in H_2O, pH 9.0
Mobile phase B:	15 mM BA, 50 mM HFIP prepared in MeOH

DBA:HFIP

Mobile phase A:	15 mM BA, 25 mM HFIP prepared in H_2O, pH 9.5
Mobile phase B:	15 mM BA, 25 mM HFIP prepared in MeOH

Mobile phases prepared gravimetrically.

Detector conditions

Detector:	ACQUITY QDa
Sample rate:	2 points/sec
Mass range:	410–1250 Da
Mode:	Negative
Collection mode:	Continuum
Cone voltage:	20 V

Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt1

Results and Discussion

Developing chromatographic methods is a process that involves decisions based on a myriad of factors including assay robustness, resolution, and selectivity. For many analysts, developing a method typically starts with a literature search to determine if there is precedence for the desired separation to establish a baseline for comparison. In the case of IP-RPLC/MS-based oligonucleotide analyses, buffers comprised of TEA:HFIP have become the "gold standard" for comparison due to its high separation efficiency and MS compatibility.[1,2] In this regard, a comparability test was initially performed to see if alternative amines could produce similar chromatographic profiles with adequate MS response as in the case of mobile phases comprised of TEA:HFIP.

Previous work has shown that a gradient of 0.5% B/min with a mobile phase comprised of 15 mM TEA and 400 mM HFIP is sufficient in separating a set of polyT standards ranging from 15 nt to 35 nt in length.[6] Using this separation as a baseline, a comparability study was performed using butylamine and dibutylamine as shown in Figure 1. Concentration of the alternative amines was held constant at 15 mM while the HFIP was adjusted for optimum MS response as previously published by Gong, et al.[4] In the case of butylamine and dibutylamine, the concentration of HFIP used was 50 mM and 25 mM, respectively.
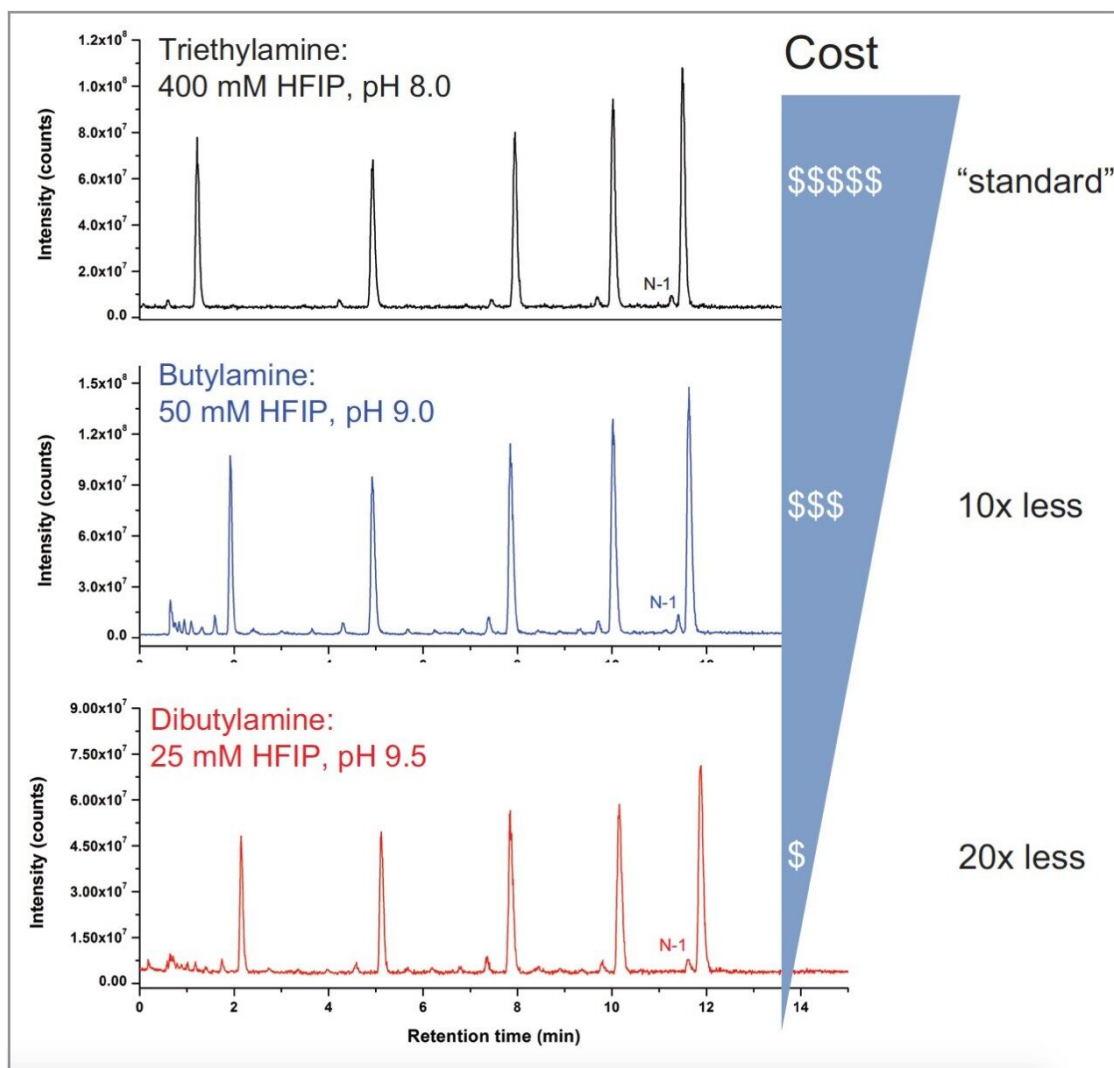
Figure 1. Evaluation of alternative IP reagents. ACQUITY QDa response for separation of the MassPREP OST polyT standard mixture using trimethylamine, butylamine, and dibutylamine buffered with 400 mM, 50 mM, and 25 mM HFIP, respectively.

As shown in Figure 1, comparable selectivity was achieved with the two alternative amines. Furthermore, butylamine was able to achieve similar selectivity as the TEA:HFIP with a gradient of 0.46% B/min, but with a lower starting organic composition; whereas dibutylamine required doubling the gradient to 0.80% B/min with similar starting organic composition as TEA:HFIP. These results suggest the concentration of HFIP and hydrophobicity of the amine impact oligonucleotide retention on the column. However, "tuning" of the separation conditions can be performed to achieve the desired separation, which in this case was to match the selectivity of the TEA:HFIP separation.

Mass detector response was shown to be similar between TEA:HFIP and BA:HFIP, with BA:HFIP showing

marginally better signal response. This was in contrast to the DBA:HFIP run, which showed approximately a 2-fold drop in signal intensity, despite having an identical mass load. The reduced signal intensity in the DBA:HFIP separation does not hinder detection of the failed sequence peak (N-1, Figure 1), indicating alternative amines such as butylamine and dibutylamine can produce adequate MS signal response for analysis when buffered with HFIP.

Interestingly, an unintended corollary with assay cost was observed during the comparability test. Cost-prohibitive MS-grade purity reagents such as HFIP are often cited as a concern in oligonucleotide assay development. Figure 1 demonstrates that IP reagents such as butylamine and dibutylamine can reduce assay costs through reduced HFIP use while maintaining assay selectivity when compared to TEA:HFIP, making them an appealing alternative in IP-RPLC/MS-based techniques. As indicated in Figure 1 though, lowering the concentration of HFIP results in a mobile phase with a pH >8.0, which can be a concern with respect to column longevity.

To test the impact of a modest increase in pH on column longevity, the mobile phase prepared with butylamine (15 mM BA:50 mM HFIP, pH 9.0) was selected for evaluation. For this study the gradient was extended to 30 minutes with a slope of 0.5% B/min. Mobile phases were prepared in 200 mL batches and refreshed every 24 hours over the course of the time study. To conserve samples, four water blanks were injected in succession using the method followed by an injection of the mixture of polyT standards. As shown in Figure 2, the PolyT standards were separated with a high degree of repeatability over 400 injections with nearly identical chromatographic profiles. Selectivity was calculated for the first four peaks relative to the last peak as a means to probe column robustness in the presence of elevated pH and temperature. Calculated values based on the experimental data were averaged over 24-hour time blocks and reported with corresponding error bars as shown in Figure 3. Selectivity values were determined to have less than 1% RSD across 400 injections over 200 hours.
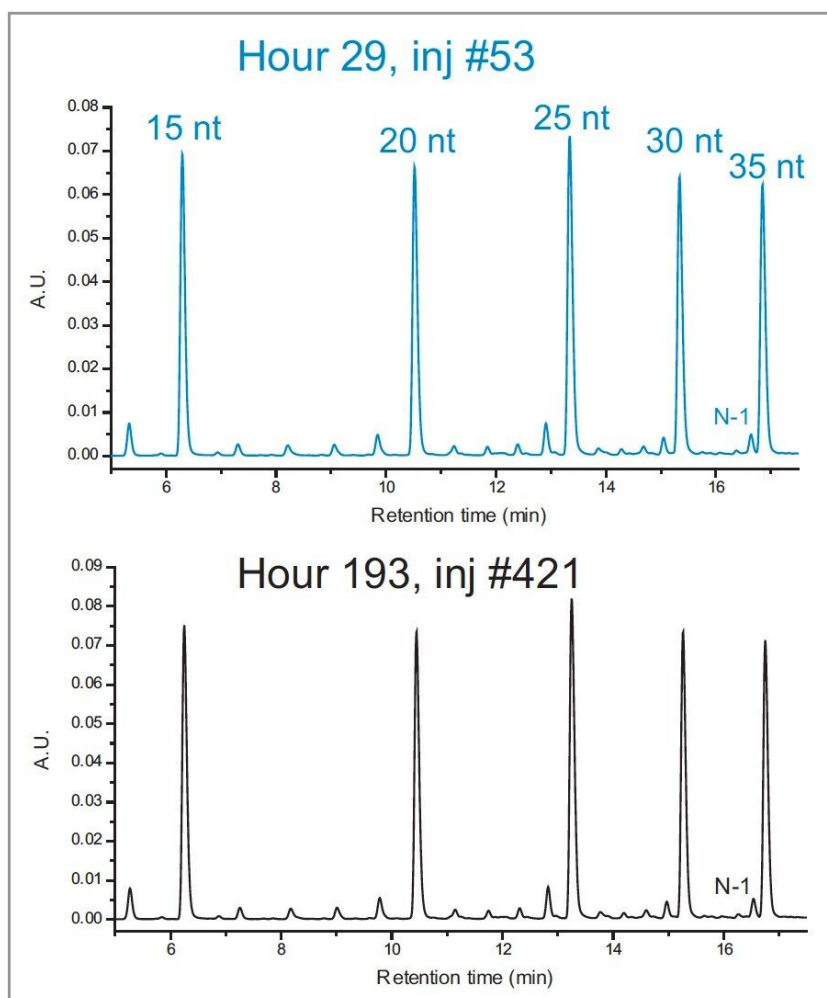
Figure 2. Column longevity evaluation at elevated pH. The MassPREP OST polyT standard mixture was separated using a 30-minute method across 400 injections over 200 hours. The PolyT standards were separated with a high degree of repeatability observed in the chromatographic profile of the standards, as well as the failed sequences (N-X).
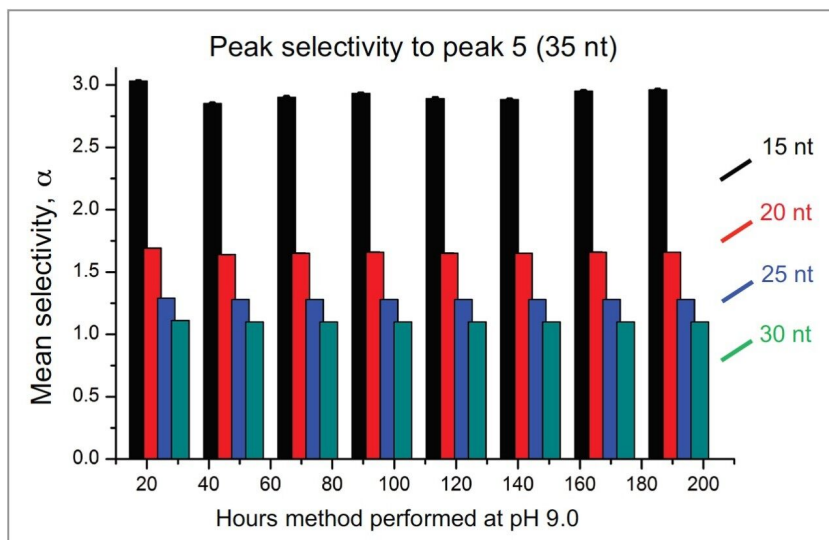
Figure 3. Column selectivity evaluation. Peak selectivity was calculated for the first four standard peaks (15–30 nt) relative to the last peak (35 nt) from a separation using elevated pH and temperature. Calculated values based on the experimental data were averaged over 24-hour time blocks and reported with corresponding error bars. Selectivity values were determined to have less than 1% RSD.

To further probe column performance, interassay variability was evaluated for retention time and peak width at half height. As shown in Table 1, retention times were stable across the injection series with RSDs ≤1.66%. Peak width was also highly stable with average peak width at half height ≤0.08 minutes with RSDs ≤1.49%. Collectively, this data confirms that the Waters OST column exhibits a high degree of ruggedness when using methods that require high pH and elevated temperatures over extended time periods. With comparability and column longevity confirmed when using alternative IP mobile phase compositions, a sample more representative of an oligonucleotide was used to test the applicability of the proposed method.

Inter-assay variability					
Peak	Avg. MW	RT (min)	RSD (%)	W_{50}	RSD (%)
15 nt	4500.9	6.15	1.66	0.077	0.90
20nt	6021.9	10.34	0.97	0.078	0.70
25 nt	7542.9	13.16	0.72	0.079	0.68
30 nt	9063.8	15.16	0.65	0.078	1.49
35 nt	10584.8	16.65	0.59	0.078	0.83

Table 1. Inter-assay variability was evaluated for retention time and peak width at half height (W50) across the injection series. Mean retention times were observed with RSDs ≤1.66%. Mean peak widths at half height were observed to be ≤0.08 minutes with RSDs ≤1.49%.

A comparison was made between TEA:HFIP and BA:HFIP mobile phases in the separation of a 21 nt ssRNA as shown in Figure 4. Using a high-resolution gradient, the N-1 and N+1 impurities were separated from the target peak using both mobile phase compositions with a high degree of comparability. Similar to before, the initial starting %B was lower for the BA:HFIP mobile phase in comparison to the TEA:HFIP (7% vs. 13%). The gradient slope for the BA:HFIP was slightly shallower at 0.6% B/min in comparison to the 0.8% B/min for TEA:HFIP, most likely due to the butylamine being less hydrophobic and requiring less eluent to desorb from the ligand surface.[7,8] In addition, the ACQUITY QDa response was observed to be highly comparable across both mobile phases for the ssRNA (1.68×10^8 TEA:HFIP vs. 1.43×10^8 BA:HFIP). The ability to separate the impurity sequences from a more representative oligonucleotide using BA:HFIP further corroborates the notion that IP reagents other than TEA:HFIP offer analysts the ability to explore alternative IP-RPLC/MS solutions for oligonucleotide analyses that enable high MS sensitivity and potentially reduce assay costs.
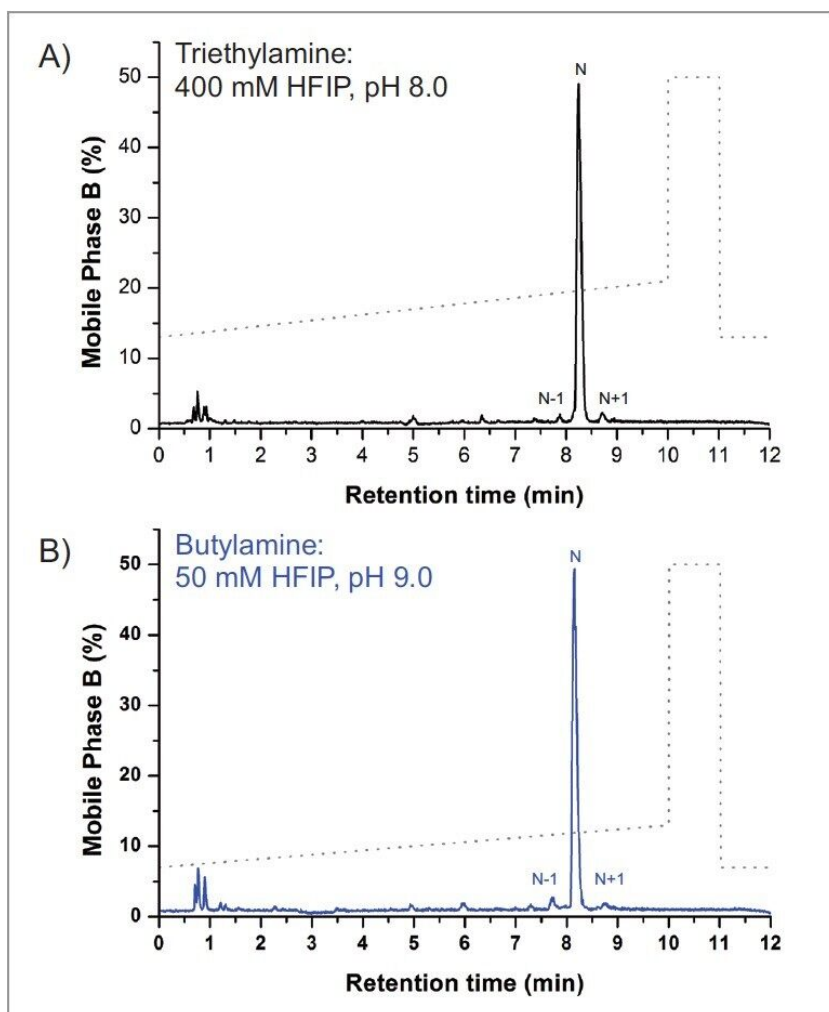
Figure 4. Impurity profiling of ssRNA. A 21 nt ssRNA (5'-UCGUCAAGCGAUUACAAGGTT-3') was separated from its N-1 and N+1 impurities using a high-resolution gradient with mobile phases comprised of A) 15 mM TEA : 400 mM HFIP, and B) 15 mM BA : 50 mM HFIP.

Conclusion

As new therapeutic oligonucleotides come to market with novel physicochemical properties, IP-RPLC/MS based techniques that incorporate alternative IP buffers to TEA:HFIP may be required to achieve optimal chromatography conditions. The Waters OST columns exhibit a high degree of ruggedness when using

alternative methods that require high pH and elevated temperatures over extended time periods, and can be incorporated into existing ACQUITY QDa workflows. Collectively, this work demonstrates that Waters offers a cost-effective solution to the challenges facing today's analysts in oligonucleotide analyses that is flexible, robust, and readily deployable.

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