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Separation and identification of oligonucleotides impurities and degradation products by reversed phase ultra-high performance liquid chromatography using phenyl-bonded stationary phases without ion pairs - A step towards sustainability

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ABSTRACT

This manuscript discusses the development of a reversed-phase ultra high-performance liquid chromatography (RP UHPLC) method based on phenyl-bonded stationary phases without ion-pairs for the separation and identification of oligonucleotides. The elimination of ion-pair reagents makes the proposed protocol as more compliant to the principles of green chemistry, compared to the traditional ion-pair reversed-phase liquid chromatography methods (IP RP LC). In detail, three phenyl-based stationary phases were tested, namely a C18/AR (a C18 stationary phase with the addition of aromatic groups), a Phenyl-hexyl, and a Diphenyl. Generally, the retention of oligonucleotides increases with the increase of salt concentration and the decrease of the pH, thus confirming the significant impact of van der Waals interactions, salting-out effect, and π-electrons interactions in the retention mechanism. The highest retention and best peak symmetry were observed for the C18/AR stationary phase, while the lowest retention for the Phenyl-hexyl, with retention influenced by the type of salt in the mobile phase. The obtained methods using C18/AR stationary phases allow for the effective separations of positional isomers and for identifying impurities and degradation products using RP UHPLC Q-TOF-MS in a comparatively short time. The application of RP UHPLC Q-TOF-MS provides reasonable selectivity for the resolution of 33 impurities and two degradation products. Both groups of compounds are mainly 3′N and 5′N-shortmers, but in the case of impurities, modifications of cyclic phosphate and phosphate groups were also identified. Nevertheless, Diphenyl and Phenyl-Hexyl may be applied to separate modified oligonucleotides with higher salt concentrations. The proposed separation methods without ion-pair reagents contribute to a more sustainable approach in oligonucleotide analysis.

1. Introduction

The most common method for oligonucleotide analysis is ion pair reversed-phase liquid chromatography (IP RP LC). Non-polar octadecyl stationary phases are the most popular [1–5]. As ion-pair reagents, amines with a higher number of carbon atoms (6 or more) are usually exploited [6,7], with evident problems concerning their biodegradability. Additionally, alkylamines mostly exhibit toxic properties (toxic upon inhalation, ingestion, or absorption through the skin). For example, commonly used hexylamine can be severely toxic or even fatal if swallowed or comes in contact with the skin [8]. Moreover, acidic modifiers are added to mobile phases containing alkylamines IP RP HPLC. These are acetate, bicarbonate, formic acids, and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). HFIP is toxic and hazardous to the environment [9,10]. Nevertheless, these substances offer excellent properties in terms of the obtained resolution of separation and sensitivity of detection in mass spectrometry (MS). However, its application limits the effective lifetime of the chromatographic column and causes MS contamination [3,9–11].

Phenyl-bonded stationary phases may be an interesting alternative to octadecyl stationary phases for separating oligonucleotides. The presence of an aromatic ring offers a different selectivity compared to C18

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material. In recent years, stationary phases for liquid chromatography have been developed, containing aromatic systems. Beyond the typical phenyl-hexyl and phenyl-propyl phases, biphenyl and diphenyl phases have become commercially available [12,13]. Phases with a C18 chain terminated by a phenyl ring or material containing both C18 groups and ligands with a phenyl group also seem interesting [14]. Among various applications, these materials can be used for the separation of ion-pair oligonucleotides [15–17].

The aryl and pentafluorophenyl stationary phases were already used in oligonucleotides separation by IP RP LC [11,15,18,19]. Usually, the retention at the surface of aryl stationary phases is higher compared to octadecyl one, due to the electron-rich aromatic ring [18–20]. The application of pentafluorophenyl stationary phase provides lower retention of oligonucleotides because of electronegative fluorine atoms. Nevertheless, the successful separation of sequence isomers for phenyl and pentafluorophenyl stationary phases was shown [18], resolution of antisense oligonucleotides mixture with different types of modifications for pentafluorophenyl [11], and antisense oligonucleotides metabolites for aryl [18]. Oligonucleotides were also analysed with similar aryl groups bonded to long octadecyl groups but in the RP LC [19]. Greater retention was noticed for C18 with terminal phenyl group, and therefore it was used for the separation of different mixtures: modified, unmodified of various lengths or sequences [7,19]. The polybutylenterephthalate-bonded silica-based stationary phase was also applied in RP LC for the separation of a high number of impurities of siRNA Patisiran. It provided sufficient retention and adequate selectivity without any presence of ion-pairing reagents, but results depended on the type, concentration, and pH of salt in the mobile, as well as on the type of organic solvent [21,22].

Moreover, the effect of the spacer between the aryl group and silica surface was tested for phenoxy-propyl, phenyl-propyl, and phenyl-hexyl stationary phases in the IP RP LC [15]. The orientation of the phenyl ring is perpendicular when the spacer contains an even number of carbon atoms promoting π-π interactions, contrary to the uneven number of carbon atoms (parallel orientation of aryl group) [15]. Changes in the conformation of aryl groups are reflected in the retention of oligonucleotides with different lengths (retained mainly by π-π interactions) and sequences (retained mainly by electrostatic and hydrophobic interactions) in IP RP LC [15].

Despite the undoubted advantages of the ion-pair system for separating oligonucleotides according to selectivity, a disadvantage of ion-pair usage is MS signal suppression [23]. Additionally, this approach is rather at odds with the principles of green chemistry and sustainable design [8]. Green analytical methods have become increasingly popular in recent years, thus suggesting its application also to oligonucleotide analysis. Hence, the research for stationary phases that allow efficient separation of oligonucleotides without the addition of ion-pair reagents or large volumes of acetonitrile (as in the case of hydrophilic interaction liquid chromatography to bring oligonucleotide analytics closer to the principles of green chemistry) is highly demanding.

Based on the above-mentioned assumptions, the main aim of this work was to develop a robust and selective reversed-phase ultra high-performance liquid chromatography (RP UHPLC) method based on phenyl-based stationary phases for separating and qualifying oligonucleotides impurities without ion-pair reagents addition to the mobile phase, thus promoting sustainable separation methods. Three non-polar, phenyl-based stationary phases were selected for the study. Deoxyribooligonucleotides and ribooligonucleotides were used, both purified after their synthesis, as well as non-purified ones. The comprehensive retention studies were preliminary performed to identify the LC conditions suitable for the analysis of oligonucleotides. The resolution of impurities was additionally investigated, implementing the method in a RP UHPLC Q-TOF-MS system. The novelty is utilizing the monodisperse material to drive high resolution of oligonucleotide mixtures while further exploring the pi-stacking mechanism utilizing different phenyl stationary phases in RP LC. Monodisperse material was applied for the first time in oligonucleotide analysis since it should be more polar retentive due to possibly higher surface area than polydisperse commercially available materials.

2. Materials and methods

2.1. Materials and reagents

Acetonitrile, methanol, and water (LC-MS grade) were supplied by Merck (Warsaw, Poland). Ammonium formate (purity > 99.995 %), ammonium acetate (purity ≥ 99 %), ammonium bicarbonate (purity ≥ 99 %), 25 % ammonium hydroxide solution, formic and acetic acids (purity > 95 %) were purchased from Sigma-Aldrich (St. Louis, USA). Deionized water was purified with the Milli-Q system (Millipore, El Passo, TX, USA).

Unmodified and phosphorothioate ribooligonucleotides standards were purchased from Sigma Aldrich (Dorset, UK). 2′-O-(2-methoxyethyl) (MOE) and 2′-O-methyl (ME) oligonucleotides were obtained from Eurogentec (Seraing, Belgium). All the oligonucleotides were provided in lyophilized form and dissolved in deionized water to 25 μM, except for OL11 (dissolved to 100 μM). Some were purified using RP LC (by producers), while the others were delivered as nonpurified (desalted) compounds (OL12). Additionally, aqueous solutions of 2′-O-methyl oligonucleotide OL13 were frozen and thawed 10 times to degrade oligonucleotides. OL14, on the other hand, was degraded by adding hydrochloric acid to the sample. The sequences and molecular masses are reported in Table 1, together with information about the type of purification or degradation.

2.2. LC-MS instrumentation and columns

Preliminary retention chromatographic experiments were performed using a Thermo Scientific™ Vanquish™ Horizon ultra-high-performance liquid chromatography (UHPLC) system with a DAD-diode array detector (Thermo). Chromeleon 7 chromatography data system was used for data acquisition and integration. For the separation and identification of oligonucleotides impurities, a 1260 Infinity Quaternary System (Agilent, Waldbronn, Germany) high-performance liquid chromatography (UHPLC) system with a binary pump, vacuum chambered microdegasser, thermostatically controlled autosampler, column compartment, was used. An Agilent 6540 UHD Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Waldbronn, Germany) with electrospray ionization was coupled. Data were collected using Agilent Mass Hunter Software (version B.04.01).

Three stationary phases were tested during the study: octadecyl with aryl group (Evosphere C18/AR), phenyl (Evosphere Phenyl-Hexyl), and diphenyl (Evosphere Diphenyl). They were produced by Fortis Technologies Ltd and provided by Mac-Mod (Pennsylvania, USA). The particle size of stationary phases was equal 1.7 μm (monodisperse), while the dimensions of the columns were 2.1 × 100 mm. Characteristics of tested columns, as well as structures of chemically bonded phases are reported in Table S1 and in Fig. S1, respectively.

2.3. Chromatographic conditions

The retention studies were carried out using mobile phases containing mixtures of methanol (MeOH) or acetonitrile (ACN) with proper salt: ammonium formate, ammonium acetate, and ammonium bicarbonate. Different salt concentrations (5–50 mM) and pH (4.5–7.5) were tested. The pH of salt in the mobile phase was corrected using formic acid, acetic acid, or ammonia solution. During this step of investigations, a gradient elution mode was applied following two different programs: 1. 10–30 % v/v MeOH in 10 min for unmodified oligonucleotides; 2. 15–50 % v/v MeOH in 10 min for modified oligonucleotides. The other chromatographic parameters were as follows: flow rate 0.3 ml/min; column temperature 30 °C or 60 °C; autosampler temperature 4 °C; UV

Table 1
Sequences, molecular masses, modification type and purification type of all the oligonucleotides used in the study.

Shortcut	Sequence (5′-3′)	Modification type	Molecular mass [Da]	Purification type
SEQUENCE ISOMERS				
OL1	AAAAAAAAAAAAAAAAAA	unmodified	5573.075	RP HPLC
OL2	ATCGATCGATCGATCGATC**A**	unmodified	6098.055	RP HPLC
OL3	ATCGATCGATCGATCGATC**G**	unmodified	6114.050	RP HPLC
OL4	ATCGATCGATCGATCGATC**C**	unmodified	6074.043	RP HPLC
OL5	ATCGATCGATCGATCGATC**T**	unmodified	6089.043	RP HPLC
OL6	ATCGATCGA**G**CGATCGATCG	unmodified	6139.056	RP HPLC
OL7	ATCGATCGA**A**CGATCGATCG	unmodified	6123.061	RP HPLC
OL8	ATCGATCGA**T**CGATCGATCG	unmodified	6114.050	RP HPLC
OL9	ATCGATCGA**C**CGATCGATCG	unmodified	6099.050	RP HPLC
OL10	ATCGATCGAT**A**GATCGATCG	unmodified	6138.061	RP HPLC
OL11	ATCGATCGATCGATCGA**A**CG	unmodified	6123.061	RP HPLC
DNA	GCCCAAGCTGGCATCCGTCA	unmodified	6060.039	RP HPLC
MODIFIED OLIGONUCLEOTIDES				
PS	GCCCAAGCTGGCATCCGTCA	phosphorothioate (each phosphate group)	6363.606	RP HPLC
ME	GCCCAAGCTGGCATCCGTCA	2′-O-methyl (each ribose molecule)	6621.149	RP HPLC
MOE	GCCCAAGCTGGCATCCGTCA	2′O-methoxyethyl (each ribose molecule)	7655.8354	RP HPLC
UNPURIFIED OLIGONUCLEOTIDES				
OL12	UCACUUUCAUAAUGCUGG	unmodified	5651.733	desalted after the synthesis, without any additional purification
OL13	GCCCAAGCTGGCATCCGTCA	2′-O-methyl (each ribose molecule)	6621.149	*degradation* by 10 freeze-thaw cycles
OL14	GCCCAAGCTGGCATCCGTC	2′-O-methyl (each ribose molecule)	6277.067	*degradation* by the addition of acid

detection at 260 nm; injection volume 1 μL. The void (t_0) of the column was measured by methanol injection.

The separation and identification of oligonucleotide impurities and degradation products were carried out using LC-MS, and only two different salt concentrations (10 and 25 mM) and pH values (6.0 and 7.5) were tested. Moreover, the gradient elution program was changed.

2.4. Mass spectrometer settings

All MS measurements were performed in negative mode, and the full scan mass spectra were recorded within the m/z 500–3200 mass range. Most of Q-TOF-MS parameters were applied based on our earlier studies [19,24]; consequently, capillary voltage was equal to 4000 V, drying gas flow 10 L min^{-1}, shielding gas flow 10 L min^{-1}, octopole voltage 800 V, drying gas temperature 350 °C, shielding gas temperature 400 °C, nebulizer gas pressure 20 psi, skimmer voltage 100 V, and fragmentor voltage 250 V.

The masses of impurities and degradation products were calculated using deconvolution in Mass Hunter Software and by assigning charge states of ions observed at the full scan spectra. Next, the sequence assignment was done in two steps. Firstly, the use of Mongo Oligo Mass Calculator v2.06 program [25] allows to determine the sequence, masses, and CID-product ions of oligonucleotides provided by the MS analysis of multi-charged ions. The other step of sequence determination was the application of oligonucleotide fragmentation.

3. Results and discussion

3.1. Retention studies

All three stationary phases (C18/AR, Phenyl-hexyl, and Diphenyl) were used to study the retention properties of oligonucleotides. The studies were conducted in solutions of three salts: ammonium bicarbonate (AB), ammonium formate (AF), and ammonium acetate (AA) with different concentrations. Measurements were carried out for unmodified (OL1-OL4, DNA) and modified oligonucleotides (ME, MOE, PS) (Table 1). Due to the exceptional efficiency of the columns and the reduction of solvents for the study, we chose to use ultra high-performance liquid chromatography (UHPLC).

A typical way to achieve oligonucleotide retention in an RP LC system is to add ion pair reagents, such as amines at pH below their pKa (by adding hexafluoroisopropanol, among others). This has a very good effect on the results obtained, but amines, as well as HFIP, are toxic and generally hazardous to the environment. Therefore, in this work, we decided to test the retention of oligonucleotides in the RP LC system without the use of ion-pair reagents in order to develop separation methods more compliant with the principles of green chemistry.

3.1.1. The impact of stationary phase and salt type on the retention of oligonucleotides

Based on the retention data shown in Fig. 1, it is easy to see that the highest retention of oligonucleotides was obtained using the C18/AR column. A Phenyl-hexyl column offers the smallest retention. It should also be noted that retention significantly depends on the type of salt used in the mobile phase. Its effect is significantly different for different phenyl columns. The use of ammonium bicarbonate for the Phenyl-hexyl column results in a loss of retention for unmodified oligonucleotides. In addition, retention on the Diphenyl stationary phase using ammonium bicarbonate is also the lowest compared to other salts. Differently, the low retention using ammonium bicarbonate is not observed on the C18/AR column, where the effect of salt type on retention is negligible. The retention of all oligonucleotides did not change significantly when salt was added to the mobile phase. Tested salts are similar in their chaotropic and kosmotropic properties. Ammonium cation is chaotropic, whereas all anions are kosmotropes. However, in this measurement, the pH of the solution was not adjusted. Although the pH of ammonium acetate is neutral and ammonium formate is acidic, the ammonium bicarbonate is alkaline, with a pH around 7.8, thus justifying the lower retention or retention loss of some oligonucleotides in the ammonium bicarbonate mobile phase. Based on the above-mentioned results, ammonium acetate was selected for further study.

The stationary phases tested fundamentally differ in their structure. While the Phenyl-Hexyl and Diphenyl phases contain a phenyl ring or two phenyl rings on a fairly short (6 carbon atoms) alkyl chain, the C18/AR phase has two types of ligands: C18 alkyl and phenyl (see Fig. S1). In each case, the stationary phase can interact with oligonucleotides through the π electrons of the aromatic ring, and in the case of the C18/AR phase, interactions of a typically hydrophobic nature become important through the presence of C18 ligands. In addition to the difference in the structure of the bound ligands, the stationary phases also

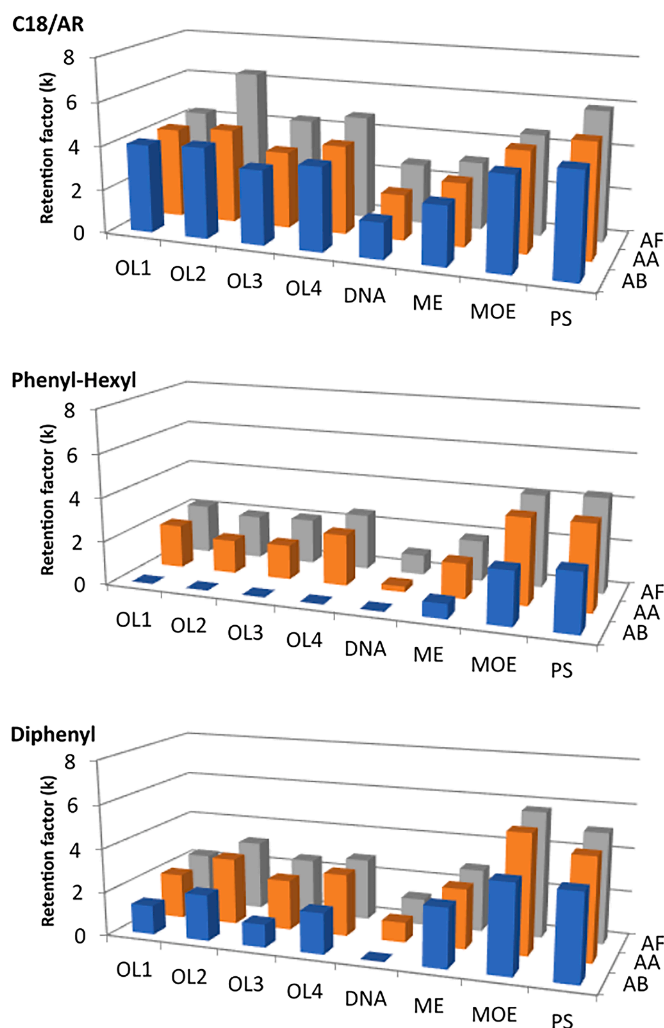

Fig. 1. Retention of tested oligonucleotides on the three tested stationary phases for mobile phases containing ammonium bicarbonate (AB), ammonium acetate (AA), and ammonium formate (AF). Conditions: mobile phase composition 25 mM salt solution and MeOH; gradient elution program: 10–30 % v/v MeOH in 10 min (for OL1, OL2, OL3, OL4); 15–50 % v/v MeOH in 10 min (for DNA, ME, MOE, and PS).

differ in carbon percentage. The C18/AR phase has the highest carbon content (Table S1), and it is in this stationary phase that the highest retention of all tested analytes is observed. For some oligonucleotides (e.g., PS, OL3, OL4, and DNA), a correlation of the retention with the carbon content in the stationary phase was found, thus suggesting that van der Waals forces could play an important role in the retention in addition to obviously significant impacts of $\pi - \pi$ interactions. This dependence is shown in Fig. S2. However, some oligonucleotides, e.g., OL1, behave in nonlinear correlation with the local minimum for Diphenyl (15 % C) stationary phase.

The main difference between the C18/AR stationary phase compared to the other tested columns is the presence of long alkyl chains (C18). Hence, the C18/AR phase is "thicker" and better insulates the silica surface from repulsive interactions resulting from the ionization of the silica surface at pH above 3.5. According to the literature, the pKa of silanols is in the range 3.5–4.6 and 6.2–6.8 [26]. Hence, phenyl and C18 stationary phase particles behave negative zeta potential values [27]. Negative surface zeta potential causes electrostatic repulsion with negatively charged oligonucleotide molecules without the ion-pair reagent in the mobile phase. Admittedly, it is difficult to clearly determine the extent of the zeta potential of the stationary phase, but it is possible that in the case of the C18/AR phase, its thickness exceeds the slipping plane [28]. Therefore, oligonucleotides can interact with the organic part of the stationary phase without the repulsion effect resulting from the silica support. Loss of the retention using ammonium bicarbonate on Phenyl-Hexyl stationary phase may also result from higher repulsion in the mobile phase of higher ionic strength.

3.1.2. *The impact of the type of oligonucleotide modification*

Comparing different oligonucleotides (Fig. 1), the greater retention on the Phenyl-Hexyl and Diphenyl phases is exhibited by far by modified oligonucleotides (ME, MOE, and PS) with the retention of MOE and PS being the greatest (about 2 times higher than for unmodified oligonucleotides). It has to be emphasized that the elution of modified and unmodified oligonucleotides was performed using different gradients (10–30 % v/v MeOH and 15–50 % v/v MeOH in 10 min for unmodified and modified oligonucleotides, respectively). Therefore, it should be noted that despite the higher elution strength used to elute the modified oligonucleotides, they nevertheless showed greater retention.

In the case of the C18/AR phase, the difference between k for unmodified DNA and modified ME, MOE, or PS is not so significant as for Diphenyl and Phenyl-Hexyl, which proves that in its case, the retention of unmodified oligonucleotides is higher, and it will be possible to use it for separation of such compounds. Moreover, in the case of C18/Ar retention, we have a clear increase in the retention depending on the modification in the DNA<ME<MOE<PS series. A different trend is observed for the other two columns: in the case of Phenyl-Hexyl, there is no difference in retention for PS and MOE, while MOE has higher retention than PS in the case of Diphenyl. These results indicate that the selectivity of these stationary phases is different for modified oligonucleotides in RP UHPLC. Moreover, for the modified oligonucleotides with ME and MOE modification, the peak shapes were symmetrical for each column (Figure S3A), indicating that they are likely to be able to be used in reversed-phase mode for the analysis of therapeutic modified oligonucleotides. The increase in retention of modified oligonucleotides is a direct result of the increase in the hydrophobicity of the molecules through the introduction of methyl and methoxyethyl groups, and by changing the polarity of the phosphate group by introducing a sulfur atom in place of an oxygen atom.

Unmodified oligonucleotides (OL1-OL4) contain many polar and ionized functional groups. Therefore, they exhibited low hydrophobicity and had low retention at the hydrophobic stationary phase surfaces. Among them, the highest hydrophobicity may be attributed to OL2 due to additional adenine (3′). This results in the highest retention among the unmodified oligonucleotides. The retention of unmodified nucleotides is always smaller than that of more hydrophobic modified oligonucleotides. The retention of ME on the C18/AR phase should be noted as an exception. In this case, the retention of unmodified oligonucleotides is noticeably higher.

In addition, for unmodified oligonucleotides, the peak shape was decidedly worse than for modified oligonucleotides in the case of Phenyl-Hexyl and Diphenyl phases. The chromatographic bands were wide and unshaped despite the use of UHPLC and very small particle size (see Figure S3B). In our opinion, this effect is a consequence of interactions between oligonucleotides and phenyl rings, as well as residual silanols at the surface of the stationary phase. Here, we primarily refer to the impact of $\pi \ldots \pi$ interactions. This effect was not observed on the C18/AR column, which is by far the best of the tested materials for oligonucleotide analytics in the RP UHPLC system without ion pairs. Peak symmetry improved significantly with increasing salt concentration in the mobile phase.

3.1.3. *The influence of salt concentration*

In addition to improving symmetry, an increase in the concentration of ammonium acetate in the mobile phase increased the retention of all oligonucleotides on all stationary phases tested (Fig. 2). While the C18/AR phase showed retention above 2 ($k > 2$) over the entire range of

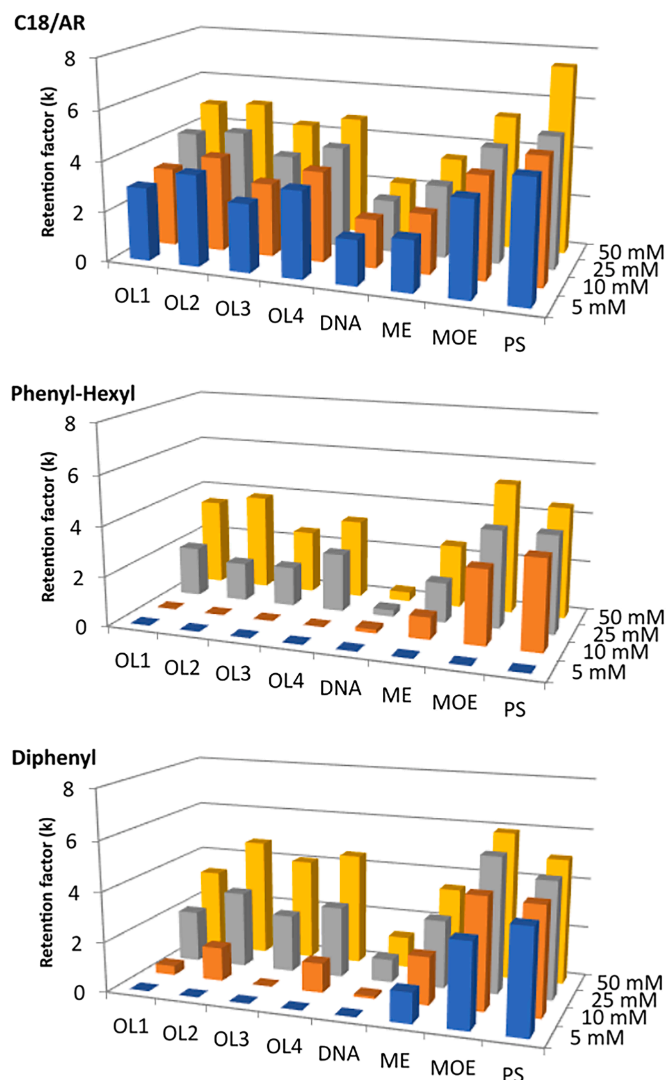

Fig. 2. Effect of ammonium acetate concentration (without adjusting of pH) on oligonucleotides retention. Conditions: mobile phase composition ammonium acetate and MeOH; gradient elution program: 10–30 % v/v MeOH in 10 min (for OL1, OL2, OL3, OL4); 15–50 % v/v MeOH in 10 min (for DNA, ME, MOE, and PS).

ammonium acetate concentrations tested (5–25 mM), no retention of oligonucleotides was observed for the Phenyl-Hexyl phase at an ammonium acetate concentration of 5 mM, and at 10 mM concentration only the more hydrophobic modified oligonucleotides showed retention. In the case of the Diphenyl phase, modified oligonucleotides showed retention even at an ammonium acetate concentration of 5 mM, but achieving retention for unmodified oligonucleotides requires the use of concentrations greater than 10 mM. This confirms the involvement of the salting-out effect in the retention mechanism of oligonucleotides, which is more noticeable for molecules with higher hydrophobicity.

An increase in ammonium acetate concentration caused a significant narrowing of the peaks, as shown in Fig. S4. On Phenyl column, symmetrical peaks for unmodified oligonucleotides were observed only for 50 mM concentration of ammonium acetate (Figure S5). Such conditions are acceptable when a detector other than MS is used.

All indications are that the mechanism of oligonucleotide retention is a combination of several effects, mostly van der Waals interactions and electrostatic repulsion. The salt concentration influences both effects by changing the dielectric constant of the mobile phase. This causes changes in the zeta potential on the one hand and increases retention through a mechanism similar to salting out on the other.

3.1.4. The impact of salt pH

The retention of oligonucleotides was tested in the pH range 4.5 to 7.5. Unfortunately, for pH 3, eluting oligonucleotides from any chromatographic columns was impossible. It should be noted that both the three tested columns and the chromatographic system were bioinert, which precludes adsorption on stainless steel surfaces. Nevertheless, for all tested oligonucleotides and stationary phases, an increase in retention was observed as the pH of the mobile phase decreased. It should be noted that a change in pH from 6.0 to 4.5 resulted in a greater increase in retention than a change from 7.5 to 6.0. This is the effect of the gradual ionization of residual silanols on the surface of stationary phases as the pH of the mobile phase increases. As a result, the contribution of electrostatic repulsion increases in the retention mechanism of oligonucleotides, resulting in a decrease in the retention factor (Fig. 3).

It is, therefore, possible that further reducing the mobile phase pH to 3 caused such a large increase in retention that the elution strength of the mobile phase was too weak. Perhaps at pH 3 the residual silanols have a partial positive charge, which increases the contribution of electrostatic attraction to retention. In that case, the concentration and type of salt used for elution would need to be changed. This, however, was not the subject of the study.

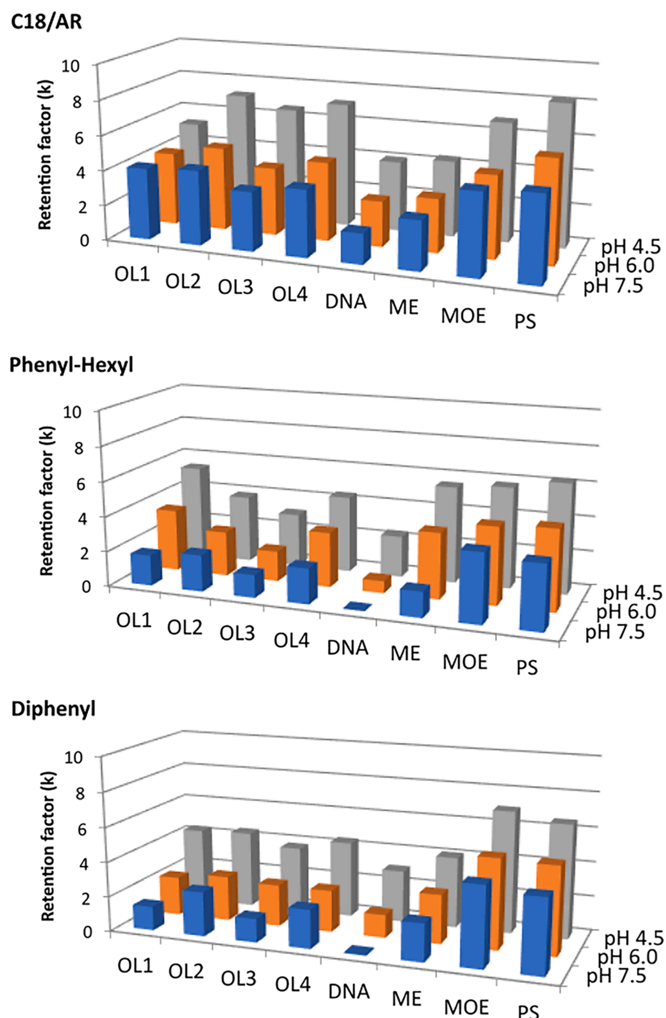

Fig. 3. Effect of the pH of the ammonium acetate solution (25 mM) on the oligonucleotides retention. Conditions: mobile phase composition 25 mM ammonium acetate and MeOH; gradient elution program: 10–30 % v/v MeOH in 10 min (for OL1, OL2, OL3, OL4); 15–50 % v/v MeOH in 10 min (for DNA, ME, MOE, and PS).

In summary, considering retention, peak symmetry, and peak width, the most effective stationary phase among those tested is the C18/AR phase. It offers the ability to separate oligonucleotides over the widest range of salt type, concentration, and mobile phase pH, providing the most symmetric and narrowest peaks.

3.2. Separation of positional isomers

This study investigates the sequence mismatches' impact on the separation selectivity in RP HPLC. The aim was to check if our method can be used to separate positional isomers, which is of paramount importance during the analysis of impurities of oligonucleotides or during the study of nonspecific hybridization.

For the study, we selected several oligonucleotides that differed in the position and type of one nitrogenous base in the sequence, with each having 20 nucleotides. We tested the separation of three mixtures, allowing us to determine three effects: 1. OL2, OL3, OL4, OL5 - the effect of the type of nitrogenous base at the 3′end; 2. OL6, OL7, OL8, OL9 - the effect of the type of nitrogenous base in the middle of the sequence; 3. OL2, OL7, OL10, OL11 - the effect of the position of the same nitrogenous base in different positions of the sequence (Table 1). Results are presented in Figure. The separations were carried out at a high temperature (60 °C) to avoid the influence of the formation of secondary structures (such as hairpins) on the separation. Note that the sequence differences are slight, which means that the mass differences are also minor, in the range between 10 and 50 Da, about 1 % of the mass of the whole compound. Therefore, separating these mixtures is challenging. In the case of the first mixture (Fig. 4A), all oligonucleotides were separated almost completely in a short time (3.5 min). This proves that RP UHPLC can be successfully used to separate positional isomers that differ by nucleotides at the ends of the sequence. The opposite situation appeared for the second mixture (Fig. 4B), where only partial separation was obtained for oligonucleotides differing by one nucleotide in the central position of the sequence. Full separation in just 4 min was obtained for a mixture of oligonucleotides differing in the position of the adenosine in the sequence, with positions number 10, 11, 18, and 20 (Table 1 and Fig. 4C). It is noteworthy that two of the separated oligonucleotides had the same masses (OL7 and OL11). In this case, the method we developed proved to be selective.

To sum up, despite the failure to separate all mixtures, it should be emphasized that RP UHPLC is an effective technique for separating mixtures of positional isomers of oligonucleotides if the changes involve the type of nucleotide at the end of the sequence or changes in the position of one nucleotide in the sequence. This effect probably could not have been obtained if UHPLC with high efficiency had not been used, which is reflected in the quality of the separation and the shapes of the oligonucleotide peaks (Fig. 4). The results obtained in this stage of the study pointed out that the method will likely be applied to separate and analyze impurities.

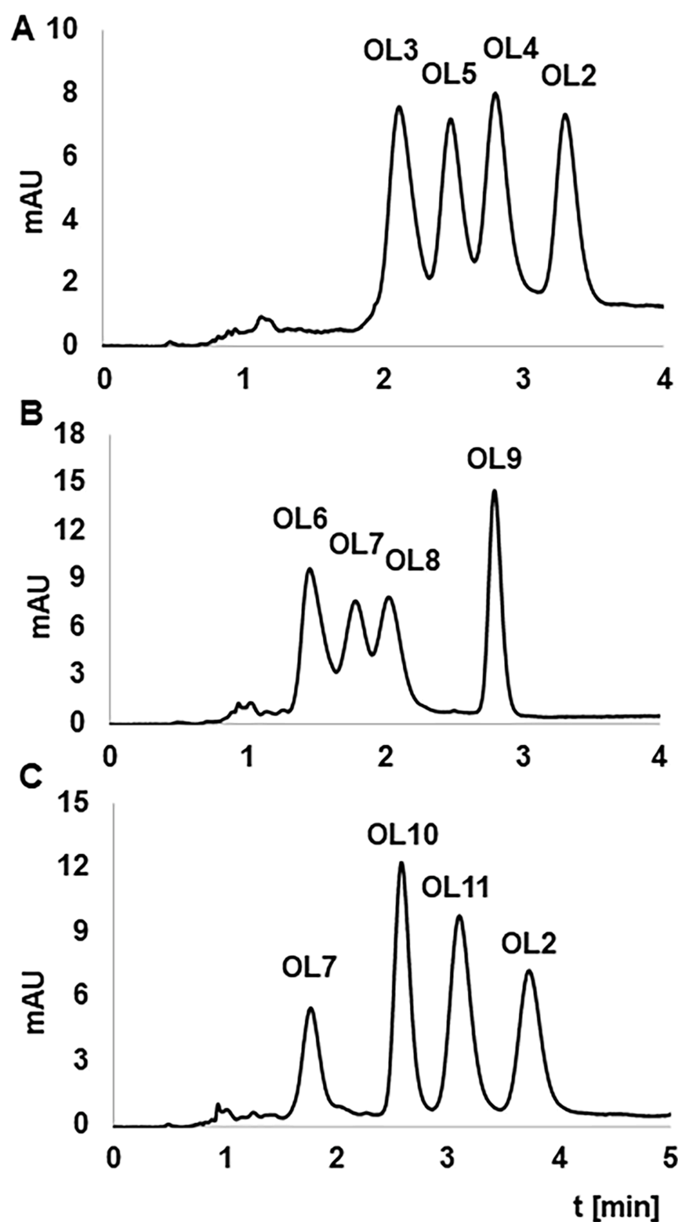

Fig. 4. Separation of positional isomers mixtures. Experimental conditions: column C18/AR (1.7 µm, 2.1 × 100 mm); mobile phase composition 25 mM ammonium acetate (pH 6) and MeOH; gradient elution 12–20 % MeOH in 10 min; flow rate 0.3 ml/min; detection UV–Vis 260 nm; column temperature 60 °C; autosampler temperature 10 °C; injection volume 1µl.

3.3. Separation and identification of oligonucleotide impurities and degradation products

The method developed here was used to separate and identify impurities of OL12 and OL13. Moreover, it was also used to resolve and analyse the degradation products of OL14 after its forced degradation with freeze-thaw cycles. These attempts should verify the applicability of RP UHPLC for separating and identifying oligonucleotide impurities.

In the first step, we tested the effect of the concentration and pH of ammonium acetate on the MS detector response for 10 and 25 mM AA and pH equal to 6.0 and 7.5. The results for OL12 and its several impurities are summarized in Table 2 and Figure S6. As described earlier, all these parameters affect the retention times of oligonucleotides and the sensitivity of MS determinations (Table 2, Figure S6). We compared the detector response based on the peak area data at EIC, and the lowest values were obtained for 25 mM AA pH 6.0 (Table 2). For an analogous salt concentration but with higher pH, the peak areas were several times higher, indicating the importance of the corresponding mobile phase pH, which is probably a consequence of more efficient ionization of the oligonucleotides. This effect was observed regardless of the length of the oligonucleotide. A lower salt concentration (10 mM) yielded greater EIC peak areas (Table 2) due to lower ionization suppression. However, a concentration of 25 mM was finally chosen for the study of impurities and oligonucleotide degradation products by RP UHPLC Q-TOF-MS, due to the better resolution of OL12 impurities. The analysis time was longer, but at the same time, the separation was complete, and this was the desired result. The difference in MS detector response between 10 mM and 25 mM AA pH 7.5 was not as significant as for pH 6, indicating a greater effect of pH than salt concentration on the intensity of impurity signals in MS (Table 2). Hence, despite the greater suppression of oligonucleotide signals noticed, 25 mM AA pH 7.5 was chosen for other

Table 2
Comparison of the impact of salt type and pH on the peak area at extracted ion chromatograms for selected impurities. Experimental conditions: 2–10 % v/v MeOH in 30 min.

Impurity	Ion *m/z*	10 mM AA pH 6		25 mM AA pH 6		10 mM AA pH 7.5		25 mM AA pH 7.5	
		t_R	A_{EIC}	t_R	A_{EIC}	t_R	A_{EIC}	t_R	A_{EIC}
5′ N-14	618.5889	4.62	23,759	5.69	9639	3.53	40,352	5.36	33,299
5′ N-13	791.1096	5.75	112,153	7.52	45,522	4.11	171,402	7.01	134,210
3′ N-8C	1033.7695	7.43	48,698	10.37	19,717	5.87	63,450	9.16	58,309
5′ N-11	738.7628	10.85	26,284	13.70	9807	8.22	45,195	13.12	37,822
3′ N-7C	1143.4564	11.47	53,057	14.07	19,831	7.59	61,387	12.95	60,886
3′ N-2C	1255.3884	14.75	28,446	17.50	5492	11.15	31,233	16.24	28,913
3′ N-1	1326.1591	17.07	12,142	19.98	3383	13.53	15,431	18.65	12,979
OL12	1412.4191	17.76	1,213,689	20.52	456,718	14.19	1,523,741	19.26	1,296,642

studies as a compromise between MS detector response and impurity resolution.

The masses of impurities and degradation products were determined using deconvolution in Mass Hunter software. It was also helpful to determine the ion charge state in the full scan mass and then calculate the masses. Examples of full scan spectra, isotopic charge distributions, and deconvolution results are shown in Supplementary materials (Figure S7-S9). Furthermore, the masses of possible impurities or degradation products were determined using the Mongo Oligo Mass Calculator v2.06 program. These masses were compared with the masses of oligonucleotides identified in OL12, OL13, and OL14. In this way, the oligonucleotides were preliminarily identified. It was assumed that the degradation products would be oligonucleotides with shorter sequences (cleavage of consecutive nucleotides), and the post-synthesis impurities would include N-shortmers, N-longmers (at 5′or 3′end), shortmers with additional phosphate (PO) 2′,3′-cyclic phosphate (CYC) groups. Such impurities are the most common.

The characteristic trend was observed for the results obtained with

Table 3
Impurities and degradation products identified in OL12, OL13, OL14.

***m/z* values for the most abundant ion**	**Charge**	**Impurity**	**Impurity sequence (5‘ – 3‘)**	**Deconvoluted mass [Da]**	**Retention time [min]**
OL12					
783.5819	−2	3′ N-13P	UCACU - PO	1568.1865	2.24
936.5965	−2	3′ N-12P	UCACUU - PO	1874.2114	2.63
774.5800	−2	3′ N-13C	UCACU - CYC	1551.1916	2.77
1012.0149	−1	5′ N-15P	GGU - PO	1013.1218	2.95
927.5894	−2	3′ N-12C	UCACUU - CYC	1856.2165	2.99
1089.6059	−2	3′ N-11P	UCACUUU - PO	2180.2363	3.24
1080.6089	−2	3′ N-11C	UCACUUU - CYC	2164.2414	3.73
827.7464	−3	3‘ N-10P	UCACUUUC - PO	2486.2770	3.87
822.0771	−3	3‘ N-10C	UCACUUUC - CYC	2469.2823	4.47
618.5889	−2	5‘ N-14	GGUC	1239.1965	5.39
1317.1025	−1	5‘ N-14P	GGUC PO	1318.1627	5.39
944.1220	−2	5‘ N-12	GGUCGU	1890.2585	6.56
791.1095	−2	5‘ N-13	GGUCG	1584.2336	7.12
848.4416	−3	5‘ N-10	GGUCGUAA	2548.3723	7.20
1033.7695	−3	3′ N-8C	UCACUUUCAU - CYC	3104.3593	9.75
1188.4754	−3	5‘ N-7P	GGUCGUAAUAC - PO	3568.4569	12.59
1148.9615	−3	3‘ N-7P	UCACUUUCAUA - PO	3449.4066	13.05
738.7628	−3	5‘ N-11	GGUCGUA	2219.3206	14.58
1143.4564	−3	3′ N-7C	UCACUUUCAUA - CYC	3433.3912	14.76
1259.1409	−3	3‘ N-6P	UCACUUUCAUAA - PO	3779.4585	16.59
950.7885	−3	5‘ N-9	GGUCGUAAU	2855.3883	18.24
1253.1384	−3	3‘ N-6C	UCACUUUCAUAA - CYC	3762.4370	18.27
1355.1466	−4	3‘ N-17P	UCACUUUCAUAAUGCUG - PO	5386.6484	18.70
1016.1080	−4	3‘ N-5C	UCACUUUCAUAAU - CYC	4068.4886	18.79
1178.6278	−4	3‘ N-3C	UCACUUUCAUAAUGC - CYC	4719.5767	18.95
1255.3884	−4	3‘ N-2C	UCACUUUCAUAAUGCU - CYC	5025.6016	19.11
1341.3932	−3	3‘ N-1C	UCACUUUCAUAAUGCUG - CYC	5369.6485	19.55
1264.1599	−3	5‘ N-6	GGUCGUAAUACU	3795.5158	21.00
1162.1529	−3	5‘ N-7	GGUCGUAAUAC	3488.4907	21.10
1326.1591	−4	3‘ N-1	UCACUUUCAUAAUGCUG	5308.6773	21.96
1024.3759	−4	5‘ N-5	GGUCGUAAUACUU	4101.5343	22.26
1412.4191	−4	**OL11**	**5′ UCACUUUCAUAAUGCUGG 3′**	5653.7321	22.90
1060.4738	−3	5‘ N-8	GGUCGUAAUA	3184.4425	23.74
1259.6543	−4	3‘ N-2P	UCACUUUCAUAAUGCU - PO	5042.6442	26.06
OL13					
1568.2643	−4	3‘ N-1	GCCCAAGCTGGCATCCGTC	6277.0862	15.70
1654.2805	−4	OL12	GCCCAAGCTGGCATCCGTCA	6621.1498	16.78
1564.2658	−4	5′ N-1	CCCAAGCTGGCATCCGTCA	6261.0923	19.31
OL14					
1488.4973	−4	3′ N-1	GCCCAAGCTGGCATCCGT	5958.0184	14.60
1568.2643	−4	OL13	GCCCAAGCTGGCATCCGTC	6277.0862	15.70
1479.0002	−4	5′ N-1	CCCAAGCTGGCATCCGTC	5920.0299	18.11

RP UHPLC Q-TOF-MS is the presence of relatively high additional peaks of potassium adducts (Figure S8, S9). Nevertheless, it is possible to accurately determine the mass of OL12, OL13, OL14 and their impurities or degradation products (Figure S8, S9), although certainly, the adducts reduce the sensitivity of the analyses, due to the decrease of intensity of oligonucleotide signal. The total ion current is usually shared over a greater number of signals when alkali adducts are present, making it a disadvantage of RP UHPLC application for oligonucleotide analysis. Reducing the number of adducts would probably increase the sensitivity of oligonucleotide determination by Q-TOF-MS. On the other hand, the accurate mass assignment is still possible.

The most abundant ions of impurities and degradation products, their charge state, retention time, deconvoluted mass, and probable sequence were presented in Table 3. One trend related to charge state distribution at the full scan spectra was observed: it increased with the increase of the length and mass of oligonucleotide (Table 3, Figure S7-S9). This tendency is related to the number of phosphate groups.

Thirty-three impurities were identified for OL12, and two degradation products for OL13 and OL14 (Table 3). Figs. 5 and 6 present EIC for each impurity and degradation sequence. In the case of OL11, twenty impurities were 3′N shortmers, while thirteen were 5′N shortmers. Non longmers were identified. Shortmers formed as an ON degradation product at the 3′ end position mostly have a cyclic phosphate (CYC) and phosphate (PO) group (Table 3). Shortmers created as degradation at the 5′ end mostly have OH group, rarely PO group (Table 3).

Regarding the elution order and its comparison only for impurities that are products of 3′ end degradation, it can be assumed that retention increases with increasing sequence length. Nevertheless, for impurities of the same sequence, oligonucleotides with a CYC group, as opposed to PO have a higher retention, but the differences are not big (Table 3). The retention of 5′ N-shortmers also increases with the increasing number of nucleotides.

A different trend was observed for the comparison of 5′N-shortmers and 3′ N-shortmers. The first ones always have greater retention compared to 3′ ones of the same length. This may be a consequence of the type of nucleotides in impurity sequence, e.g., 5–6mers 3′ N-shortmers (3′ N-13P, 3′ N-12P, 3′ N-13C) are eluted first, followed by 5′N-15P, which is the shorter 3-mer (Table 3). However, its structure contains two guanosines that are more hydrophobic than U, C, A. A similar situation applies to 3′ N-shortmers built of 7–8 nucleotides (3‘N-12C, 3′N-11P, 3′N-11C, 3′N-10P, 3,N-10C), which are eluted earlier than the shorter 4–5mers of 5′ N-shortmers (5′N-14,5′N-14P, 5′N-12, 5′N-13, 5′N-10) (Table 3). Here again, the hydrophobicity of the oligonucleotide sequence and the presence of guanosines play a major role. The cause of these effects may also be related to an additional phosphate group or a cyclic phosphate in the structure of 3′ N-shortmers, causing an increase in their polarity and, at the same time, a decrease in retention in RP UHPLC.

The results of the separation of OL12 impurities (Fig. 5) showed that the application of RP UHPLC Q-TOF-MS method with C18/AR stationary phase provides reasonable selectivity for the resolution. The significant advantage is the lack of coelution of OL12 impurities together with the main compound, although not all of the 33 impurities were separated completely (e.g. 5′N-14 and 5′N-14P, 3′N-3C and 3′N-2C) (Fig. 5). Nevertheless, the analysis time is <30 min; there are 33 compounds in the mixture that differ in both size and polarity; we use a simple reversed-phase system; and yet we can separate and identify these compounds without using ion pair reagents (ion pair chromatography) or a large proportion of acetonitrile (hydrophilic interaction liquid chromatography). What's more, the peak shapes are symmetrical

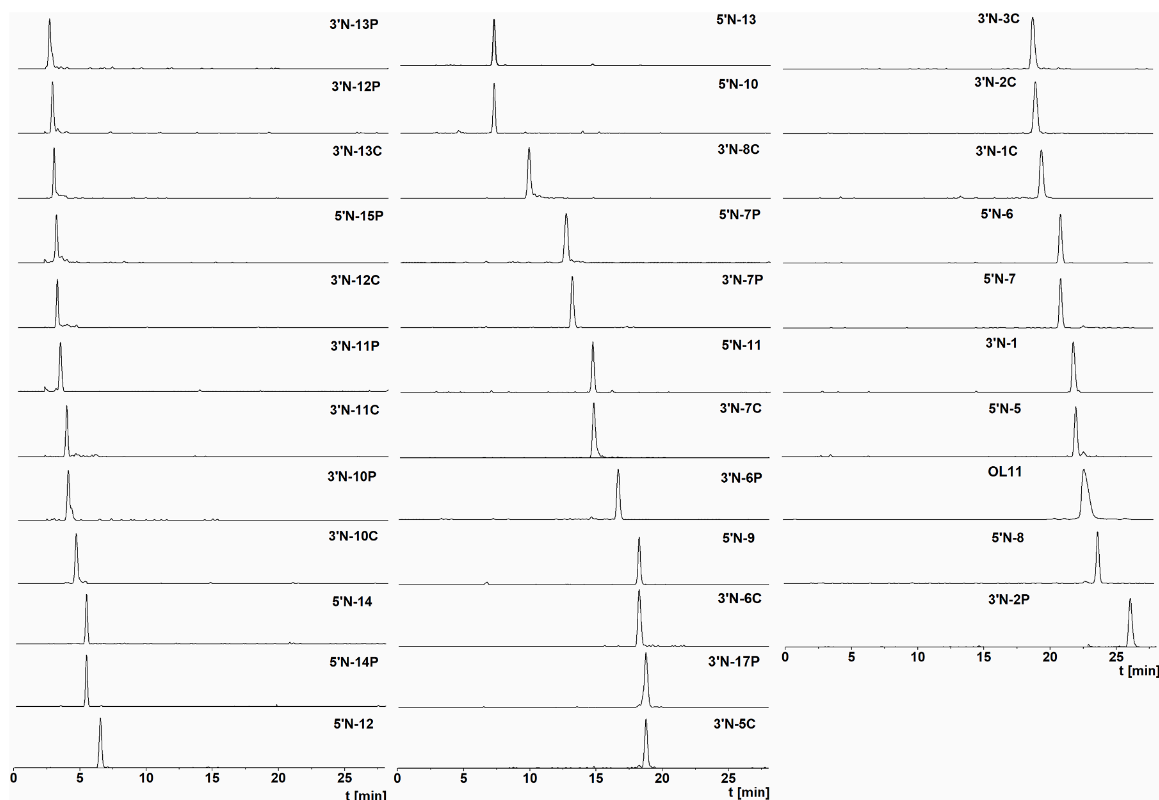

Fig. 5. Extracted ion chromatograms for impurities of OL12. Experimental conditions: Fortis C18/AR column; mobile phase composition 25 mM ammonium acetate (pH 7.5) and MeOH; gradient elution program: 2–8 % v/v MeOH in 30 min.; column temperature: 60 °C; autosampler temperature 10 °C; flow rate: 0.3 mL min^{-1}; injection volume 10 μL; MS conditions: nebulizer gas pressure 20 psi, skimmer voltage 100 V, capillary voltage 4000 V, fragmentor voltage 250 V; drying gas flow 10 L min^{-1}, shielding gas flow 10 L min^{-1}, octopole voltage 800 V, drying gas temperature 350 °C, and shielding gas temperature 400 °C. Impurities notation is similar as in Table 3.

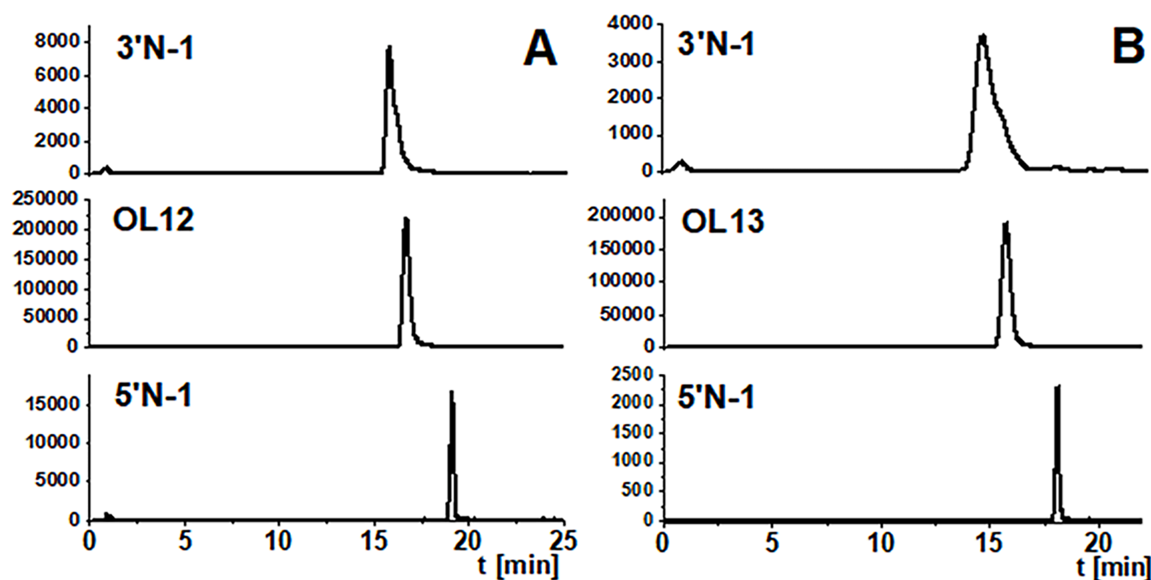

Fig. 6. Extracted ion chromatograms for impurities of OL13 (A) and OL14 (B). Experimental conditions: Fortis C18/AR column; mobile phase composition 25 mM ammonium acetate (pH 7.5) and MeOH; gradient elution program: 10–25 % v/v MeOH in 30 min.; column temperature: 60 °C; autosampler temperature 10 °C; flow rate: 0.3 mL min^{-1}; injection volume 10 μL; MS conditions: nebulizer gas pressure 20 psi, skimmer voltage 100 V, capillary voltage 4000 V, fragmentor voltage 250 V; drying gas flow 10 L min^{-1}, shielding gas flow 10 L min^{-1}, octopole voltage 800 V, drying gas temperature 350 °C, and shielding gas temperature 400 °C. Impurities notation is similar as in Table 3.

(despite the polarity of the compounds), a result of using UHPLC. In our opinion, these are major achievements and undoubtedly advantages of the developed method.

In the case of OL13 and OL14 (modified with methoxy group in 2′ position), only two degradation products each were identified, in both cases arising in the same way: by deleting one nucleotide from the 3′ and 5′ ends of the sequence. These are the most typical products of stepwise oligonucleotide degradation (regardless of whether acid or thawing/freezing was used). Interestingly, in both cases, 5′ N-1 was eluted after the peak of the main compound. In this case, however, the separation of OL13 or OL14 and respective 3′N-1 degradation products was incomplete (Fig. 6).

To sum up, the developed RP UHPLC Q-TOF-MS method using a C18/Ar allows the separation and identification of impurities and degradation products of oligonucleotides (both ribooligonucleotides and deoxyribooligonucleotides) in a relatively short time. It has to be emphasized that developed methods do not use ion-pair reagents. This approach offers a significant advantage in terms of environmental friendliness compared to traditional methods of oligonucleotides separations.

3.4. Comparison with other reversed phase methods

RP HPLC was the first separation technique applied for the analysis of oligonucleotides [29,30]. Nevertheless, the time of analysis was long (up to 50 min), and resolution was limited. Furthermore, high concentrations of salts (e.g., phosphates, acetates) were applied in the mobile phase, providing retention based on the salting-out effect. It may be detrimental for chromatographic columns in some cases [29,30]. Our method requires the application of low concentrations of salts, the time of analysis is shorter, and selectivity is greater due to the application of UHPLC. Nowadays, RP HPLC is used mainly for the purification of oligonucleotides, or for the analysis of molecules with e.g., terminal hydrophobic-protecting groups [31,32]. Our method provides good selectivity of separation of both unmodified, polar oligonucleotides, as well as modified antisense ones, therefore, we may conclude that we have extended the applicability of the reversed-phase mode. We believe this was possible not only because of the use of a stationary phase other than octadecyl, but also because of the use of UHPLC. To date, RP UHPLC analysis of oligonucleotides has used octadecyl stationary phases with terminal aryl groups and polybutylene terephthalate-based [19,21,22]. Utilization of octadecyl stationary phase with aryl rings allowed the separation of oligonucleotides differing in length, sequence, and modification type [19]. In the case of the present study, the use of a monodisperse stationary phase allowed us to combine all the advantages: increased resolution due to the use of UHPLC and monodisperse materials and increased selectivity due to the change within the stationary phase. As a result, the method developed in this research can be successfully applied to the separation of impurities after the synthesis and degradation of oligonucleotides, which was not achieved in earlier studies [19]. On the other hand, the use of polybutylene terephthalate-based stationary phase allowed almost complete separation of siRNA strand impurities; nevertheless, it was not possible to achieve complete separation of the main compound from closely related impurities (eluted in one peak) [21,22]. Therefore, it was necessary to use 2D-LC. In the case of the method developed during present research, this situation does not occur, proving its superiority over previously developed methods for reverse-phase analysis of oligonucleotides.

4. Conclusion

Retention of oligonucleotides on three phenyl-based stationary phases was compared in the RP UHPLC system without ion-pair reagent, which is a step toward sustainable chromatographic methods. Phenyl-hexyl and Diphenyl stationary phases differ significantly in retention from the C18/AR stationary phase. Generally, the retention of oligonucleotides increases with the increase of salt concentration, and the decrease of the pH confirms the significant impact of van der Waals interactions, salting-out effect, and π-electronic interactions in the retention mechanism.

Changing the salt type, salt concentration, and pH of the mobile phase, the C18/AR stationary phase proved to be superior in achieving optimal retention, peak symmetry, and resolution compared to other tested phases. Nevertheless, Diphenyl and Phenyl-Hexyl may be applied to separate modified oligonucleotides with higher salt concentrations. In such a condition, symmetrical peaks of modified oligonucleotides were

obtained.

The developed RP UHPLC Q-TOF-MS method using a C18/AR allows the separation and identification of impurities and degradation products of oligonucleotides in a comparatively short time. This also applies to the very challenging separation of positional isomers. Even if the mass differences are minor (lower than 1 %), the complete separation may be obtained for oligonucleotides differing with the last nucleotides in the sequence or with the position of one nucleotide in the sequence. Therefore, the method we developed proved to be selective. Moreover, the method was used to separate and identify impurities and degradation products. The mobile phase salt concentration and pH impact the final results (retention time and sensitivity). The 25 mM ammonium acetate of pH 7.5 was chosen for the analysis despite the more significant suppression of oligonucleotide as a compromise between MS detector response and impurities separation. The application of RP UHPLC Q-TOF-MS provides reasonable selectivity for the resolution of 33 impurities and two degradation products. Both groups of compounds are mainly 3′N and 5′N-shortmers, but in the case of impurities, modifications of cyclic phosphate and phosphate groups were also identified. Generally, the retention increases with increasing sequence length of impurity in RP UHPLC and 3′ N-shortmers always have greater retention than 5′ N-shortmers. Our method's significant advantage, except for the lack of ion pairs, is the complete separation of closely related impurities. This ion-pair-free approach offers a substantial advantage in terms of environmental friendliness compared to traditional methods.

CRediT authorship contribution statement

Sylwia Studzińska: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Szymon Bocian:** Writing – original draft, Methodology, Data curation. **Luca Rivoira:** Writing – review & editing, Data curation. **Ed Faden:** Writing – review & editing. **Geoff Faden:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465380.

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