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# The Impact of Adducts and Strategies to Control Them in IP-RPLC Based Oligonucleotide Analyses

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

## Abstract

This work demonstrates how a rapid low-pH regeneration step incorporated into the oligonucleotide analysis method is able to maintain assay performance over time with minimal impact on productivity.

### Benefits

The accumulation of trace salts in IP-RPLC based analyses of oligonucleotides is a well-known phenomenon requiring efficient mitigation strategies to maintain consistent and comparable results.


## Introduction

Due to the negatively charged phosphodiester backbone, charge-based separations such as ion-pairing reversed phase chromatography (IP-RPLC) have become a popular choice for the analysis and characterization of oligonucleotides. When adsorbed onto hydrophobic bonded phases, IP agents such as n-alkyl amines, provide a means to separate oligonucleotides with high separation efficiency based on charge interaction of the phosphodiester backbone. However, as a charge-based separation, positively charged cations of alkali metal salts such as sodium ($Na^+$) and potassium ($K^+$), are electrostatically attracted to the negatively charged polyanionic backbone of oligonucleotides which can interfere with the separation mechanism and impact assay performance. A common practice to address this known phenomenon is to periodically take the system off-line and clean using mobile phases containing metal chelators such as EDTA or CDTA to reduce trace salt concentrations. While this strategy can be effective, it is less than ideal as it requires system downtime and resources. By understanding the formation of alkali metal salts in oligonucleotide analyses, we have developed a mitigation strategy that maintains consistent chromatographic performance with minimal impact on productivity.

## Results and Discussion

An eight hour time study was conducted using a 21-mer ssRNA sample (5′-UCGUCAAGCGAUUACAAGGTT-3′) to evaluate the impact of alkali metal salts on assay performance. To establish baseline performance, a

Waters ACQUITY UPLC H-Class Bio System was passivated using an acidic mobile phase and conditioned with freshly prepared conventional IP-RPLC mobile phase comprised of 15mM TEA: 400 mM HFIP, pH 8.0. An ACQUITY QDa Mass Detector was configured in-line post UV detection to monitor alkali metal salt adducts from deconvoluted spectrum. The Oligonucleotide BEH $C_{18}$ Column was selected for this work based on its superior column life time and performance for oligonucleotide separations. Two selected chromatograms representing the initial injection (blue trace) and end injection (red trace) of the time study are shown in Figure 1. From the deconvoluted spectrum (Figure 1, inset) a significant increase in the relative amount of adduct ions (6% to 63%) was observed over time due to accumulation of trace alkali metal salts. The shift in retention time and peak deterioration observed correlates with the degree of alkali metal adducts (Figure 2, red trace). These observations demonstrate that salts, when present at elevated concentrations, can severely disrupt the ion-pairing equilibrium and interfere with the charge-based ion-pairing retention mechanism leading to decreased chromatographic performance and MS sensitivity. Considering initial conditions were acceptable after passivating the system, the study was repeated incorporating a one minute acidic column regeneration step in the method after the separation gradient using 0.1% FA to displace non-specifically adsorbed metal salt cations. As shown in Figure 2, the low-pH regeneration method (blue trace) was sufficient in mitigating alkali metal adducts resulting in high spectral abundance (>92%) and retention time stability (mean 2.44 min, RSD 0.57%) of the target oligonucleotide (M). This effective mitigation strategy can be built into the chromatographic method and run on a regular basis, thus avoiding system downtime and yielding greater consistency in results.

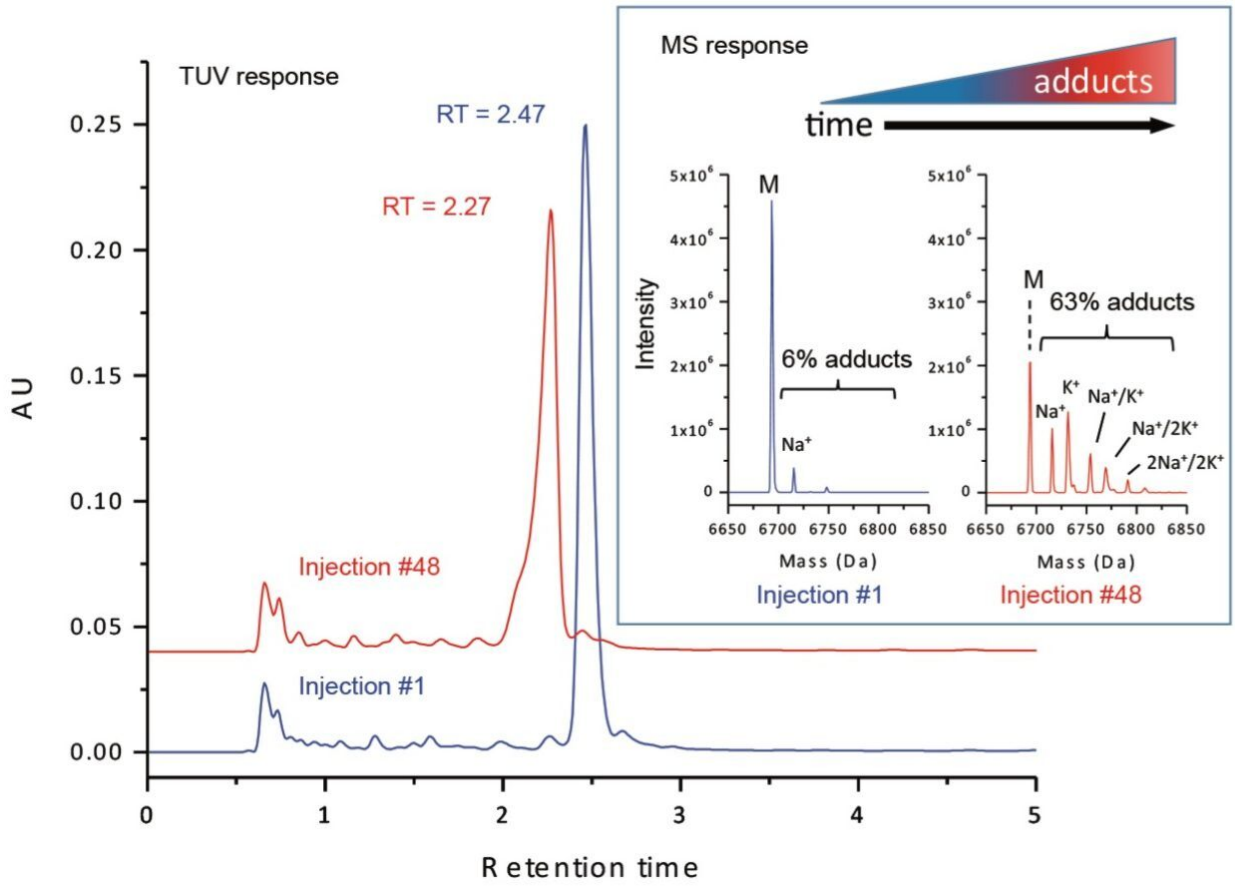


*Figure 1. Impact of alkali metal salts in oligonucleotide analyses. Using a four minute separation gradient from 18%B to 20%B (10-minute total run-time), a 21-mer ssRNA sample (5'-UCGUCAAGCGAUUACAAGGTT-3') was separated using an OST BEH $C_{18}$ Column (130 Å, 1.7 µm, 2.1 mm x 50 mm) over an eight hour time study. An increase in adducts relative to the neutral target peak (M) was observed over time due to accumulation of trace alkali metal adducts which correlated to a retention time shift of 0.2 min and noticeable peak shape deterioration in the chromatogram (red trace).*

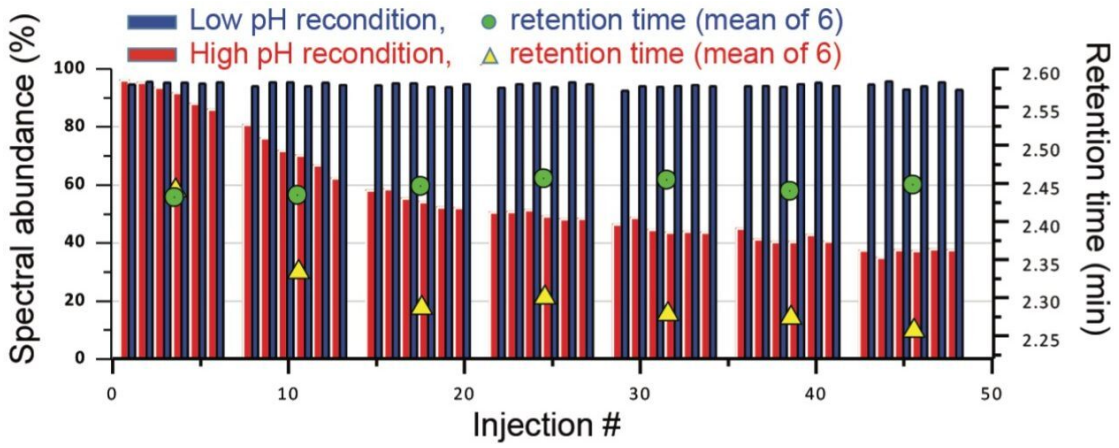


*Figure 2. Comparison of low and high pH reconditioning methods. Alkali metal adducts were kept at a minimum levels using the low pH reconditioning method (blue trace) with spectral abundance for the deconvoluted neutral peak [M] maintained above 92.5% over an eight hour time study with stable retention time (mean 2.44 min, RSD 0.57%). Assay performance using the low pH reconditioning method was significantly improved when compared to the original high pH regeneration method (red trace) which exhibited a high degree of adduct formation and retention time drift over the same amount of time.*

## Conclusion

Understanding the formation of alkali metal salts in oligonucleotide analyses is necessary for the development of effective mitigation strategies. This work demonstrates how a rapid low-pH regeneration step incorporated into the oligonucleotide analysis method is able to maintain assay performance over time with minimal impact on productivity.

## References

1. Birdsall, et al. Reduction of metal adducts in oligonucleotide mass spectra in ion-pair reversed-phase chromatography/mass spectrometry analysis. *Rapid Commun. Mass Spectrom*. 2016; 30(14):1667.

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ACQUITY UPLC H-Class PLUS Bio System <https://www.waters.com/10166246>

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