Evaluation of reversed phase liquid chromatography columns, ion pair reagents and separation conditions - for stability studies of modified RNA

Master’s thesis within the Biotechnology master programme

Jacob R. Dahlberg
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Abstract

mRNA for therapeutic purposes is a new and exciting area on the verge of being accepted as an established technique for treating human diseases. Because of this it is vital to develop effective and reliable methods for analyzing the mRNAs stability related to storage and handling during pharmaceutical development. This project assesses the main parameters for developing such a method using IP RP HPLC. In total four ion pair reagents were evaluated for influence on separating a RNA ladder: n-HAA, TEAA, TPAA and TBAA where the latter three were subject to an experimental design. Results favoured TPAA, which then was used as an IPR in degradation studies of a 858 nt RNA molecule. The degradation studies covered conditions: Heat-, oxidation-, hydrolysis- and RNase A treatment, where fragmentation could be seen to a varying degree for all cases.

Keywords: Ion pair, reversed phase, HPLC, RNA, design of experiments, forced degradation, heat, oxidation, hydrolysis, RNase A.
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List of Abbreviations

ACN Acetonitrile
DOE Design of experiments
EDTA Ethylenediaminetetraacetic acid
HPLC High performance liquid chromatography
IP Ion pair
IPC Ion pair chromatography
IPR Ion pair reagent
n-HAA n-Hexylammonium acetate
nt Nucleotides
RNase Ribonuclease
RP Reversed phase
RT Retention time
ss Single strand
TBAA Tripropylammonium acetate
TEAA Triethylammonium acetate
TPAA Tributylammonium acetate
UTR Untranslated region
1 Introduction

Nucleic acids were used for the first time as drugs almost three decades ago when Wolff et al. successfully demonstrated that plasmid DNA and mRNA injected into the skeletal muscles of mice resulted in protein expression [1]. From the 1990s and onwards effort has been put into preclinical exploration of in vitro transcribed mRNA for purposes such as vaccination, protein substitution, infectious disease- and cancer treatment. As a result of the increase in research, recent methods for overcoming difficulties related to short half-life and immunogenicity were able to be developed. The most important step lies in the alteration of mRNA structural elements, namely the 5’ cap, 5’- and 3’ untranslated regions (UTRs), the coding region and the poly(A) tail. Some of the crucial advantages with using mRNA-based therapeutic approaches instead of other nucleic-acid based approaches include its effectiveness outside of the nucleus and the negligible risk for insertional mutagenesis [2].

The highly sensitive RNA molecules, especially compared to DNA, may be provoked into a degradation event under pharmaceutical development when subject to conditions such as heat, hydrolysis, oxidation, UV or omnipresent ribonucleases. RNases are often introduced as a result of sloppy lab handling and great care is therefore required when handling samples [3]. For these reasons are analytical tools, and stability indicating methods, that effectively separate and analyse RNA becoming increasingly sought after. Ion pair (IP) reversed phase (RP) high performance liquid chromatography (HPLC) proved promising results at an early stage for DNA analysis, and recent studies involving RNA have demonstrated the platforms versatility and complexity, requiring careful decision making regarding IPRs, temperature and mobile phase composition [4]. The technology can be practised for purification quantification, as well as qualification of labeled or unlabeled RNA samples including mRNA, rRNA, discrete transcripts and total RNA [5].

1.1 Aim of Study

The project aim to construct and evaluate an ion pair reverse phase liquid chromatography method with UV detection applicable for stability studies of mRNA. Focus will be directed towards finding critical separation- and preparation conditions. Interesting aspects include usage of different columns, ion pair reagents and gradient programming.
1. Introduction
2 Theory

2.1 mRNA as a Therapeutic Agent

In the early 1980s discoveries were made regarding catalytic RNA, and later in the 1990s the phenomenon known as RNA interference. This has made way for today’s curiosity and appreciation of RNA for therapeutic applications [6]. An mRNA-oriented approach has advantages over DNA-based approaches as it poses a negligible risk of genomic integration. mRNA need no promoter and is required to cross only one cellular membrane for its applications, while DNA has to travel into the cell nucleus for permanent integration [7].

The major hurdles for mRNA techniques include efficient delivery to the targeted cells as well as potential immunogenicity and shifting stability. The latter one relates in part to the overabundance of ribonucleases, which degrade RNA, in serum and in cells. This can however be prevented with use of chemical modifications, which also has been known to reduce immunogenicity [6]. Modification of regions that has shown improved stability and translational efficiency include the 5’-cap, the coding region, 5’- and 3’-UTRs, and the poly(A) tail [2].

2.2 Stability of mRNA

There are two overall types of mRNA degradation that is of relevance. One is the chemical degradation, which is tightly linked to this project. The other one is the degradation within living tissue, which is important for understanding the final applications of modified mRNAs for therapeutic purposes.

2.2.1 Chemical Degradation

Because of the fact that the field of mRNAs as therapeutic agents are still in its infancy, little is known about its stability outside of the body, but polynucleotides are known to show significant chemical degradation under certain environmental conditions. Some of these degradation assisting conditions include alkaline, acidic, oxidative stress, thermal stress and photolytic stress. However, most of the data available addresses degradation patterns during synthesis, which is not very representative for what might occur during development, manufacturing processes, storage and handling [8].
2.2.2 In Vivo Degradation

mRNAs are known to exist with significantly different half-lives in vivo, ranging all the way from seconds, to minutes, to several days [9]. The degradation rates of mRNA is of importance to gene expression where differential mRNA turnover can lead to shifting levels of translated protein [10]. Logically, short-lived mRNA strands usually exist in lower concentration where translated protein levels respond quickly to transcription levels, while the more long-lived mRNA strands accumulate in high concentrations and respond slowly to transcriptional regulations [9].

A number of eukaryotic mRNA decay pathways have been described in literature (Figure 2.1). The left pathway describes a process where the poly(A) tail is shortened, followed by decapping and 5’ to 3’ degradation of the mRNA transcript. A slightly different pathway involves the transcript undergoing 3’ to 5’ decay after poly(A) tail shortening. The right part of the figure illustrate how decay is initiated prior to poly(A) tail shortening. Certain transcripts can for example be degraded through deadenylation independent decapping before 5’ to 3’ degradation. Finally, a pathways is shown where degradation is initiated in the transcript body through endonucleolytic cleavage. Additional routs for degradation include recognition of specific sequences which trigger degradation with little dependency on deadenylation events [11].

![Figure 2.1: Common mRNA degradation pathways in eukaryotes.](image)

2.3 Ion-Pair Reversed-Phase LC

The traditional RP LC exploits the hydrophobic interaction between the non-polar stationary phase and the non-polar analytes. If the analytes have a sufficiently hydrophobic nature, retention will occur. The retention event may in turn be modified by varying the aqueous-to-organic composition of the mobile phase. When the sample consist of ionic constituents, as is the case with RNA, they lack affinity for the stationary phase. Ion suppression has previously been the method of choice.
for chromatographic separation of charged analytes, which relies on pH-adjustments resulting in a non-ionized analyte. The many disadvantages of ion suppression, such as extensive method development and complications with multi-component samples, led to development of the more generally applicable approach of ion-pair chromatography (IPC) [12].

IPC was developed in 1973 and depend on ion pairs that are formed between charged analytes and counter ions added to the mobile phase. The ion-pair complex, where counter ions often contain alkyl chains, displays an increased affinity for the stationary phase, facilitating retention on RP columns [12].

2.3.1 The Retention Mechanism

A detailed understanding on a molecular level for how the retention works has been sought by analytical chemists for decades. Several theories has been proposed that rely on both theory and experimental observations. Some of these are however in direct conflict with each other regarding fundamental aspects of RP LC. For example, it is presently unclear whether if the retention process is best described by a partitioning process where the solutes are embedded into the the bonded phase, or if interface adsorption is of greater importance [13].

One of two simple rules is that hydrophilic analyte ions are best separated using a hydrophobic IPR and vice versa. An additional, but debatable, rule is that smaller reagents usually result in better separation than larger ones. These allow the analyte to have a greater contribution from how the IP complex behaves in the system [14].

In contrast to Ion-exchange methods, the selectivity for IP separation is foremost determined by the mobile phase. The main components of the aqueous mobile phase are the organic solvent and the IPR. The type and concentration for each component can be altered in order to modify the separation event [14].

2.3.2 UV Detection

Measuring the UV absorption of the sample analytes is one of the common detection techniques associated with LC systems. The relation between concentration and UV absorbance relies on Beer’s law, some rearrangement lead to the equation stated below:

\[
c = I_{UV} \cdot \frac{UV_{RF}}{\epsilon \cdot l}
\]  

(2.1)

\(c\) correspond to the concentration of the analyte, \(I_{UV}\) the intensity of the UV signal, \(UV_{RF}\) the response signal of the detector, \(\epsilon\) the molar extinct coefficient and \(l\) is the length of the detector cell. Oligonucleotides, with their purine and pyrimidine bases, have their UV absorption maximum between 255 and 265 nm. Usually detection is set for 260 nm, even though it can be optimized for the specific analyte beforehand [15].

As described in Equation (2.1), the concentration readings are dependent on UV absorption and the extinct coefficient is unique depending on analyte. For oligonucleotides the coefficient is related to the specific base sequence, however to a
limited extent, and can be calculated using the "nearest-neighbour model" for single strands. Significant impact on the absorption characteristics can instead be seen between single strands and double helices, duplex forming lead to decreased UV absorption, a phenomenon known as hypochromicity. Therefore it is vital to have knowledge about the analytes annealing and melting properties and work under controlled analytical conditions [8].

2.4 Analysis of Single Stranded RNA

The main properties to utilize when separating single-stranded (ss) nucleic acids are size, conformation and hydrophobicity. Negatively charged phosphate groups present in the RNA molecule lead to hydrophilic properties. Another important factor is the size and specific base composition of the RNA. Cytosine (C), Guanine (G), Adenine (A) and Uracil (U) display different levels of hydrophobicity, where A is the most and G is the least hydrophobic base [16]. As a result, strands rich in A elute slower than strands rich in G when an ACN gradient is enforced. This effect can however be reduced or eliminated by using a more hydrophobic IPR. The hydrophobic interaction between ion-pair and stationary phase in IP RP HPLC depend on the number of methyl groups constituting the alkyl chain(s) of the IPR. TBAA for example, compared to TEAA, will not allow the RNA to interact directly with the stationary phase due to complete coverage from the IPR. The result is very low sequence dependency on the separation, but rather separation based merely on size [4]. IPRs utilized within the scope of this project are presented in Figure 2.1 together with an acetate ion (deprotonated acetic acid).

![Figure 2.2: Display of a) Triethylammonium b) Tripropylammonium c) Tributylammonium and d) Acetate ion [17].](image)

2.5 Design of Experiments

Statistical design of experiments constitute the course of planning a set of experiments so that relevant data can be collected and evaluated by statistical tools, and thereby resulting in unbiased and valid conclusions. A statistical approach to design of experiments is vital in order to draw correct conclusions from the data, especially when the problem include data that are subject to errors. Every experimental prob-
lem can be said to include at least two aspects, the design of experiments (DOE) and the following statistical analysis of data. [18].

Each experiment brings results, commonly numerical values, for the response variables which are evaluated by regression analysis. The following model relate changes in factors to changes in responses and provide information on the factors relative importance as well as combined influences over the responses. Commonly DOE is symmetrically performed around an interesting reference experiment which constitute the center-point [19].
2. Theory
Experimental

The following sections aim to state all equipment and materials used during the course of the project, as well as the used methodology.

3.1 Equipment and Chemicals

The HPLC system used for all experiment consisted of six components, all belonging to the Agilent (Santa Clara CA, USA) 1200 Series, namely vacuum degasser, quaternary pump, autosampler thermostat, high performance autosampler, thermostatted column department (allowing for temperatures up to 100°C) and diode array multiple wavelength detector (range 190-950 nm). Two columns were donated from Agilent Technologies. The first one titled PLRP-S consisting of macroporous styrene/divinylbenzene polymer particles, pore size 4000Å, particle size 8 µm, serial# 0006209035-49, part# PL1912-3803 and length/I.D. 150x2.1 mm. The second one titled AdvanceBio Oligonucleotides consisting of superficially porous Poroshell particles with an end-capped C18 phase, pore size 100Å, particle size 2.7 µm, serial# USHRR01022, part# 653750-702 and length/I.D. 150x2.1 mm. A third column was donated from Waters Corporation (Milford MA, USA) titled XBridge Peptide BEH C18, pore size 300Å, particle size 3.5 µm, item# WT186003609 and length/I.D. 150x2.1 mm.

For all of the experiments during the course of this project deionized water was used that had also been treated to remove RNases. This was done by filtering the already ionized water through a Biopak® (Sydney, Australia) Polisher, catalog# CDUFBI001. The organic solvent in all mobile phases consisted of Acetonitrile purchased from SIGMA-ALDRICH CHROMASOLV® (St. Louis MO, USA) purity of ≥ 99.9%, Lot# STBF8871V and Stock Keeping Unit (SKU)-Pack Size 34851N-2.5L. For cleaning equipment, mainly bottles and volumetric flasks, from RNases and other impurities methanol was used as organic solvent. The methanol used was purchased from SIGMA-ALDRICH CHROMASOLV® purity of ≥ 99.9%, Lot# STBF7572V and SKU-Pack Size 34885-2.5L-R.

Acetic acid (glacial) was purchased from MERCK (Darmstadt, Germany) purity ≥ 99.8%, catalog# 1000631000. Triethylamine (TEA) was purchased from SIGMA-ALDRICH®. Analytical purity ≥ 99.5%, Lot# STBF8135V and SKU-Pack Size 90335-100mL. Tripropylyamine (TPA) was purchased from Fluka® (Buchs, Switzerland). Analytical purity ≥ 98.0%, Lot# 1228680 and SKU-Pack Size 93240-100mL. Tributylamine (TBA) was purchased from SIGMA-ALDRICH®. Analytical purity ≥ 99.5%, Lot# BCBQ9979V and SKU-Pack Size 90781-50mL. n-Hexylamine
3. Experimental

(n-HA) was purchased from SIGMA-ALDRICH®. Analytical purity ≥ 99%, Lot# STBF1201V and SKU-Pack Size 219703-100mL.

Hydrogen peroxide 30%(w/w) solution was purchased from Fluka® (Buchs, Switzerland). Lot# BCBH3297V and SKU-Pack Size 95321-500mL. Sodium hydroxide solution were ordered from and prepared by AstraZeneca media lab at 1M. Hydrochloric acid 25%(w/w) (8.11 M) solution was purchased from MERCK (Darmstadt, Germany), catalog# 1003161000. RNase A (biochemistry grade) was purchased from Thermo Fisher Scientific (Waltham MA, USA) at 1 µg/mL, catalog# 15623100 and pub.# 4393904.

The RNA ladder used for method development came from Invitrogen (Carlsbad CA, USA) at 1 µg/µL in 10 mM HEPES (pH 7.2), 2 mM EDTA. Catalog# 15623100 and Lot# 1740923. Storage occurred at -80°C (-20°C short term). It consisted of ten bands of single stranded RNA (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5 and 2.0 knt) with polyA-tails of 40 nucleotides. The single-stranded polyadenylated mRNA of 858 nucleotides was purchased by TriLink Biotechnologies (San Diego CA, USA), Lot# T50-E01A and stored at -80°C (-20°C short term). Modifications: ARCA capped and fully substituted with 5-Methyl-C and Pseudo-U. Delivered as 5 x 1.00 mL of 1.00 mg/mL in RNase-free H2O.

3.2 Methods

The project can preferably be divided into three major areas; Familiarization Trials, Experimental Design and Degradation Studies.

All separation experiments were performed on the Agilent 1200 system stated under "Equipment and Chemicals". The majority of the experiments used flow rate of 0.2 mL/min, column PLRP-S, temperature 80°C and analyte concentration 0.1 mg/mL except for the flow-, column, temperature- and degradation studies respectively. Injection volume of 10 µL, ACN as organic solvent and detection at 260 nm were used for all experiments throughout the project. Vials put in the autosampler awaiting injection were subjected to a temperature of 15°C for 2-12 hours depending on queuing order. Samples not subject to analysis were kept in a freezer at -20°C and carefully mixed by pipetting upon thawing. Gradient settings varied depending on the experimental purpose and the used IPR, actual conditions are described when referred to in figures under "Results and Discussion". Evaluating data and programming the instruments was made through the chromatography data software Empower 3, produced by Waters Corporation.

3.2.1 Familiarization Trials

Column PLRP-S and AdvanceBio came from Agilent, while XBridge came from Waters. Respective manufacturer recommended these columns to fit a project separating large RNA molecules in the range of ~500-2000 nt. Both AdvanceBio and XBridge were used only for a few experiments included in the familiarization trials while PLRP-S were consistently used for both DOE and forced degradation studies.

Literature searches were made in order to find suitable IPRs for further studies, TEA being the first one evaluated on the system. TEA was initially evaluated
at concentration 50, 100 and 200 mM. The analyte for both familiarization trails and design of experiments consisted of the 10 peak mRNA Ladder, where the 0.3 kb band was more intense than the other bands in order to simplify interpretation of results. The initial concentration of 1 µg/µL was diluted ten times by addition of RNase free water into 200 µL vials for all experiments.

Mobile phases A and B differed from each other only by the ratio of H2O:ACN, where B always contained the higher amount of ACN. The phases were always mixed in the following order: 1. ACN 2. Acetic acid 3. Amine 4. H2O, where the amine and acetic acid ratio were kept at a 1:1 molar ratio.

Additional IPRs to TEAA assessed during the familiarization trials included TBAA, TPAA and n-HAA. Concentrations ranged 2.5-5 mM (TBAA), 5-20 mM (TPAA) and 15-100 mM (n-HAA). Five blank injections, containing only H2O and no analyte, were included in every run in order for the column to reach equilibrium.

Separation columns (Figure 4.6 and 4.7), flow rate (Figure 4.8), column temperature (Figure 4.9) and the effect of EDTA (Figure 4.10) were evaluated under conditions described in figures.

### 3.2.2 Design of Experiments

Information gained from the familiarization trials together with further literature studies were combined in order to construct a concentration scheme for DOE (Table 3.1) on the PLRP-S column. Gradients were then calculated and constructed based on the findings. For TEAA the mobile phases consisted of 0% ACN (A) and 50% ACN (B), the gradient was programmed to cover the range 0.5-50% ACN over 36.8 min. For TBAA and TPAA the mobile phases consisted of 10% ACN (A) and 90% ACN (B), the gradient was programmed to cover 10.8-70% ACN over 44 min. Each of the two gradient resulting in a slope of 1.35% ACN increase per minute. Graphical presentation of the gradients can be seen in Figure 4.5. Sample- and mobile phase preparations were conducted in the same manner as in the familiarization trials.

<table>
<thead>
<tr>
<th>IPR</th>
<th>low</th>
<th>mid low</th>
<th>mid</th>
<th>mid high</th>
<th>high</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAA</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>TPAA</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>TBAA</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>12.5</td>
<td>25</td>
</tr>
</tbody>
</table>

The DOE involved 18 runs in total, including center points (TPAA 7.5 mM), performed in the order starting with TPAA, followed by TBAA and last TEAA. Center points were conducted as runs 1, 8 and 18 in order to determine the systems variance. For detailed run order, see Figure 4.11. Assessed responses included the retention time (RT) difference between peak one and peak eight, RT for peak three and the width at half peak height (w½) for peak three. Statistical evaluations related to the DOE were made using MODDE 11, developed by Umetrics (Umeå, Sweden).
3. Experimental

3.2.3 Forced Degradation Studies

As a final part of the project forced degradation studies were initiated, including heat-, oxidative-, hydrolytic and RNase treatment. The TriLink RNA were subject to different conditions and analyzed using 12.5 mM TPAA as IPR, which originate from TPAA \textsubscript{mid high} in the DOE. The gradient was flattened compared to the DOE, covering 10-54\% ACN over 45 min. Analyte concentration were diluted from the original 1 mg/mL to 0.5 mg/mL by addition of RNase free water, resulting in a five times higher concentration compared to the RNA ladder.

3.2.3.1 Heat Treatment

Vials containing 200 µL TriLink RNA solution at concentration 0.5 mg/mL were heat treated for 50 °C respectively 80 °C, both for 120 min followed by a 30 min resting period in a 5 °C fridge in order to halt degradation. While waiting for column equilibration, the vials then spent 5 hours at 15 °C in the autosampler before injection.

3.2.3.2 Oxidative Treatment

A 200 µL vial was prepared by mixing the components in the following order: 1. 100 µL TriLink RNA at concentration 1 mg/mL 2. 98.18 µL RNase free H\textsubscript{2}O 3. 1.82 µL H\textsubscript{2}O\textsubscript{2} solution. The resulting vial contained 0.5 mg/mL TriLink RNA in a 0.3\%(w/v) H\textsubscript{2}O\textsubscript{2} solution, a concentration recommended by literature covering pharmaceutical stress testing [20]. While waiting for column equilibration, the vials then spent 6-12 hours at 15 °C in the autosampler before injection. 3 injections in total were performed, at 6, 9 and 12 hours after sample preparation.

3.2.3.3 Hydrolytic Treatment

A 200 µL vial was prepared by mixing the components in the following order: 1. 100 µL TriLink RNA at concentration 1 mg/mL 2. 80 µL RNase free H\textsubscript{2}O 3. 20 µL NaOH stock solution (1 M). The resulting vial contained 0.5 mg/mL TriLink RNA in a 100 mM NaOH solution. In the same manner a 200 µL vial was prepared in the following order: 1. 100 µL TriLink RNA at concentration 1 mg/mL 2. 97.53 µL RNase free H\textsubscript{2}O 3. 2.47 µL HCl stock solution (~8.11 M). The resulting vial contained 0.5 mg/mL TriLink RNA in a 100 mM HCl solution. 100 mM for both NaOH and HCl originated from recommendations in literature covering pharmaceutical stress testing [20]. Later two additional vials were prepared in the same way as before but at a lower concentration, namely 10 mM solutions of NaOH and HCl respectively. While waiting for column equilibration, the samples spent 2-11 hours at 15 °C in the autosampler before injection. Three injections were conducted for each sample (NaOH: 5, 8, 11 h and HCl: 2, 5, 8 h after sample preparation).

3.2.3.4 RNase Treatment

The RNase A, delivered at 1 µg/µL, was diluted 50x resulting in a concentration of 20 ng/mL. A 200 µL vial were then prepared by mixing the components in the following order: 1. 100 µL TriLink RNA 2. 80 µL RNase free H\textsubscript{2}O 3. 20 µL of
the diluted RNase A. The resulting vial contained 0.5 mg/mL TriLink RNA in a solution with 2 ng/mL RNase A. While waiting for column equilibration, the vials then spent 5-11 hours at 15°C in the autosampler before injection. 3 injections in total were performed, at 5, 8 and 11 hours after sample preparation.
3. Experimental
Results and Discussion

4.1 Familiarization Trials

In order to construct an experimental design to distinguish the importance of the different parameters, initial trials were executed. These built on the early established limitations for the project, where the most important was the analytical method of IP RP HPLC. Further boundaries came to include the column temperature, which operated at high temperatures (80°C for PLRP-S, 65°C for AdvanceBio and 80°C for XBridge) in order to have the RNA molecules denatured, the flow rate at 0.2 mL/min, the sample injection volume of 10 µL, the DOE gradient slope (Figure 4.5), ACN for organic solvent and the UV detection at 260 nm.

Parameter estimates relied on previous work known from literature. However, due to lack of literature in several areas related to analyzing the stability of nucleic acids in HPLC systems, much of the early work came to build on vaild estimates.

The temperature study and the experiment involving column XBridge was conducted at a later stage of the project than the other elements under this section but were deemed to fit best to be represented under Familiarization Trials.

4.1.1 Exploration of IPRs for Further Studies

The most extensively used IPR for separation of large nucleic acids is TEAA, where the concentrations range from 100-400 mM [21]. For this reason the very first experiments constituting this project were conducted with TEAA as IPR (Figure 4.1). Unlike the later tested IPRs, full separation of the ten peak ladder proved to be insufficient.

n-hexylammonium acetate (n-HAA) were next in line to be investigated as a candidate for further studies (Figure 4.2). The results were promising, but n-HAA as an IPR was abandoned due to the different nature compared to the other IPRs (TEAA, TPAA and TBAA) that later were assessed in the experimental design. n-HAA did simply not fit in the series of analogies for making a fundamental chromatography study.

For TBAA the literature suggested a concentration of 2.5 mM, which proved to be below optimum for this project [4]. 2.5 and 5 mM TBAA (Figure 4.3) were tested during the familiarization trials. These concentrations later came to be included in the experimental design.

Lastly, TPAA was evaluated for its ability to act as an IPR. Concentrations between 5 and 30 mM were investigated and optimum were thought to be found somewhere in between 10 and 20 mM concentration (Figure 4.4).
4. Results and Discussion

Figure 4.1: RNA Ladder with 200 mM TEAA. Sample injection volume 10 µL, column PLRP-S, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-25% ACN over 30 min.

Figure 4.2: RNA Ladder with 15 mM n-HAA. Sample injection volume 10 µL, column PLRP-S, column temperature 80°C, flow rate 0.2 mL/min and gradient 15-100% ACN over 60 min.

4.1.2 Gradient and Mobile Phases

One of the more extensive and important steps was to develop a functional gradient that later could be used in the experimental design. The consistently used organic solvent in the mobile phases was ACN. This way fair conclusions could be drawn, compared to if multiple organic solvents would have been involved. Different IPR:s
4. Results and Discussion

Figure 4.3: RNA Ladder with 5 mM TBAA. Sample injection volume 10 µL, column PLRP-S, column temperature 80°C, flow rate 0.2 mL/min and gradient 5-100% ACN over 60 min.

Figure 4.4: RNA Ladder with 20 mM TPAA. Sample injection volume 10 µL, column PLRP-S, column temperature 80°C, flow rate 0.2 mL/min and gradient 25-100% ACN over 60 min.

led to various results for retention time and resolution depending on gradient slope and boundary values. The gradient was therefore kept at a broad range during most of the early trials, before finalizing what became the experimental design gradients for the three IPRs (Figure 4.5). Covering a wide span of ACN carries the side benefit of preventing accumulation of hydrophobic as well as hydrophilic impurities.
Because of the differences between the IPRs, especially TEAA, a separate gradient scheme was developed for TEAA (Figure 4.5) together with a different mobile phase composition (Table 4.1). RNA paired with TEAA elute at lower concentrations of ACN than RNA paired with TPAA or TBAA. Furthermore, the solubility for TPAA and TBAA are poor in water solutions. The gradient slope, which is the mobile phase composition of ACN over time, was kept the same for all three IPRs.

**Figure 4.5:** Gradient scheme used in the DOE for TEAA (left), TPAA and TBAA (right). The y-axis represent the percentage fraction of ACN while the x-axis represent time in minutes.

**Table 4.1:** Amount of ACN in mobile phases used in the experimental design.

<table>
<thead>
<tr>
<th>IPR</th>
<th>% ACN Mobile Phase A</th>
<th>% ACN Mobile Phase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAA</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>TPAA</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>TBAA</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

### 4.1.3 Separation Columns

Two types of columns, PLRP-S and AdvanceBio, were considered during early trials. These columns came with recommendations from the manufacturer, Agilent Technologies, to fit this particular project where one important requirement was high temperature tolerance. An experimental design for each column would have been too time consuming. Instead comparative studies, using TPAA as IPR, were made between the two columns where PLRP-S proved to be superior for separating RNA under given conditions (Figure 4.6). Consequently, the AdvanceBio column was put to the side early on while focus was directed towards PLRP-S.

A third column, Waters XBridge Peptide, was donated during the final stages of the project. The results were not satisfying for larger RNA molecules even thou the column proved to separate smaller molecules (<300 nt) sufficiently (Figure 4.7).

### 4.1.4 Flow rate

The very first flow rate evaluated for PLRP-S, which came to be the consistently used flow rate throughout the project, was at 0.2 mL/min. This originate from Agilent
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Figure 4.6: RNA Ladder comparison between column PLRP-S (black) and AdvanceBio (blue) with 20 mM TPAA. Sample injection volume 10 µL, column temperatures 80°C/65°C, flow rate 0.2 mL/min and gradient 25-100% ACN over 60 min.

Figure 4.7: RNA Ladder on column XBridge with 12.5 mM TPAA. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10.8-70% ACN over 44 min.

Technologies own protocols where precisely 0.2 mL/min is used for analysis of nucleic acid polymers in excess of 500 nt. One argument for operating at a low flow rate is the mass transfer behaviour for large oligonucleotides, where a fast flow rate and low diffusion coefficients result tend to result in peak broadening. That being said,
0.2 mL/min is still well below what is used in some related protocols where the used flow rates are in excess of 1.0 mL/min. Therefore a comparison were made between 0.2 mL/min and 0.5 mL/min in order to evaluate whether to continue conducting experiments at 0.2 mL/min (Figure 4.8). The results proved that a higher flow rate gave comparatively poor response and no further investigations were made at the time [22].

![Figure 4.8](image)

**Figure 4.8:** RNA Ladder comparison between flow rate 0.2 mL/min (black) and 0.5 mL/min (blue) with 5 mM TPAA. Sample injection volume 10 \(\mu\)L, column PLRP-S, column temperature 80°C and gradient 25-100% ACN over 60 min.

### 4.1.5 Temperature Studies

As one of the early established limitations the column temperature was set at 80°C for PLRP-S. Literature expressed the importance of denaturing the RNA molecules to promote chromatographic uniformity and resolution [23]. Reports on negative resolution effects from intra- an intermolecular forces at low temperatures for ssDNA confirmed the theory of conducting separation at an elevated temperature [5].

Later in the thesis project, subsequent to the experimental design, a temperature study was conducted to investigate the effect of on separation more in detail. RNA ladder runs were performed at temperatures in the range of 40, 50, 60, 70, 80 and 90°C on the PLRP-S column (Figure 4.9). The results proved to differ from expectations since separation and resolution, even in the lower temperature range, showed promising results. Fact is that one can argue that the results from low temperature runs is an improvement, for larger RNA molecules (1000-2000 nt), compared to the results from higher temperature runs.
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Figure 4.9: RNA Ladder comparison between column temperature 40°C (black) and 90°C (blue) with 12.5 mM TPAA. Sample injection volume 10 µL, column PLRP-S, and gradient 10.8-70% ACN over 44 min.

4.1.6 Column Equilibration

Working with several IPRs at different concentrations, efforts were made to determine a capable way of reaching column equilibrium while removing residues from previous injections. An efficient way of doing so was to conduct a number of blank injections ahead of each series of injections. Tests revealed that five blank injections fulfilled the requirements on reaching column equilibrium. The equilibration capability were verified continuously for every series of chromatographic runs and five blank runs proved to be effective throughout the project.

4.1.7 The Effect of EDTA

It has been reported in literature that metal ions may interfere with the chromatography, which led to experiments involving EDTA [24]. The compound has the ability to isolate and dismantle possible metal ions, and the probable outcome would have been to witness a slightly improved chromatogram when added to the mobile phases. Addition of EDTA proved to have little effect, only a slight shift in retention time could be observed (Figure 4.10).

4.2 Design of Experiments

The Familiarization Trials had already given an indication of what might be a suitable IPR for separating RNA molecules at sizes ~100-2000 nt. TPAA were early on the candidate for giving the best separation and resolution among the tested IPRs (Figure 4.4). In order to verify this and make a thorough investigation into the sepa-
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Figure 4.10: RNA Ladder comparison between runs with (black) and without (blue) EDTA on column PLRP-S with 20 mM TPAA. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 25-100% ACN over 60 min.

ration and resolution capabilities among TEAA, TPAA and TBAA an experimental design was constructed. Each IPR were evaluated at five concentrations (low, mid low, mid, mid high and high) for three responses, retention time difference between peak 1 and peak 8, retention time for peak 3 and \( w_{1/2} \) for peak 3 (Figure 4.11).

Differences in retention time and concentration intervals made non-obvious statistical comparisons between the IPRs troublesome. Also, for TPAA (Figure A.2) and TBAA (Figure A.3) there were disturbance from a bumpy baseline which interfered with the RNA Ladder response, especially for higher concentrations of TBAA. The behaviour of the baseline had a clear correlation with type of IPR, its concentration and the gradient slope. Experiments during the familiarization trials, with a flattened gradient slope compared to the one used during DOE, showed promising baseline behaviour. Accurate retention times could be obtained regardless of the baseline quality, but peak integration became less reliable. Experiments with TEAA (Figure A.1) resulted in a high quality baseline, but instead showed poor peak separation. In retrospect it would had been beneficial to use a different gradient for TEAA, similar to one of those used during the familiarization trials (Figure 4.1). Because of the different behaviour from the IPRs, much of the focus was shifted from comparing the IPRs against each other to evaluating each IPR separately.

4.2.1 Concentration Intervals and Responses

Knowing that the common concentration of TEAA lies in the range 100-400 mM, there was interest in covering this range while also testing some ambient concentrations. For TPAA some literature states concentrations in the range 5-20 mM and by the same logic as for TEAA there was interest in evaluating ambient concentrations...
The concentration resulting in optimal responses for TBAA was thought to lie slightly lower than TPAA due to increased hydrophobicity, which was supported by literature using concentrations around 2.5 mM [4]. However, the familiarization trials led to improved results for 5 mM compared to 2.5 mM TBAA and the concentration range for TBAA was therefore kept the same as for TPAA, namely 2.5-25 mM.

The responses assessed in the experimental design were the RT difference between peak 1 and peak 8, the RT for peak 3 and width at half peak height ($w_{\frac{1}{2}}$) for peak 3. The reason for inspecting the RT at peak 8 and not peak 10 of the RNA ladder was that the peak resolution for peak 10 in some cases came in conflict with the bumpy baseline or gave too poor response to be identified. Peak 3 was always easy to identify due to its relatively high intensity and gave information on retention time independent on the quality of separation. $w_{\frac{1}{2}}$ provides information on the peak resolution, a response that became difficult to measure due to baseline disturbances.

**Figure 4.11:** Experimental design including concentration intervals and compiled responses for retention time difference between peak 1 and peak 8, retention time for peak 3 and width at half peak height ($w_{\frac{1}{2}}$) for peak 3.
for high concentrations of TBAA, and due to insufficient peak separation for TEAA. Efforts were made to integrate peaks in an as uniform way as possible, resulting in integration of adjacent peaks together with peak 3 for TEAA.

4.2.2 Statistical Evaluation

A summary of fit plot for all three responses (Figure 4.12) display a high model fit, where an R2 below 0.5 would have indicated otherwise. Q2, which is an estimate of future prediction precision, should be greater than 0.1 for a significant model and greater than 0.5 for a good model. This proves to be the case for Delta RT and RT peak 3, but not for \((w_{1/2})\) where Q2 obtains a value of 0.48. The model validity value below 0.25 indicates statistically significant model problems, which could be due to a transformation problem or presence of outliers. Both Delta RT and RT Peak 3 show values below 0.25. All responses prove to have high reproducibility, i.e. the variation of the replicates compared to the overall variability. Also here a value greater than 0.5 is preferred.

![Summary of Fit - Experimental Design (PLS)](image)

**Figure 4.12:** Summary of Fit plot displaying four columns for each response. Columns correspond to R2, Q2, model validity and reproducibility.

The coefficient plots (Figure 4.13) shows coefficients relating to the scaled and centered variables, where the scaling makes the coefficients comparable. Coefficient size represents the change in the response when a factor varies from 0 to 1 and the remaining factors are kept at their averages. A coefficient is said to be significant when the confidence interval does not cross the zero line. TPAA and TBAA can be said to influence a higher value for Delta RT, TBAA also has an affect on RT peak 3. TEAA have a significant negative effect on all responses. Few of the concentrations prove to have have a significant effect.
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Figure 4.13: Coefficient plots with confidence intervals for the three responses and the influence of IPR and concentration.

The Observed vs. Predicted plots (Figure 4.14) indicate how well the model can make predictions. With an optimal model all the points would fall on the 1:1 line. Delta RT and RT peak 3 give a clearer results compared to \( w_{1/2} \) because of the latter ones several outliers.

4.3 Forced Degradation Studies

At the final stage of the project forced degradation studies was initiated. The analyte subject to degradation were not the RNA ladder, but the RNA provided by TriLink Technologies. The objective was to introduce breaks in the ribose-phosphate backbone, something that the developed method is able to detect, rather than more subtle structural changes. Sample concentration was increased five-fold (0.5 mg/mL) compared to the consistently used concentration for the RNA ladder (0.1 mg/mL) in order to further increase the response. Figure 4.15 display a sample of TriLink RNA as it looks untreated and Figure 4.16 display an overview for all assessed degradation treatments, but not all runs, as well as the full chromatographic baselines. The latter figure proves that the method does not only detect larger fragments from the parent molecule, but also small fragments. Note that all experiments in the degradation studies uses 12.5 mM TPAA as IRP and uniform gradient conditions.
Figure 4.14: Observed vs. Predicted plots for the three responses where each dot represents an IPR at a certain concentration.

Figure 4.15: TriLink RNA (blue) together with RNA Ladder (black) with 12.5 mM TPAA. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.

4.3.1 Heat Treatment

Heat degradation experiments for TriLink RNA were conducted at 50°C and 80°C respectively, both treatments lasting 120 minutes. The 50°C treatment showed no
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**Figure 4.16:** Degradation results compiled into one chromatogram for comparison. TriLink RNA treated with heat at 80°C for 120 min (black), 0.3%(w/v) H₂O₂ for 12 hours (blue) 10 mM HCl for 8 hours (green), 10 mM NaOH for 11 hours (teal) and 2ng/mL RNase A for 11 hours (pink). a) representing the full chromatogram and b) representing the region between 11 and 23 minutes. 12.5 mM TPAA, sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.

degradation compared to the untreated sample, while the 80°C treatment (Figure 4.17) demonstrated extensive RNA degradation. The pattern of degradation appears to be mostly random, but diffuse peaks and fluctuations in the baseline suggest some statistical tendency for breaks at certain positions in the TriLink RNA molecule. Es-
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Especially interesting are the two rightmost peaks in the chromatogram which most likely represent the un-degraded parent molecule and some distinct degradation product of high concentration. The fastest eluding of the two could possibly represent cleavage at the 5’-cap or the 3’-poly(A) tail. The results from this method confirm that this method can be used to detect heat degraded RNA.

![Chromatogram](image)

**Figure 4.17:** TriLink RNA heat treated at 80°C for 120 min with 12.5 mM TPAA. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.

4.3.2 Oxidative Treatment

Addition of H$_2$O$_2$ gave relatively modest degradation (Figure 4.18). Three runs at different times were conducted in order to detect any changes over time. The main peaks from the treated samples eluted approximately 10 seconds before that of the untreated sample of TriLink RNA, indicating either that the H$_2$O$_2$ itself has an influence over the separation event or that one or both ends of the molecule is consistently assaulted, losing only a few bases. Fragments in the range of 100-500 bases, presumably originating from nonspecific cleavage, can also be seen after comparisons with the untreated molecule and RNA ladder.

4.3.3 Hydrolytic Treatment

Experiments with 100 mM NaOH and HCl resulted in too extensive degradation to allow for meaningful evaluation. Treatment with 10 mM on the other hand gave relevant results for both NaOH (Figure 4.19) and HCl (Figure 4.20). The effect of alkaline conditions were evaluated at 5, 8 and 11 hours which gave drift towards faster elution times and therefore more extensive fragmentation with elapsed time. The acidic treatment from injections at 2, 5 and 8 hours after sample preparation
Figure 4.18: TriLink RNA treated with 0.3% (w/v) H$_2$O$_2$ and analyzed at 6 (black), 9 (blue) and 12 (green) hours. 12.5 mM TPAA, sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.

led to much the same conclusions as for NaOH, the developed method is capable of detecting major degradation patterns after hydrolytic sample treatment.

Figure 4.19: TriLink RNA treated with 10 mM NaOH and analyzed at 5 (black), 8 (blue) and 11 (green) hours. 12.5 mM TPAA, sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.
Figure 4.20: TriLink RNA treated with 10 mM HCl and analyzed at 2 (black), 5 (blue) and 8 (green) hours. 12.5 mM TPAA, sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.

4.3.4 RNase A Treatment

The concentration of RNase at 2 ng/mL lies significantly below the, by Thermo Fisher Scientific, recommended concentration at 1-100 µg/µL. The purpose for which these concentrations are recommended for are however to remove RNA contamination from DNA samples, something that requires high amounts of RNase in order to be sufficiently efficient. RNA degradation experiments at AstraZeneca similar to the ones conducted within this thesis project has used 20 ng/mL, which led to extensive fragmentation. Consequently it was decided to make experiments at an even lower concentration of RNase A. The results from chromatographic runs at 5, 8 and 11 hours after sample preparation showed a comparatively modest degradation (Figure 4.21). It is however clear that the used method is able to detect RNase degradation even at concentrations 500-50 000 times lower than above mentioned recommendation. The results also serves as an indication of how potent RNases can be when acting as contaminants in a RNA environment.
Figure 4.21: TriLink RNA treated with 2ng/mL RNase A and analyzed at 5 (black), 8 (blue) and 11 (green) hours. 12.5 mM TPAA, sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10-54% ACN over 45 min.
4. Results and Discussion
Literature studies complimented by familiarization trials enabled for early conclusions to be drawn. Because of the extent to which TEAA is used as an IPR in oligonucleotide analysis it was though that this IPR would be the main candidate for further studies. This was not the case, instead TPAA proved to be the most promising IPR to be utilized for degradation studies. It is possible however that the experimental conditions did not favour TEAA in the same way as for TPAA. In order to improve separation resolution for TEAA the foremost measure would be to optimize a different gradient.

The choice of column relied on the expertise of the column manufacturers. All three columns evaluated during the course of this project showed promising results for separating RNA. PLRP-S proved however to be the superior column in this particular project. With that being said, under different circumstances the two remaining columns might have shown improved separation results. With more time it safe to say that effort should be invested in assessing the remaining columns by optimizing parameters differently, especially the AdvanceBio column which demonstrated promising results also for larger molecules.

The fact that the project has assessed numerous parameters and conditions made the evaluations complex, and even thou it would have been desirable to look at as many situations as possible, it would have increased the complexity exponentially. The DOE gave foremost information on TPAA and TBAAs ability to facilitate a high delta RT. Poor peak separation for TEAA and, in some cases, an interfering baseline for TBAA resulted in difficulties in evaluating some responses for the two. Given the circumstances, all three IPRs showed significant effects for at least one response and TPAA at 12.5 mM came to be the IPR and concentration used in the degradation studies.

All four categories of degradation treatment resulted in unquestionable fragmentation, proving the fragility of RNA and the methods competence in detecting such fragmentation. Both heat and hydrolytic treatment gave extensive degradation while oxidative and RNase treatment gave a comparatively modest, but clearly visible degradation at the tested conditions.

Succeeding research in the area could very well extend the framework which constitute this project. Results indicate that there are IPRs better suited than TEAA for separation of RNA $\geq 500$ nt. Further optimization of key parameters such as flow rate, choice column, temperature and gradient together with TPAA as an IPR would logically yield even more promising results.
5. Conclusion
Bibliography


Figure A.1: RNA Ladder on column PLRPS from the DOE, TEAA_{mid high} (250 mM TEAA), where a) represent the full and b) represent a zoomed in region at RNA ladder elution. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 0.5-50% ACN over 36.8 min.
Figure A.2: RNA Ladder on column PLRPS from the DOE, TPAA$_{\text{mid high}}$ (12.5 mM TPAA), where a) represent the full and b) represent a zoomed in region at RNA ladder elution. sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10.8-70% ACN over 44 min.
Figure A.3: RNA Ladder on column PLRPS from the DOE, TBAA<sub>mid high</sub> (12.5 mM TBAA), where a) represent the full and b) represent a zoomed in region at RNA ladder elution. Sample injection volume 10 µL, column temperature 80°C, flow rate 0.2 mL/min and gradient 10.8-70% ACN over 44 min.