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Optimization of High-Performance Liquid Chromatography Parameters for Purification of Oligonucleotide-A

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Abstract

The modification of high-performance liquid chromatography parameters leads to a more effective oligonucleotide-A purification process. Using various experimental parameters such as buffer, concentration, and pH, a method for optimizing the purification of an oligonucleotide-A on a reverse-phase C18 column was created. To purify oligonucleotide-A, High-Performance Liquid Chromatography (HPLC), Ultraviolet-Visible Spectrophotometry (UV-Vis), Liquid Chromatography-Mass Spectrometry (LC-MS), and lyophilization were used. Chromatographic data were collected with a semi-prep HPLC system, quantified with the UV-Vis technique, and validated with the LC-MS method. The most optimized parameters found to obtain the purity of 93.0% are 40 mM triethylammonium bicarbonate (TEAB) buffer with pH 7, which is approximately 6.0% higher than the reported method of which the purity is 87.0%. However, the yield under these conditions was reduced by about 5%. The worst possible optimized settings that resulted in the lowest purity (84.0%) and yield (69.0%) are 10 mM ammonium acetate ($NH_4CH_3CO_2$) with pH 7.

Keywords

Purification, High-Performance Liquid Chromatography, Oligonucleotide-A, Dipotassium Phosphate, Triethylammonium Acetate, Triethylammonium Bicarbonate, Ammonium Acetate

1. Introduction

Synthetic oligonucleotides have evolved into important biomolecules with a wide range of applications. Purification and modification of synthetic oligonucleotides are critical technologies for a wide range of applications in the life sciences [1].

Synthetic oligonucleotides have been used in DNA sequencing as hybridization probes, linkers, and primers, as well as in amplification procedures and antisense therapies [2]. One of the most serious issues with synthetic oligonucleotides is impurity, which can result from poor synthesis, de-protected products, short sequences, and/or changed sequences [3] [4]. These contaminants can jeopardize an experiment or assay in some cases by competing with full-length products or inhibiting reactions [5].

Oligonucleotide purification approaches (optimizing pH, concentration, mobile phase, and chain) have been developed to increasing functionalization in a variety of genomic assays and pharmacological treatments [6]. The most common purification processes are Thin-Layer Chromatography (TLC), purification cartridges, Polyacrylamide Gel Electrophoresis (PAGE) gels, desalting, and High-Performance Liquid Chromatography (HPLC). As laboratories strive for greater efficiency, a focus on increasing yields and shortening purification times has grown in importance [7]. Several parameters, including the application, oligonucleotide length, and oligonucleotide alterations, determine the best approach [8] [9]. HPLC is one of the purification methods that can be used on a large scale to separate oligonucleotides. Among the many HPLC procedures available are Reverse-Phase (RP), Normal-Phase (NP), Hydrophilic Interaction (HI), Ion-Exchange (IE), and Size-Exclusion (SE). All of these strategies apply to the stationary phase of the system.

To fully comprehend the retention mechanism influencing a separation, the chemistry of the bonded phase, the nature of the silica surface treatment, and the surface accessibility must all be considered and classified, which will then influence the initial column selection or method optimization [10] [11] [12]. HPLC parameters have an effect on the chromatogram, which is the final product of the HPLC experiment. The detector response is a time-dependent function of the chromatogram [13]. In HPLC, the resolution of the chromatogram is the most important factor. The goal is to achieve the best possible resolution in the shortest possible time. A resolution of 1.5 or higher ensures that the sample components are well separated enough to determine the area under the curve correctly [14].

When developing HPLC procedures, the first approach frequently fails to separate key components of a mixture. To achieve the required separations, the mobile phase, stationary phase, and column temperature can all be adjusted [15]. Changing the particle size of the column is another useful parameter. Greater plate numbers are produced by smaller particle sizes, resulting in sharper peaks and the ability to resolve closely eluting peaks [16]. Some components, such as proteins and oligonucleotides, may be difficult to separate on smaller columns but can be resolved fast on a larger column [17]. Other factors that determine chromatogram resolution include selectivity, efficiency, and retention, which are regulated by HLPC parameters (solvent strength, pH, column material, and temperature) [18].

This paper provides a thorough assessment of oligonucleotide-A purification

in terms of purity and yield. HPLC parameters such as buffer, concentration, and pH were used to find the best purification procedure.

2. Experimental

2.1. Materials

The sample in all separation experiments was a 30-mer oligonucleotide-A. The crude oligonucleotide-A obtained from IDT (USA) had a molecular weight of 9513. Reagent grade 1 M Tris HCl, acetonitrile, phosphate-buffered saline (PBS), and NaCl were supplied by VWR (USA). The on-site MilliQ system provided deionized water for the research (NANOPure). The chromatography stationary phase for the initial research was a Dionex DNAPac PA200 oligonucleotide column (4 × 250 mm). Before purification, all samples were examined on an Agilent 1260 analytical HPLC system. A 500 nmol of stock oligonucleotide-A was diluted to 1 mM in 10× PBS. To attain incoming purity, one nanomole of oligonucleotide-A was run on the PA200 column. After running the sample through the HPLC system, a mass was recorded using an Agilent 6100 series SQ system to confirm the product. The initial parameters in Table 1 show the analysis method used to record initial purity. The purity was documented, and the sample was readied for purification.

2.2. Initial Control

The preparative chromatographic studies were carried out on a ChemStation Work Station that was linked to an Agilent 1260 semi-prep HPLC system equipped with a diode array detector. Table 2 shows the experimental parameters that are being modified and tested. Each variant change was tested three times, with the given result being the average of the three runs. The first stage in the optimization process was to replicate the results provided in the literature, which showed that HPLC employing potassium phosphate buffer at pH 7 and acetonitrile yielded 88.0% purity and 80.00% recovery [1]. For peaks greater than 300 mAU, a shallow gradient was applied to an XTERRA C18 column (19 × 100 mm), and fraction collection was utilized.

Table 1. Initial IEC analysis gradient.

Time, min	K_2HPO_4%: 100 mM Tris HCl + 25% Acetontrile	TEAB %: 100 mM Tris HCl + 25% Acetonitrile + 1.25 M NaCl
0	100	0
30	50	50
31	50	50
33	100	0
36	100	0

Table 2. Testing parameters for each experiment.

Constant	Constant	Variant	Variant	Variant	Variant	Variant
K_2HPO_4	10 mM	pH 5	pH 6	pH 7	pH 8	pH 9
K_2HPO_4	20 mM	pH 5	pH 6	pH 7	pH 8	pH 9
TEAB	40 mM	pH 5	pH 6	pH 7	pH 8	pH 9
TEAB	pH 7	1 mM	10 mM	20 mM	30 mM	40 mM
K_2PO_4	pH 7	1 mM	10 mM	20 mM	30 mM	40 mM
10 mM	pH 7	K_2PO_4	TEAA	TEAB	$NH_4CH_3CO_2$	
TEAB	pH 7, 40 mM	Oligo-20	Oligo-25	Oligo-30	Oligo-35	Oligo-40

Note: Each variant was run three times, the average of the three is reported in this work.

2.3. Rotary Evaporation

In-process samples from potassium phosphate buffer preparative runs at pH 5 to 9 were kept in the refrigerator at 4˚C. After that, the samples were concentrated using a Heidolph Precision rotary evaporator. The bath temperature was set at 30˚C, and the rotation speed was set to 100 rpm. The pressure was manually controlled, and it took roughly 75 minutes for one sample to evaporate from 40 mL to 2 mL.

2.4. Lyophilization

A FreeZone Freeze Dry System was used to lyophilize the material. The leftover sample is frozen using dry ice and isopropanol alcohol after each sample has been evaporated close to dryness, 2 mL. This procedure takes approximately 5 minutes. The sample is placed on the Freeze Dry System overnight when it has totally frozen. The material was returned to powder form the following morning. After that, the sample is reconstituted in 10× PBS buffer.

2.5. Quantification Using Spectrophotometer

After the material has been restored to liquid form, it is quantified. The quantification is done using a Biotek Synergy H1 spectrophotometer with the extinction coefficient of the oligonucleotide-A provided by IDT. The coefficient of extinction was 363,400·M^{-1}·cm^{-1}. The following calculation was used to determine concentration:

$$\text{Concentration in uM} = \left(\left(\frac{\text{Absorbance} * \text{Dilution Factor}}{\text{Extinction. Coefficient}} \right) \Big/ t \right) * 1{,}000{,}000$$

2.6. Reanalysis

The final step in the procedure is reanalysis on an Agilent 1260 analytical HPLC system and verification on an Agilent 6100 Series SQ LC/MS system.

3. Result and Discussion

Table 2 shows the experiment designed to investigate this work. Buffer type, buffer concentration, and buffer pH are factors that affecting the purification of oligonucleotide-A. Keeping two factors constant and changing either buffer type, buffer concentration, or buffer pH, purity and yield were determined.

3.1. pH Optimization

Table 3 lists all % purity values and yields with different pH values (5 to 9) at constant 10 mM K_2HPO_4, 20 mM K_2HPO_4, and 40 mM TEAB. Figure 1(a) compares the percent purity and yield of K_2HPO_4 at 10 mM, Figure 1(b) for K_2HPO_4 at 20 mM, and Figure 1(c) for TEAB at 40 mM, with pH values ranging from 5 to 9.

When the concentration of K_2HPO_4 was kept constant at 10 mM and pH 7, the purity was 87.5% and the yield was 79.0%, which stayed at the optimum level. However, lowering the pH results in a decrease in purity and yield. The purity of the sample was 86.0% at pH 6 and 85.9% at pH 5, with yields of 78.8% and 78.0%. This value is 1% - 2% lower than pH 7. The purity was 86.9% and 87.0%, respectively, and the yield was 79.1% and 78.9% at higher pH 8 and 9 but did not produce a greater purity and yield at this pH.

Table 3. Comparison of purity and yield with different pH values for Oligonucleotide-A at constant 10 mM and 20 mM concentrations of K_2HPO_4 and constant 40 mM concentration of TEAB with pH ranging from 5 to 9.

pH	Buffer	Concentration, mM	Purity, %	Yield, %
5	K_2HPO_4	10	85.9 ± 0.2	78.0
	K_2HPO_4	20	86.3 ± 0.2	78.1
	TEAB	40	93.0 ± 0.3	75.1
6	K_2HPO_4	10	86.0 ± 0.1	78.8
	K_2HPO_4	20	86.7 ± 0.2	77.9
	TEAB	40	93.7 ± 0.3	75.7
7	K_2HPO_4	10	87.5 ± 0.5	79.0
	K_2HPO_4	20	87.7 ± 0.2	77.9
	TEAB	40	93.9 ± 0.1	74.5
8	K_2HPO_4	10	86.9 ± 0.3	79.1
	K_2HPO_4	20	87.4 ± 0.3	77.8
	TEAB	40	93.3 ± 0.1	74.5
9	K_2HPO_4	10	87.0 ± 0.1	78.9
	K_2HPO_4	20	87.3 ± 0.1	77.5
	TEAB	40	93.1 ± 0.1	74.1

Note: Each value is obtained based on the average of three runs.

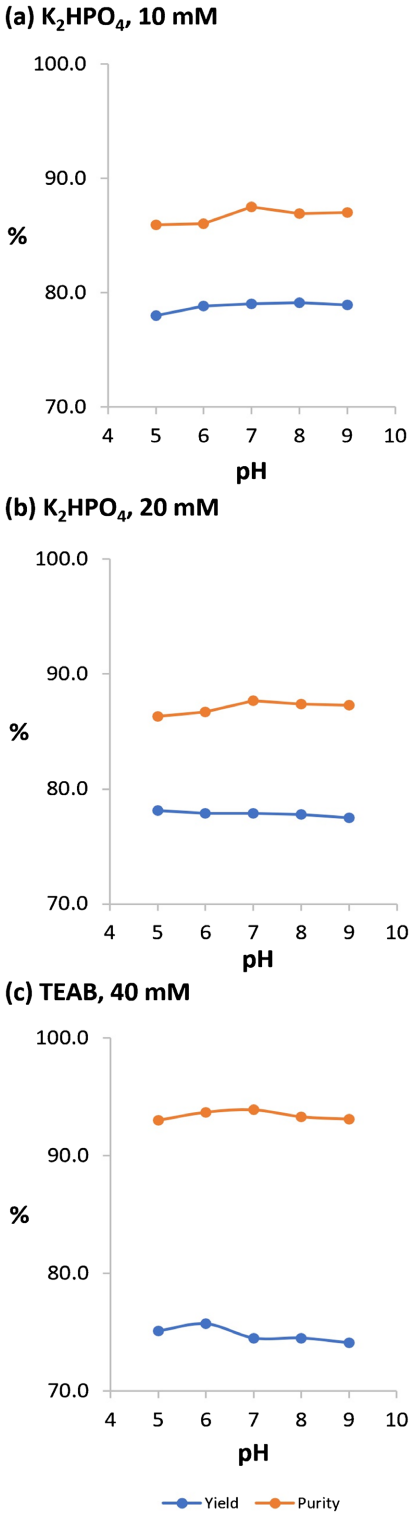

Figure 1. Comparison of the purity and yield percentages of K_2HPO_4 (10 and 20 mM) and TEAB (40 mM) with pH ranging from 5 to 9.

Changing the concentration of K_2HPO_4 from 10 to 20 mM and then varying the pH from 5 to 9 resulted in no improvement in oligonucleotide-A purification at a constant 20 mM concentration of K_2HPO_4. The pH 7 sustained optimum

values reveal the same purity and yield as 10mM in the previous method as 87.7% and 77.9%, respectively. The overall purity at pH 6 and 5 was 86.7% and 86.3%, respectively, in the experiment, although the overall yield was 77.9% and 78.1%.

Yield was shown to decrease by 1.6 percent when the pH climbed from pH 5 to pH 9. The purity of oligonucleotide-A increased by 0.2 percent at 20 mM concentration and pH 7 compared to 10 mM concentration and pH 7. Yield, on the other hand, fell by 0.5 percent.

When the dipotassium phosphate buffer pH variation was compared, the results from the 20 mM concentration studies were all slightly higher than the results from the 10 mM concentration experiments. There was a 0.2% - 0.7% higher in purity at 20 mM K_2HPO_4 across pH 5 - pH 9. However, increased K_2HPO_4 concentrations resulted in a 0.2% - 1.4% decline in output.

When the buffer was changed from K_2HPO_4 to TEAB at 40 mM, the pH had a significant impact on the results. The purity and yield (93.3% and 74.5%, respectively) at pH 8 were equivalent to pH 9 (93.15% and 74.1%) at the same concentration; however, when compared to K_2HPO_4 at 10 mM, the purity increased by 6.1% while the yield decreased by 4.8%. When compared to K_2HPO_4 at 20 mM, the purity increased for TEAB at pH 8 and 9, but the yield was somewhat reduced.

When pH 5 and 6 were used with TEAB, the yields (93.0% for pH 5 and 93.7% for pH 6) were significantly higher (7.1 to 7.8%) than when pH 5 and 6 were used with K_2HPO_4. However, at the same pH levels, the yields were roughly 2.0% lower than with K_2HPO_4. TEAB at 40 mM and pH 7 produced the highest purity (93.9%); nonetheless, the percent yield is comparable to other pH values of the same buffer.

The standard deviation was calculated in these tests to quantify the amount of variation across all three trials at each pH level. All results were considered low, with the maximum standard deviation in the data set being 0.36 at pH 8 with 10 mM K_2HPO_4. The data set's lowest standard deviation was 0.10, which was observed at pH 8 and pH 9.

3.2. Concentration Optimizations

According to the data in **Table 3**, at pH 7, both buffers (K_2HPO_4 and TEAB) produce the best outcomes when compared to the same concentration at other pH levels. This section explains the findings obtained when the buffer concentration varies from 1 to 40 mM while maintaining a constant pH of 7 (see **Table 4** and **Figure 2**). At 1 mM concentration, K_2HPO_4 produced higher purity than TEAB (90.8% vs. 86.1%). TEAB, on the other hand, outperformed K_2HPO_4 by 5.4% in yield. At 10 mM concentration, TEAB produced 5.1% more purity than K_2HPO_4; nevertheless, TEAB produced 4.1% less yield than K_2HPO_4. Buffer K_2HPO_4 had around 5.1% less purity than TEAB at 20 mM concentration but had 2.8% higher yield. At 30 mM, TEAB has 6.0% higher purity than K_2HPO_4 and just 0.6% lower yield. Buffer K_2HPO_4 had 5.7% less purity than TEAB and 1.6% higher yield than TEAB at 40 mM. There were no significant variations in

Table 4. Comparison of purity and yield for Oligonucleotide-A with different concentration values at a constant pH of 7 for K_2HPO_4 and TEAB.

pH	Concentration, mM	Buffer	Purity, %	Yield, %
7	1	K_2HPO_4	86.1 ± 0.9	79.8
		TEAB	90.8 ± 0.1	74.4
7	10	K_2HPO_4	87.5 ± 0.4	79.0
		TEAB	92.6 ± 0.1	74.9
7	20	K_2HPO_4	87.7 ± 0.3	77.9
		TEAB	92.6 ± 0.2	75.1
7	30	K_2HPO_4	87.1 ± 0.1	77.5
		TEAB	93.1 ± 0.1	74.9
7	40	K_2HPO_4	86.9 ± 0.2	76.1
		TEAB	93.3 ± 0.1	74.5

Note: Each value is obtained based on the average of three runs.

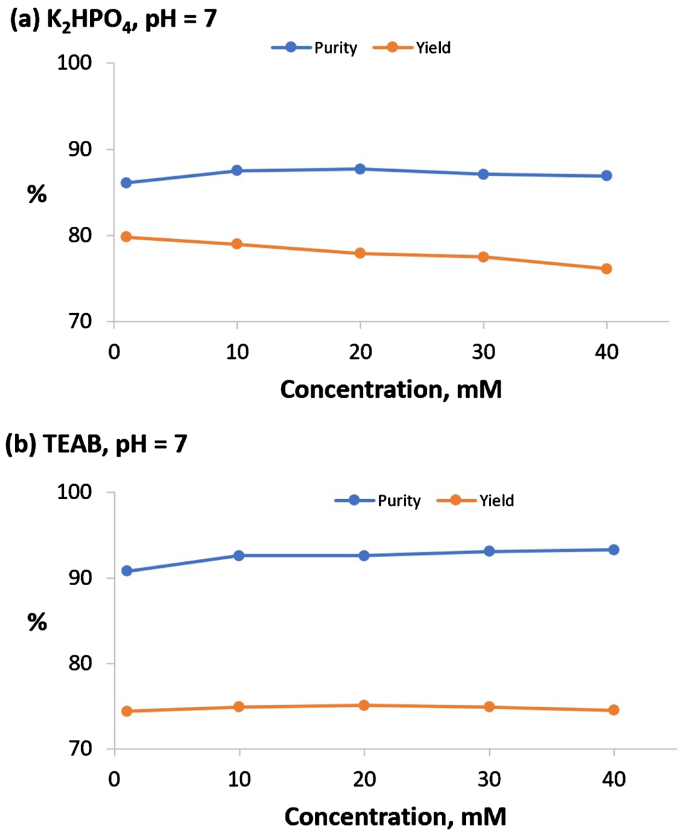

Figure 2. Comparison of the purity and yield percentages of K_2HPO_4 and TEAB with a constant pH of 7 and concentrations ranging from 1 to 40 mM.

purity or yield during pH tuning. However, increasing the concentration of K_2HPO_4 and TEAB had a greater impact on purity and yield. The drop-in yield during the pH experiment was not as extreme as the concentration decrease, but the purity

increased dramatically.

The standard deviation was calculated based on all three experiments at each concentration level to measure the degree of variation. All concentrations were considered modest, although the data set's biggest standard deviation was at 1 mM K_2HPO_4. With a standard deviation of 0.96, this experiment had the largest standard deviation of all the experiments. The data set's lowest standard deviation was 0.10, which was observed at 1 mM and 10 mM for TEAB.

3.3. Mobile Phase Optimization

To optimize oligonucleotide-A purification, two additional buffers, TEAA and $NH_4CH_3CO_2$, were used in this study, and the experiment was performed at a constant pH of 7 and a constant concentration of 10 mM. The most change was generated by changing the nature of the buffer. Table 5 and Figure 3 show that TEAB had the highest purity at 92.6% (yield, 74.9%), followed by TEAA at 90.6% (yield, 72.1%), K_2HPO_4 at 87.5% (yield, 79.0%), and $NH_4CH_3CO_2$ at 84.3% (yield, 69.9%). According to the published literature [1], 88.0% purity and 80.0% yield were obtained by HPLC using K_2HPO_4 buffer at pH 7 (10 mM), while in this experiment, K_2HPO_4 purity was 87.5% and yield was 79.0%. There was a 5 -

Table 5. Comparison of purity and yield with different buffers at constant pH 7.

pH	Concentration, mM	Buffer	Purity, %	Yield, %
7	10	K_2HPO_4	87.5 ± 0.4	79.0
7	10	TEAB	92.6 ± 0.1	74.9
7	10	TEAA	90.6 ± 0.	72.1
7	10	$NH_4CH_3CO_2$	84.3 ± 0.5	69.9

Note: Each value is obtained based on the average of three runs.

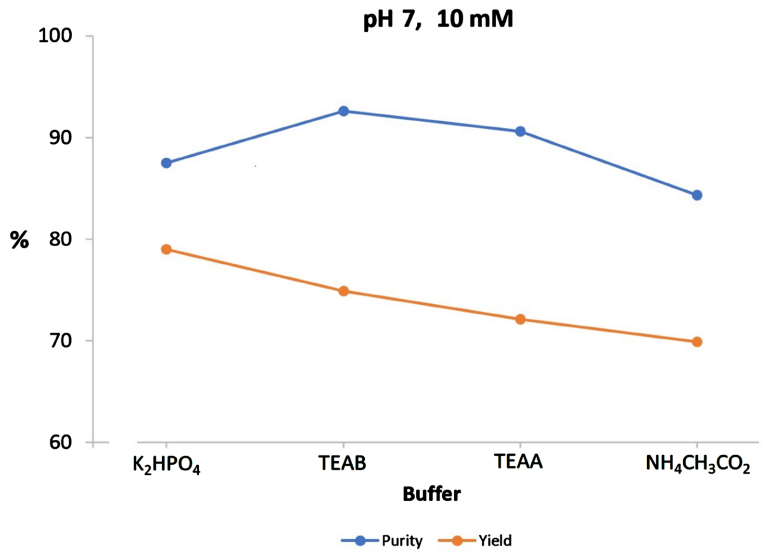

Figure 3. Comparison of the purity and yield percentages of four different buffers at constant pH 7 and concentration of 10 mM.

6 percent higher from the earlier reported approach when TEAB was used instead of K_2HPO_4, and a 2.6 percent increase when TEAA was used instead of potassium phosphate. In all situations, the yield was reduced. The use of $NH_4CH_3CO_2$ reduced yield by 10.1%. TEAA reduced production by 7.9%, TEAB by 5.1%, and potassium phosphate by 1%. TEAB has the highest purity and yield of the four buffers tested; the purity is 5.4% greater than previously reported data, but the yield is lower. TEAB has the largest concentration, followed by TEAA and $NH_4CH_3CO_2$.

The standard deviation for the mobile phase experiment change was recorded, and the findings are shown in Table 5. The data set's largest standard deviation was 0.66 at 10 mM TEAA. The data set's lowest standard deviation was 0.10, which was seen at 10 mM TEAB buffer.

3.4. Optimization in Oligonucleotide-A Size

With purity improved from 88 to 94 percent, further investigation into expanding the study to different oligonucleotide-A chain lengths was pursued. Chain lengths ranged from 20 to 40 oligonucleotide-A. The same optimized procedure was used to treat these chains with TEAB at a constant pH of 7 and a concentration of 40 mM. Table 6 and Figure 4 summarize the outcomes of these experiments. In

Table 6. Comparison of purity and yield with different Oligonucleotide lengths with constant pH 7, concentration of 40 mM, and buffer TEAB.

Length	Buffer	pH	Concentration, mM	Purity, %	Yield, %
Oligonucleotide-20	TEAB	7	40	92.6 ± 0.2	76.3
Oligonucleotide-25	TEAB	7	40	93.3 ± 0.4	76.1
Oligonucleotide-30	TEAB	7	40	93.7 ± 0.1	75.7
Oligonucleotide-35	TEAB	7	40	93.9 ± 0.3	74.5
Oligonucleotide-40	TEAB	7	40	94.0 ± 0.2	74.8

Note: Each value is obtained based on the average of three runs.

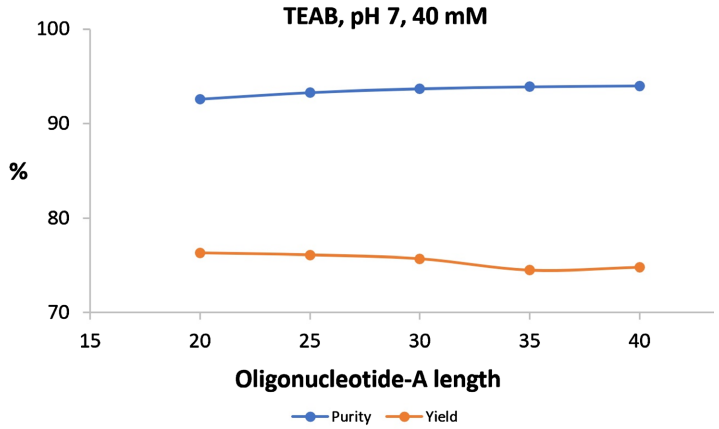

Figure 4. Comparison of purity and yield of Oligonucleotide-A lengths range from 20 to 40 with constant pH 7, concentration of 40 mM, and buffer TEAB.

chains longer than or equivalent to 30 oligonucleotide-A, the approach proved sufficient. Purity was found to be larger than the figure stated in the literature. The optimized process achieved a purity of 94% and a yield of 75%.

4. Conclusion

The proposed approach for purifying oligonucleotide-A by HPLC was rapid, accurate, and precise. This method is based on the usage of a basic working procedure for an oligonucleotide-A chain that was tuned among several parameters based on purity and yield. The process was evaluated for oligonucleotides-A ranging from 25 - 40 nucleotides in length, utilizing HPLC capabilities in the laboratory, followed by rotary evaporation and lyophilization, and products were produced with a purity of 92 - 94 percent and a yield of 75 - 79 percent. Researchers can choose the best buffer and pH for their work based on the application and sensitivity of the work.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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