# Development of a hydrophilic interaction-based procedure for the extraction, separation, and determination of therapeutic oligonucleotides

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## Abstract

**Background:** For analyzing oligonucleotide impurities and metabolites in serum samples, where mass spectrometry detection is essential, hydrophilic interaction liquid chromatography utilizing fully mass spectrometry compatible mobile phases is the best choice. However, reliable analysis must be preceded by a reliable extraction method providing sufficient and repeatable recoveries. Thus, this work aimed to develop a simple and repeatable methodology for extracting and analyzing modified oligonucleotides differing in length (4mer-14mer), both under hydrophilic interaction conditions.

**Results:** A comprehensive retention study examining the effects of mobile phase composition and temperature on retention and peak shape was conducted for the zwitterionic and amide columns. Based on the results, the amide column was chosen for further study, mainly because the zwitterionic column does not permit elution of longer oligonucleotides (>10mer). In addition, the amide column exhibits greater robustness (minor effects of tested parameters on chromatographic performance), which ensures higher repeatability. The optimization of mass spectrometry parameters was performed to obtain the highest possible sensitivity, which is well well-known issue because of adducts formation. A validated procedure was applied to analyze enriched serum extracts - a newly synthesized amide-based sorbent was used for the dispersive solid-phase extraction. The conditions for adsorption and desorption, which are driven by a weak ion-exchange mechanism and thus by pH change, were optimized to obtain the highest recoveries.

**Significance:** A fully hydrophilic interaction-based procedure was utilized for the first time f extraction and analysis of a mixture of 5 modified oligonucleotides differing in length from enriched serum. This method proved to be simple, repeatable, and provides recoveries higher than 75% (RSD <5%).

**Keywords:** hydrophilic interaction liquid chromatography, dispersive solid phase extraction, mass spectrometry, modified antisense oligonucleotides, amide adsorbent

## 1. Introduction

With the increasing development of therapeutic oligonucleotides (ONs) for gene therapies, the demand for reliable analytical methods enabling proper identification and quantification of impurities (in drug development processes) and metabolites (after drug administration) is growing [1]. Nowadays, two main chromatographic approaches can be applied for LC-MS analysis: i) ion-pairing reversed-phase liquid chromatography (IP RP LC) [2–4], ii) hydrophilic interaction liquid chromatography (HILIC) [5–7]. Both approaches have limitations, especially in mass spectrometry (MS) detection, which is required to identifyimpurities and metabolites [8]. Utilization of IP RP LC leads to the contamination of LC instrument by IP agents; however, it provides higher sensitivity compared to HILIC, where sensitivity is lowered by alkali metal adduct formation [9,10]. Moreover, HILIC suffers from non-specific ONs adsorption on the metal surfaces in LC instrumentation (caused by usually lower pH than IP RP LC), which can lead to signal losses and non-repeatable results [11,12]. While IP RP LC is generally considered a gold standard for ONs analysis, only a few comprehensive studies on the analysis of therapeutic (modified) ONs in HILIC have been published [3,6,7,9,13,14]. Moreover, HILIC proved to be suitable also for separating nucleobases, nucleosides, and nucleotides themselves, which can be used for analysis of DNA epigenetic modifications [15–17].

HILIC retention mechanism is complex and combines partitioning, hydrogen bonding, and electrostatic interactions. Because of the higher water content in the mobile phase (MP) necessary for elution of (longer) ONs, HILIC of ONs utilizes a unique MP composition compared to "traditional" HILIC [5]. Under these conditions (30-70% of water in MP), the water layer on the stationary phase (SP) surface is stable. Thus the changes in partitioning provide only a limited contribution to the retention mechanism [18]. The typical HILIC MP is fully MS compatible, and as it was demonstrated earlier, using ammonium acetate over ammonium formate is beneficial in terms of MS sensitivity [6]. Amide SPs are generally considered as a gold standard for analysis of ONs under HILIC conditions, since it was shown that amide SPs exhibit a thick water layer on the SP surface [19] and contain both hydrogen bond donors and acceptors. However, to the best of our knowledge, no comprehensive study dealing with the effects of MP composition and separation temperature on retention and resolution of modified ONs (phosphorothioation, base methylation, 2′-O-methylation of ribose) and varying in length (3mer to 14mer) was performed. In addition, zwitterionic columns recently showed great potential for the separation of short 3mer ONs [20].

Since therapeutic ONs and their metabolites must be monitored in complex biological matrices of patients undergoing the therapy, the crucial step is sample preparation [21]. Usually, the first step includes removing proteins because they exhibit a strong affinity to ONs and can reduce their recovery. The protein removal can be performed in several ways: i) liquid-liquid extraction (LLE); ii) digestion of proteins by proteinase K. Both approaches provide good recoveries for ONs, but need an additional purification step (for LLE, removal of phenol, and for digestion, removal of digested peptides) [22,23]. In the next step, LLE, solid-phase extraction (SPE), dispersive solid phase extraction (dSPE), micro-extraction by packed sorbent (MEPS), or hybridization can be utilized [24–28]. Since SPE, dSPE, and MEPS are solid phase extraction, ON recovery depends on the adsorbent type and mode of extraction, namely strong ion-exchange, weak ion-exchange or ion-pair formation [23,25,27,29]. Each mode allows obtaining relatively high recoveries for most of ONs, but reproducibility and time-consumption are different. Hydrophobic adsorbents applied in ion-pair mode are the most complex methods (gravimetrical flow, long procedures, lower reproducibility) [29,30]. Adsorbents with a permanent positive charge at the surface require application of high concentrations of inorganic salts (excluding MS) [29,31]. Extraction based on weak ion exchange has lately proved to be promising (easy procedures, MS-compatible eluates) [22,32]. For example, using adsorbents containing amine and carboxyl groups provided high recovery for both standards and real samples (80-92%) [32]. Another interesting attempt is SPE extraction using polar (silica gel, aminopropyl silica) adsorbents for hydrophilic interaction-based extraction [33,34]. It has been shown that for silica sorbent, the recovery decreases with ON modification, and an additional decrease has been observed for enriched plasma, resulting in a recovery of around 50% [33]. On the other hand, aminopropyl sorbent exhibits recovery from enriched plasma around 60% [34]. Despite SPE popularity (predominantly because of the possibility of sample preconcentration by using large volumes of sample up to sorption capacity), lately, dSPE is also used for ONs extraction, as it minimizes solvent and sample consumption, shortens the procedure time, and reduces the amount of adsorbent needed for extraction [29,35].

The goal of this work was to develop a complex, hydrophilic interaction-based procedure for the extraction, separation, and determination of antisense ONs. To the best of our knowledge, this is the first study of its kind. We have shown for the first time that this approach is possible and has many advantages in the modern analysis of therapeutic ONs. The potential of HILIC in separating ONs with chemical modifications (in the phosphate group and ribose molecule) and of different lengths (relevant for both impurity and metabolite studies) was studied. Moreover, hydrophilic interactions were also used for extracting these ONs from serum samples, which can show the potential of using hydrophilic dSPE to extract metabolites. The new type of extraction adsorbent was synthesised for this purpose and efficiently applied for the first time for antisense ONs extraction.

## 2. Materials and methods

### *2.1. Materials and reagents*

Acetonitrile for MS (ACN, LC-MS Chromasolv®, ≥ 99.9%), acetonitrile (Chromasolv®, gradient grade, ≥ 99.9%), methanol (MeOH, Chromasolv®, gradient grade, ≥ 99.9%), and water for MS (LC-MS Chromasolv®) were purchased from Honeywell (Charlotte, NC, USA). Ammonium acetate (purity ≥ 99%), ammonium acetate for MS (LiChopur$^{TM}$), acetic acid (purity ≥ 99%), formic acid for MS (purity ≥ 99%), phenol-chloroform-isoamyl alcohol mixture (BioUltra purity) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Chloroform ($CHCl_3$, > 98,5%), 25% ammonium hydroxide solution ($NH_4OH$) was purchased from Avantor (Gliwice, Poland). All tested ONs (see their list in Table 1) were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Future Synthesis (Poznan, Poland). Long (12-14mer) ones were purchased from the first company, while short ones (3-5mer) from the second one. These compounds were modified simultaneously with phosphorothioate groups and 2'-O-methylation at each ribose. Deionized water was purified with the Milli-Q system (Millipore, Burlington, MA, USA).

The solid support of in-house-made amide adsorbent was porous silica gel purchased from Sigma-Aldrich (St. Louis, MO, USA). The average pore size of silica gel was 60Å, and the particle diameter range was 63-200 µm. Aminopropyltrimethoxysilane, triethylamine, and acetyl chloride were purchased from Alfa Aesar GmbH (Karlsruhe, Germany). Methanol, toluene, and hexane (all analytical grades) were obtained from J. T. Baker (Deventer, The Netherlands).

### *2.2. Instrumentation and columns*

Thermo UHPLC Vanquish Flex (Thermo, Germany) and Shimadzu Nexera-i LC-2040C 3D (Kyoto, Japan) liquid chromatographs equipped with spectrophotometric diode array detectors were used. For all MS measurements (quantitative analysis), Shimadzu Nexera X2 LC-30AD instrument equipped with Shimadzu LCMS-8050 mass spectrometer (Kyoto, Japan) with electrospray ionization (ESI) was used. The chromatograph was equipped with two LC-30AD pumps, SIL-30AC autosampler, CTO-20AC thermostat, and SPD-M20A diode array detector (Kyoto, Japan). The data were collected using Chromeleon 7 and Lab Solution chromatography data systems.

During the retention study, the following columns were tested: Acquity Premier BEH Amide column (1.7 µm, 2.1×100mm; Waters, Milford, USA), and Atlantis Premier BEH Z-HILIC (1.7 µm, 2.1×100mm; Waters, Milford, MA, USA). A pH meter CP-505 Elmetron (Zabrze, Poland) was used to reach the required pH of MPs. The suspensions during the extraction process were centrifuged using a Frontier Micro FC5515 centrifuge (OHAUS Europe GmbH, Nänikon, Switzerland).

Solid-state NMR measurements were performed on a Bruker Avance III 700 MHz spectrometer (Bruker AG, Karlsruhe, Germany) with a CPMAS broadband probe for obtaining spectra in solids, with a 3.2 mm rotor rotating at up to 20 kHz.

All data were graphically processed in software OriginLab 9.1 (Northampton, MA, USA).

### *2.3. Experimental procedures*

#### *2.3.1. Preparation of ONs standards*

Standards of ONs were provided in lyophilized form. Stock solutions were prepared by dissolution in deionized water at a concentration of 100 µM. These stock solutions were stored in a freezer and diluted by a mixture of ACN/water (80/20, *v/v*) before use (to reach the required concentration, e.g., 10 µM for retention study).

#### *2.3.2 Synthesis and characterization of amide adsorbent for hydrophilic interaction dSPE*

Before the chemical modification of bare silica gel, a sample of adsorbent was placed in a specially designed glass reactor, protecting against the contact of the reagents with the external environment. Silica gel was dried at 180 °C under vacuum for 10 h. Then, the temperature was decreased to 80 °C, and aminopropyltrimethoxysilane in toluene solution was added. After 12 h, the reaction products were washed out with toluene, methanol, and hexane and dried. Next, the aminopropyl silica was placed in a glass reactor, and acetyl chloride in toluene solution was added. The addition of triethylamine neutralized the hydrogen chloride released due to the reaction. The reactions were carried out during 12 h, and the final products were washed out as described above. The structure of the sorbent was confirmed by elemental analysis and $^{13}$C NMR spectroscopy.

#### *2.3.3. Development of a hydrophilic interaction-based dSPE procedure with the use of amide adsorbent*

Unmodified ONs with lengths 18mer and 20mer (Table 1) were used to optimize adsorption and desorption conditions during the dSPE procedure. The 2 mg of sorbent was used during each experiment and performed twice. The dSPE procedure consisted of the following steps: i) sorbent conditioning, ii) sample loading, iii) washing, iv) elution. During sorbent conditioning, 200 μL of MeOH, 200 μL of water, and finally 200 μL of the same solution, in which the ON is dissolved during sample loading, were used. After sorbent conditioning, 100 μL of ON (10 μM or 2.5 μM) dissolved in solvent for adsorption was loaded and mixed (with vortex) for 5 minutes. The following solvents for adsorption were tested: water/ACN 10/90 (*v/v*); water/ACN/formic acid 10/90/1 (*v/v/v*); water/ACN 20/80 (*v/v*); water/ACN/formic acid 20/80/1 (*v/v/v*); water/ACN 30/70 (*v/v*); water/ACN/formic acid 30/70/1 (*v/v/v*). The washing step was performed using 100 μL of ACN/adsorption solution 80/20 (*v/v*). For ON elution, several desorption solutions were tested: water/ACN 90/10 (*v/v*); water/ACN/ammonium hydroxide 90/10/2 (*v/v/v*); water/ACN 70/30 (*v/v*); water/ACN/ ammonium hydroxide 70/30/2 (*v/v/v*); water/ACN 50/50 (*v/v*); water/ACN/ ammonium hydroxide 50/50/2 (*v/v/v*), and water/ACN/ ammonium hydroxide 25/75/2 (*v/v/v*). Between each step, 10 minutes of centrifugation (14 000 rpm) was performed. The extraction process was controlled using HILIC measurements at each step (sample load, washing, elution). It is worth noting that during the recovery evaluation, the peak area of the ON standard and eluent containing ON after the desorption procedure are compared. For proper evaluation, the ON standard has to be dissolved in the same solution as the eluent. Schematic procedure is illustrated in Fig. S1.

SPE procedure was performed using the same steps as dSPE under optimized sorption and desorption conditions. 50 mg of sorbent was packed into an SPE cartridge (volume 1 mL). Using higher solvent volumes (200 μL) for conditioning, loading, and washing was necessary for SPE. Elution was performed in two different ways: i) 1×600 μL; ii) 3×200 μL. The sample load (adsorption) and elution (desorption) were performed under gravity flow to ensure sufficient contact time.

Sorption capacity was measured using 1 mg of adsorbent. The 125 μL of 100μM ON was loaded during the adsorption. The suspension was mixed for 5 minutes, then centrifuged for 10 minutes (14,000 rpm). Unadsorbed ON was measured in the supernatant using HILIC. The sorption capacity was calculated according to the following equation:

$$Q = \frac{((c_0 - \mathrm{c}) * \mathrm{V}) * \mathrm{M}}{\mathrm{m}},$$

where $Q$ is sorption capacity [μg/mg]; $c_0$ is initial ON concentration [μmol/cm$^3$]; $c$ in concentration of ON in solvent after adsorption [μmol/cm$^3$]; $V$ is volume of solution with ON from which ON was adsorbed [cm$^3$]; $M$ is molecular mass of ON [g/mol] and $m$ is sorbent weight [mg].

### *2.3.4. Enriched serum sample preparation*

The serum samples were collected according to guidelines approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022). Human serum samples were enriched with ONs in the following way. Firstly, serum was diluted with water (1:3), and next, the mixture of 5 ONs (4mer A, 5mer A, 12mer A, 13mer A, and 14mer) was added to a final concentration of 10 µM of each. Next, 500 µL of enriched serum was mixed with 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1, *v/v/v*). The sample was mixed and centrifuged at 14000 rpm for 40 minutes. The 30 µL of LLE extract (upper layer) was diluted with 70 µL of ACN and 1 µL of formic acid. Next, the sample was loaded onto 2 mg of amide adsorbent, and the dSPE procedure (section 2.3.3) was applied to purify the sample and extract ONs. After desorption, the extract was diluted 1:1 with ACN to ensure sample solvent compatibility with HILIC conditions. The final schematic procedure is summarized in Fig. S2.

### *2.3.5. Mobile phases and chromatographic conditions*

During the retention studies, several MPs were tested. MP A was always fully aqueous and consisted of ammonium acetate at various concentrations (10mM; 25mM; 50mM) and pH (pH 4.5; pH 6.5; pH 8.5). pH of MP A was adjusted by adding acetic acid (to reach pH 4.5) or ammonium hydroxide (to reach pH 8.5). MP B was composed from a mixture of ACN/water (80/20; *v/v*) with the same amount of ammonium acetate as in MP A. A gradient elution was used for all measurements: 0-10-12-27 (min), 100-64-100-100 (%B).

Since the results from the retention study did not reveal any significant differences between tested MPs (see chapter 3.1.), for the following experiments (method validation and dSPE development), the simplest possible MP composition was chosen to ensure high repeatability and feasibility, i.e., 10mM ammonium acetate without pH adjustment (pH ~ 6.8). During the development of the dSPE procedure, different gradients (0-6 min. 80-37 %B) were used to evaluate the recovery (longer ON was used for dSPE conditions optimization, and thus the gradient had to be changed to maintain the elution).

The flow rate for all experiments was 0.4 mL $\text{min}^{-1,}$ and the UV detection wavelength was set at 260 nm. The injection volume was 1 µL - 2 µL. Column temperature was 40 °C, except for the testing of the effect of temperature on retention and resolution during the initial phase of the study, where the following temperatures were tested: 20 °C, 40 °C, 55 °C, and 70 °C. The sample temperature in the autosampler was set to 10 °C for all measurements.

### *2.3.6. MS conditions*

All MS measurements were performed in negative ion mode. Nitrogen was used in the ion source, and argon was applied in the collision cell. The optimization of ESI MS parameters and collision energy for each ON was carried out without a column (with the capillary), with the use of a MP consisting of 20% MP A (10mM ammonium acetate in water; pH 6.8) and 80% MP B (10mM ammonium acetate in 80/20 ACN/water (*v/v*). Full-scan mass spectra were recorded within the *m/z* 450–2000, and fragmentation spectra for *m/z* 50–1000. The selected precursor ions (including their charge state), fragmentation ions, and corresponding collision energies are summarized in Table 1 (only for ONs analysed by MS).

The design of experiments with central composite design (CCD) was used to optimize ESI-MS parameters to obtain the highest possible sensitivity. Six different parameters were considered: interface temperature (IT; range 150-350 °C), heat block temperature (HBT; range 150-400 °C), DL temperature (DLT; range 90-250 °C), drying gas flow (DGF; range 4-10 L min-1), nebulizing gas flow (NGF; range 1-2.5 L min-1), and heating gas flow (HGF; range 4-10 L min-1). Temperatures were combined in one set, while gas flows were in the second one. They were selected as independent variables, while the SIM peak area was a response, dependent variable. A total of 14 chromatographic runs (28 in duplicates) for each set were sufficient to perform optimization. The levels of MS parameters for CCD were calculated using a free online calculator DATAtab; they are collected in Supplementary materials (Table S1). The following conditions were selected as the most optimal ones (provide the highest peak areas): IT – 400 °C; HBT – 275°C; DLT – 170 °C; DGF – 7 L min$^{-1}$; NGF – 1.8 L min$^{-1}$; HGF – 12 L min$^{-1}$.

### *2.3.7. Validation of HILIC MS/MS method*

The developed HILIC MS/MS method was validated. A calibration curve was constructed based on the results obtained with standards of tested ONs in the concentration range 0.156 – 10 µM for long ONs and 0.0049 – 10 µM for short ONs. The linearity of the method was determined using the determination coefficient R$^2$. The intraday and interday precision was determined for three (10 µM; 3.3 µM; 1.1 µM), respectively two (5 µM; 2.5 µM) different concentration levels. The degree of repeatability was determined by performing five injections on the first, third, and seventh days. The precision was achieved using even injections daily. The limit of detection (LOD) and quantification (LOQ) resulted from experimental determination based on signal-to-noise ratio. LOD was defined as the concentration at which the signal-to-noise ratio was at least 3:1, while LOQ was defined as the concentration at which the signal-to-noise ratio was at least 9:1.

The matrix effect (ME) was measured by comparing ONs peak area in a fortified blank serum extract to the peak area of the ON standard. Both standard and enriched blank serum extract had the same ON concentration. ME was calculated according to the equation:

$$ME = 100\frac{A_{\text{blank enriched by standard}}}{A_{\text{standard}}},$$

where $A$ is the peak area. For dSPE recovery and ME determination, the MS detection (MRM) was used. UV detection was used to show the effect of LLE and dSPE on protein and phenol content.

## 3. Results and discussion

The selection of ONs for the study was primarily based on the nusinersen investigation. The sequences of tested ONs are shorter fragments of nusinersen analogue. Moreover, the research involves ONs modified within two structural elements: a ribose to which a methoxy group has been introduced, and a phosphate group in which one oxygen atom has been replaced by sulfur. Different lengths of ONs were also tested: from very short (3-5mers) to long (12-14mers). The list of tested ONs is presented in Table 1, and the schematic structure of modifications in Fig. S3. Owing to this approach, the results most effectively reflect the potential of developed methods to the study of therapeutic ONs, whose modifications always involve several structural elements. On the other hand, the two different length ranges of ONs will enable us to explore the potential of the developed methods for studying impurities and metabolites. In both groups, different ONs lengths are characteristic. The study is divided into three main parts to establish a complex hydrophilic interaction-based procedure of modified ONs extraction, separation, and quantification.

**Table 1**

### *3.1. Retention study*

Since zwitterionic columns recently showed great application potential for analysis of short ONs [20,36], one of them has been chosen for comparison with amide column, which is generally considered as a gold standard for analysis of ONs [7,9]. Both columns were selected in bioinert variant (Premier), ensuring minimal non-specific ONs adsorption on metal surfaces in column hardware. Diol-based columns previously showed low selectivity and significant separation of diastereomers, resulting in lower resolution of individual ONs [37]. Additionally, bare silica gel columns provide asymmetric peaks, lowering the resolution and efficiency [14].

Thus diol-based and bare silica gel columns were not included in this study, and only two columns with great potential for analysis of ONs have been selected to study their retention mechanism in detail.

### *3.1.1 The effect of mobile phase composition on retention and resolution of modified ONs*

In the first part of our study, the effect of MP composition (pH and concentration of ammonium acetate) on retention and resolution of modified ONs differing in length (3mer, 4mer, 5mer, 12mer, 13mer, 14mer) or differing in sequence (for those with the same length) was investigated in detail for two HILIC columns, namely Acquity Premier BEH Amide and Atlantis Premier BEH Z-HILIC.

The results concerning the effect of MP composition on retention and resolution for the amide column are summarised in Fig. 1A. The change in MP pH does not significantly affect the retention; only the slight decrease in retention with increasing pH (more pronounced for longer ONs) was observed. Moreover, even the resolution (represented by the distance on *y* axis between points for individual analytes) does not change with the change of MP pH. Significantly higher resolution can be achieved for shorter ONs (3-5mer), contrary to 12-14mer. Shorter ONs are baseline resolved at all tested conditions (*Rs*>4.5), while for longer ONs, the resolution between 13mer and 14mer was only around 0.5. Changes in ammonium acetate concentration in MP revealed a slight increase in retention with increasing concentration for all tested ONs and MP pHs (Fig. 1A). These results (i.e., small changes in chromatographic behaviour with pH and concentration change) for amide SP are consistent with the assumed retention mechanism. ONs are permanently negatively charged in the whole tested pH range, and the amide SP is neutral. Thus, no significant changes resulting from the charge changes can be expected, and observed differences can probably be attributed to the changes in hydrogen bonding capacity.

**Fig. 1**

The same series of experiments was also performed for the zwitterionic column (Fig. 1B). The retention of ONs is significantly higher compared to amide SP. Thus, it was impossible to elute longer ONs (12-14mer) under these conditions, even though the gradient was prolonged to: 0-25 (min), 100-10 (%B), maintaining the same gradient slope (3.6 %B for 1 minute). This effect results from the strong electrostatic attraction between negatively charged ONs and positively charged groups on the SP surface. The higher concentration (>50mM) of ammonium acetate in MP was tested to check the possibility of elution of 12-14mer ONs, as it inhibits electrostatic attraction and decreases retention (see the example of shorter ONs in Fig. 1B). The most sufficient results were obtained for 200mM ammonium acetate in MP A and 50mM ammonium acetate in MP B (see the results in Fig. S4), however, the peaks were very broad. Moreover, this approach excludes the use of MS detection. Not surprisingly, no effect of MP pH on retention and resolution was observed (see Fig. 1B), since both column and analytes are permanently charged in the tested pH range.

Besides the separation of ONs differing in length, the separation of ONs with the same length, but different sequence, was also performed (Fig. 2). For this purpose, a mixture of 3mer A and B (mix 3mer); a mixture of 4mer A and B (mix 4mer); a mixture of 5mer A and B (mix 5mer); a mixture of 12mer A and B (mix 12mer), and mixture of 13mer A and B (mix 13mer) were prepared. Regarding the analysis of shorter ONs (3-5mer) using the amide column, the complete resolution was observed only for mix 4mer. The resolution of 4mer A and 4mer B was baseline for most of the tested conditions, independent of MP pH, but it increases with increasing ammonium acetate concentration. The same trend was observed while using a zwitterionic column, i.e., only mix 4mer exhibits baseline resolution in all tested conditions. However, no pH or concentration effect was observed.

**Fig. 2**

This phenomenon can be explained by a detailed inspection of differences in sequences between two ONs in each of the mixtures (see Table 1 and Fig. 2). In mix 3mer, the representation of individual nucleobases is the same (2×A;1×U), only their position is different. In mix 5mer, 4 of 5 nucleobases in sequence are the same (2×A;1×U; 1×C; regardless of position, see sequence in bold in Table 1), and the difference is connected with adenine (5mer A) and uracil (5mer B). However, both these nucleobases can form two hydrogen bonds, and their polarity is similar. Finally, mix 4mer contains two ONs differing also only in one nucleobase (2×A;1×U; regardless of position), but this nucleobase is adenine for 4mer A and cytosine for 4mer B. Adenine and cytosine differ in the number of possible hydrogen bonds (2 and 3, respectively) and show different polarity. This observation is supported by the work of Gilar et.al., where higher resolution of *n* and *n*-1 species was observed in the case of loss of nucleobase with three hydrogen bonds compared to two hydrogen bonds.

For longer ONs (mix 12mer and mix 13mer), the resolution was observed only for mix 12mer. The explanation is the same as for shorter ONs – 12mer A and 12mer B differ in cytosine and uracil (three *vs*. two hydrogen bonds, different polarity), while 13mer A and 13mer B differ in adenine and uracil (two hydrogen bonds, similar polarity).

Additionally, the effect of MP composition on peak width (and peak shape) has been investigated. The results for the amide column (Fig. S5) show that no significant trend concerning the effect of ammonium acetate pH or concentration on peak width was observed. On the one hand, for the zwitterionic column (Fig. S6), the impact of ammonium acetate pH on peak width is negligible, while with increasing concentration, the peak width increases. The fact that no trend for the amide column was observed can probably be attributed to partial separation of diastereomers (more pronounced for short ONs), thus the data evaluation is more challenging since the peak widths are strongly affected by this effect. On the other hand, zwitterionic SP exhibits suppression of diastereomer separation (at least the visible partial diastereomer separation) (Fig. S7).

To improve chromatographic performance, we tested the effect of gradient on the resolution of critical ONs (mix 12-14mer) using an amide column. The changes in initial conditions (maintaining the time and final conditions), final conditions (maintaining the time and initial conditions), and gradient time (maintaining the initial and final conditions) were performed. The results are summarized in Fig. S8. No significant changes in resolution were observed for different gradients, which may support the theory of the on-off retention mechanism of these large biomolecules, as described in detail for IP RP LC [2].

To sum up the effects of MP composition on chromatographic performance, the amide column exhibited great robustness, i.e., almost no changes in retention and peak shape with changes of MP pH and buffer concentration. Even changing  the gradient conditions did not significantly affect method selectivity. These observations correlate with the fact that the amide column is neutral, and under tested conditions, the water layer at the stationary phase surface remains stable. On the other hand, for the zwitterionic column, a decrease in ON retention with increasing buffer concentration was observed, indicating application of electrostatic attraction between ONs and the stationary phase – both are permanently changed. Thus no differences between MP pH were observed.

### *3.1.2 The effect of temperature on retention and resolution of modified ONs*

The effect of temperature on retention and resolution of modified ONs was tested for the following values: 20 °C, 40 °C, 55 °C, and 70 °C. The results obtained for the amide column (Fig. S9) show that retention of shorter ONs decreases with increasing temperature, contrary to longer ONs. Additionally, a slight increase in resolution with increasing temperature was observed in the range 20 °C – 55 °C; however, for 70 °C the resolution decreases (which probably correlates with increased peak widths for this temperature, see Fig. S10). The zwitterionic column exhibits a significant increase in retention with increasing temperature, resulting in higher resolution of individual ONs (mix 3-5mer and mix 4mer) (Fig. S11).

Additionally, a significant decrease in peak width with increasing temperature is illustrated in Fig. S12. Regarding the effect of temperature on retention of ONs, it has been previously demonstrated that, contrary to RP LC, a decrease in retention with increasing temperature is not typical for HILIC [6,20]. Increased temperature increases the diffusion of analytes and thus facilitates their access to the water layer and the stationary phase surface, leading to higher retention. This phenomenon is more pronounced for zwitterionic SPs, where thicker water layer can be observed (charged groups have high affinity to water) and also for longer ONs, whose diffusion is more affected by temperature compared to shorter ones (short ones have naturally higher diffusion than the longer ones) [38].

Finally, the amide column was selected for routine analysis of modified ONs under HILIC conditions. It showed greater robustness compared to the zwitterionic column, i.e., small changes in chromatographic behaviour were observed with changes of MP composition and separation temperature. This fact allows us to use the simplest MP, namely 10mM ammonium acetate; pH 6.8 (unadjusted), to ensure the highest possible repeatability and feasibility. The separation of the mixture used for subsequent experiments under these conditions is shown in Fig. S13.

## *3.2. MS detection*

### *3.2.1. Optimization of ESI source parameters*

Tandem mass spectrometry (MS/MS) was used to quantify the studied ONs. Firstly, the full scan spectra were recorded, and precursor ions were selected for six selected ONs. Typically, there is a series of multiply deprotonated ions $[M\text{-}nH]^{n-}$ with specific charge state distribution in ESI. In the case of our study, only two signals were recorded at full scan spectra for 3-5-mer ONs and two/three signals for 12-14-mer ONs under HILIC conditions (Fig. S14). The selected precursor ions are summarized in Table 1. The optimization of temperature and gas flows in the ESI source was performed to obtain the highest possible MS response employing selected ion monitoring (SIM) peak areas for precursor ions. Six different parameters were tested and changed: IT, HBT, DLT, DGF, NGF, and HGF; their values were chosen based on the design of experiments using CCD. Two different sets of variables were studied: i) temperatures (IT, HBT, DLT); ii) gas flows (DGF, NGF, HGF). For a detailed description of the CCD procedure, see section 2.3.6.1. The results were used for response surface modelling, presented in Figures S15 (for gas optimization) and S16 (for temperature optimization), as three-dimensional response surface plots (raw data are presented in Table S1).

The results were very similar for all of the ONs, indicating similar behaviour during electrospray ionization. Regarding the gas flow, using the highest possible HGF (12 L min$^{-1}$) is beneficial for MS sensitivity (in terms of peak areas), while NGF should have values in the middle range (Fig. S15). Finally, DGF 7 L min$^{-1}$ was set as optimal. Analogously, the temperatures in the ESI source were optimized, and the results are summarized in Fig. S16. It can be concluded that increasing IT (up to 400 °C) leads to a significant increase in ONs SIM peak areas. The highest values can be reached using DLT 170 °C and HBT 275 °C.

### *3.2.2. Fragmentation and MRM transitions for tested ONs*

Quantitative analysis was performed using multiple reaction monitoring (MRM) for each ON. For this purpose, firstly, collision energies were optimized to obtain an appropriate fragmentation spectrum (Table 1). The goal was to record the highest possible intensity of ions characteristic of ONs. A trend typical for ONs fragmentation was observed: for low collision energies, signals of nucleotides were observed in the fragmentation spectra; an increase in collision energy leads to a significant fragmentation, resulting in the observation of low-mass ions derived from ribose and nitrogenous bases. Simultaneously, with the rise of collision energy, the intensities of product ions increase (leading to greater MRM peak areas). For short ONs (3-5mer), the ion with the highest intensity was *m/z*=134 originating from adenine, while for long ONs (12-14mer) it was the signal of uracil (*m/z*=111). Not surprisingly, for longer ONs, the higher collision energy has to be used (30eV for 3mer and 100eV for 14mer). Fragmentation spectra under the most optimal collision energy for each ON are illustrated in Fig. S17. Typical signals of fragmentation ions for tested ON are: *m/z*=79 (phosphate group) and *m/z*=95 (thiophosphate group), *m/z*=110 (cytosine), and *m/z*=150 (guanine).

### *3.2.3. Validation of HILIC-MS/MS method*

The developed HILIC-MS/MS method was validated to confirm its accuracy and repeatability. A five ONs differing in length were selected (4mer A, 5mer A, 12mer A, 13mer A, 14mer, Table 1). The validation parameters of the HILIC MS/MS method are collected in Table 2. The method showed excellent linearity over the tested concentration range with $R^2$ higher than 0.999. The graphical illustration of calibration curves is shown in Fig. S18. For short ONs, LOD and LOQ values were lower in comparison to longer ONs, i.e., LOQ = 0.020 μM for 4mer A and LOQ = 0.625 μM for all long ONs (Table 2). One of the possible reasons may be related to the basics of the ESI MS mechanism – for longer ONs, the signal is divided into a higher number of charged states. Thus the intensities for individual charge states are lower. Reduction of LOQ  and increasing the length of ONs may result from alkali metal adducts, which reduce ONs signal intensity. This effect is known in HILIC analysis of ONs and has been described several times as the main disadvantage of HILIC application for therapeutic ONs analysis [6,9]. Probably longer ON has a greater potential for adduct formation and consequently reduces the sensitivity. An additional reason for the sensitivity differences between ONs with various lengths may be related to the composition of MP. A simple test of the impact of ACN content in MP on the precursor ions signal intensities was performed (without the column). The concentration of ammonium acetate was kept constant (10 mM, pH 6.8), while the content of ACN was changed. While for short ONs with the decreasing amount of ACN in MP (from 80 to 16%), the MS response decreases, for long ONs, the impact of ACN content in MP was negligible.

In most cases, the relative standard deviation (RSD) for peak areas during intraday experiments did not exceed 5%. For interday precision for some ONs, the RSD values were around 10%. Regarding the retention times, RSD values did not exceed 2% for intraday and interday precision measurements (data not shown) provingstationary phase stability andreusability. Moreover, repeatability of quantitative analysis was excellent because a biointert column was used. During all the measurements, no carryover effect was observed.

**Table 2**

### *3.3. Development of hydrophilic interaction-based dSPE*

The idea of developing hydrophilic interaction dSPE (i.e., dSPE under HILIC conditions) was based on a recent publication of MacNeil [34], where hydrophilic-phase extraction of ONs was introduced. To follow this idea, we first tried to reproduce the proposed procedure with a commercially available aminopropyl SPE adsorbent. We observed complete adsorption of ONs on the adsorbent surface; however, the recovery in proposed conditions (70/30/2 (*v/v/v*) water/ACN/conc. ammonium hydroxide) was extremely low (below LOD). Since, in our opinion, the adsorption/desorption mechanism is driven mainly by weak ion-exchange between negatively charged ONs and positively charged amino-propyl, we tried to elute adsorbed ON by increasing the ionic strength, but using 100mM sodium perchloride led to the recovery below 5%. Moreover, increasing the pH of the solution used for elution also provided low recoveries. Thus, we decided to utilize a sorbent containing not only aminopropyl groups (necessary for the weak ion-exchange), but also amide groups (reducing the number of strong interaction sites). This combination ensures the polar nature of the adsorbent that is typical for HILIC and gives promise for developing hydrophilic interaction-based extraction.

For this purpose, we synthesized a completely new adsorbent for extraction, in which amide groups played a key role. Its structure differs from that of Waters' amide SP. Still, its interaction with ONs should be similar, so that extraction is based on hydrophilic interactions without using amines (ionic pairs) or high concentrations of inorganic or organic salts (ion exchange).

### *3.3.1. Amide adsorbent synthesis and characterization*

As a result of the synthesis, the silica gel was modified with propylamine groups, which were converted into acetic acid amide in the second stage of the reaction. The structure of the adsorbent is presented in Fig. 3 and S18. The material was the subject of elemental analysis, containing 1.48% hydrogen, 6.23% carbon, and 1.57% nitrogen. The aminopropyl silica (after the first s synthesis step) contains 1.12% hydrogen, 3.94% carbon, and 1.77% nitrogen. The coverage density of initial amine groups was 3.02 µmol/m$^{2,}$ whereas the final coverage density of amides was 2.71 µmol/m$^2$. We believe that most of the amino groups were blocked with amide.

The structure of the adsorbent was confirmed by obtaining a $^{13}$C NMR spectrum (Fig. S19). The formation of an amide bond on the surface of the adsorbent is confirmed by the signal of the carbonyl group present at 175 ppm. Notably, the carbon atoms coupled to nitrogen in the amine and amide ligand differ significantly in chemical shift, 55 ppm and 45 ppm, respectively. The ratio of these signals also confirms that most amine ligands have been converted to amide. The signal indicated as (2) on the spectrum corresponds to two types of carbon atoms, as shown in the figure.

### *3.3.2. Development of a hydrophilic interaction dSPE method with the use of an amide sorbent*

#### *3.3.2.1. Optimization of adsorption/desorption conditions for dSPE*

18mer and 20mer (Table 1) ONs were used during the development of the extraction procedure. It is worth noting that for proper recovery evaluation, the standard solution has to be dissolved in the same solvent as the eluted sample. To calculate recovery (during the extraction method development), we performed the experiments by measuring the standard of the same ON concentration dissolved in desorption solutions. The UV peak area was approximately 30% lower for the standard dissolved in the solvent used for adsorption compared to the solvent used for desorption.

The adsorbent conditioning was done using MEOH, water, and the adsorption solution. Several solvents for adsorption differing in ACN content (90 %, 80 % and 70 % of ACN) and pH (pH unadjusted vs. addition of formic acid) were tested and compared in terms of the adsorption efficiency. Complete adsorption of ONs was observed for all tested solutions. The electrostatic attraction between the positively charged sorbent surface and negatively charged ON leads to the adsorption at acidic pH. The aminopropyl ligands (pKa~10.2) are protonated even in pure water; therefore, the ONs adsorption is complete not only for acidified solutions but also for water. However, for subsequent experiments, an adsorption solution consisting of water/ACN/formic acid in a 30:70:1 (*v/v/v*) ratio was selected; consequently, hydrophilic interaction mode was used, but with the lowest ACN content. For the washing step, a mixture of adsorption solution with high ACN content was used (adsorption solution/ACN 20/80 (*v/v*).

Regarding the ON elution, desorption solutions differing in ACN content (75 %, 50 %, 30 % and 10 %) and pH (unadjusted vs. addition of ammonium hydroxide) were compared. The results summarized in Fig. 3 show that basic pH is necessary for ON desorption. These conditions probably lead to a reduction of the positive charge of the residual aminopropyl groups. Thus, ON does not interact with the sorbent by electrostatic attraction and can be desorbed using the solution with greater water content (reducing polar interactions, e.g., hydrogen bonding). The ON recovery did not exceed 4 % without the addition of ammonium hydroxide, contrary to the mixtures of $H_2O$/ACN containing ammonium hydroxide (~80 %) (Fig. 3). Moreover, recoveries were comparable for all tested ACN contents, and only 2 % decrease in recovery with increasing amount of ACN was observed (Fig. 3). The only exception was 75% ACN, where no ON desorption was observed, probably due to the change of pH by the high amount of ACN. In addition, under these conditions (typical for HILIC), the adsorption of ONs on the sorbent surface (caused by partitioning) can occur. Preliminary, for future experiments, a desorption solution consisting of water/ACN/ammonium hydroxide 70/30/2 (*v/v/v*) was selected to maintain HILIC conditions (highest possible ACN content). However, we would like to point out that for water/ACN/ammonium hydroxide 50/50/2 (*v/v/v*), recovery was still high and acceptable ~78 %.

**Fig. 3**

The sorption capacity was calculated according to the equation given in the experimental section, and it is equal to 54 µg ON/mg, making it attractive for real samples.

In summary, the extraction method that was developed is quick and easy to perform. It is characterized by high recovery of modified therapeutic ONs and high reproducibility. Our method does not require ion-pair reagents (amines) or high concentrations of organic or inorganic salts. Furthermore, the extracts are in a solvent suitable for direct HILIC MS analysis.

#### *3.3.2.2. The attempt to apply an amide adsorbent in SPE*

Additionally, we packed our sorbent into the SPE cartridges to check the potential of the developed dSPE procedure in SPE. The same solvents but greater volumes were used (e.g., 200 µL for ON loading). Moreover, the gravity flow was applied during sample load, washing, and elution. ONs were completely adsorbed during sample load. Regarding the elution step, the recovery depended strongly on the sample volume, and utilization of 200 µL enabled obtaining only 25% of recovery. Elution of ON by a greater volume (600 µL) leads to recovery around 45 %, while applying 3×200 µL of desorption solution increased the recovery to around 75 %. Elution using three small portions of solvent maximizes ON recovery and enhances the solvent's ability to overcome interactions with amide adsorbents. On the other hand, the need to use a large volume of solvent for elution may result from the longer contact time between the sample and the solvent needed during elution. This is a typical effect for a hydrophilic interaction system where a necessary equilibrium usually needs time (e.g., during HILIC analysis). Even though the final SPE recovery was comparable to dSPE, it is worth noting that ON was diluted during the procedure (three times greater volume of elution solvent compared to sample load). Thus, other preconcentration steps (e.g., evaporation) before HILIC analysis would be necessary, which could lead to a decrease in recovery and prolongation of the procedure. For these reasons, we decided to use dSPE. Still, it should be emphasized that the developed hydrophilic interaction-based extraction procedure can also be used in SPE, which is another advantage of this method and an undoubted benefit.

#### *3.3.2.3. Optimization of dSPE procedure for mixture of modified, therapeutic 4-14mer ONs*

The developed and optimized hydrophilic interaction-based dSPE procedure was applied to the mixture of modified RNA ONs with different lengths, namely 4mer A, 5mer A, 12mer A, 13mer A, and 14mer (Table 1). Complete adsorption was reached for all of these ONs, and recoveries were higher than 80 % with RSD <2% (procedure 1 in Table S2). However, divided peak shapes were registered for the two shortest ONs (4mer and 5mer). The reason is that the solution used for dSPE elution contains a lower amount of ACN (30 % ACN) than the MP at the time of 4mer and 5mer elution (~70 % ACN). In HILIC, the different elution strengths of the sample and MP cause broad and divided peak shapes. For that reason, we decided to change the extraction procedure and various approaches were tested: i) elution to lower volume (50 µL instead of 100 µL) followed by 1:1 dilution with ACN - recovery around 65 % (RSD<2%); ii) evaporation of extract and reconstitution with MP for HILIC - recoveries up to 20% with low reproducibility; iii) change in desorption solution to water/ACN/ammonium hydroxide 50/50/2 (*v/v/v*) and additional dilution 1:1 with ACN. The last approach appeared most efficient despite the extract dilution (procedure 2 in Table S2). This approach was necessary for the proper quantification of short ONs eluting at the beginning of the gradient.

Surprisingly, a decrease in recovery for two of the tested ONs was observed (12mer A,14mer) (Table S2). Still, the overall, final recoveries were high (79.3 -93.4 %) with high repeatability (RSD for each ON < 5%), proving the utility of the developed hydrophilic interaction-based dSPE.

### *3.4. Application of developed hydrophilic interaction-based procedure to the extraction, separation, and quantification of therapeutic ONs in enriched human serum samples*

Our goal was to develop a hydrophilic interaction-based dSPE procedure followed by HILIC MS/MS analysis suitable for serum samples containing therapeutic ONs metabolites, i.e., nusinersen metabolites with various lengths. Therefore, our procedure was applied to serum enriched with five selected, modified ONs (4mer A, 5mer A, 12mer A, 13mer A, and 14mer, Table 1). The serum was first diluted with water in a ratio of 1:3 (serum:water) and enriched with ON to reach a 10 μM concentration. It was reported previously that direct application of the SPE and dSPE to diluted serum without any additional sample purification leads to the co-adsorption and co-desorption of proteins together with ONs, because of their very high affinity to each other [22,35]. Therefore, an initial step for protein removal has to be applied, usually LLE using a mixture of phenol/chloroform/isoamyl alcohol. This method does not always allow the removal of all the proteins from the serum sample. Moreover, after the first LLE, three or four additional LLEs using chloroform are required to remove residual phenol (see schematic procedure in Fig. S2). The whole procedure (several LLE steps) is time-consuming and causes loss of ONs, without guaranteeing proper purification. In our study, we utilized the developed hydrophilic interaction-based dSPE method to purify serum extracts obtained after the first LLE (Fig. 4A), to remove proteins (without any additional LLE extraction). We showed that our extraction method is suitable for removing phenol remaining in the sample after LLE, since the amide adsorbent is selective for ONs and phenol does not interact with its surface (see Fig. 4B). Residual phenol is washed during the washing step (see Fig. 4C), and thus, after desorption, no phenol and proteins are detected (see Fig. 4-CD. The quantification of the extraction procedure recovery was done by MRM spectrum measurements and evaluation (see Fig. S20). Fig. 4E shows the chromatogram for a mixture of ONs standards. This approach (LLE/dSPE in hydrophilic interaction mode) significantly simplifies the procedure, leading to smaller ONs losses and reducing chloroform consumption. The recoveries and matrix effect are summarized in Fig. 5. Values higher than 75 % with RSD < 5% were observed for all ONs. For all tested ONs, the matrix effect was in the range 95.1 % and 98.6 %, which means that the matrix causes only a slight decrease in signal intensity (=decrease in ionization). The above results suggest that the developed method is selective and repeatable, provides high recoveries and low matrix effect, making it suitable for extracting ASOs metabolites from serum samples. Nevertheless, dSPE is prone to biases, which can be overcome by precisely removing the liquid layer (to not lose ONs/decrease their concentration and without taking sorbent particles) and precise mixing and centrifuging. Moreover, the complexity of serum samples for the extraction of ONs using SPE or dSPE is also not an issue, since proteins and lipids are removed using LLE [21].

**Fig. 4**

**Fig. 5**

### 3.5. Comparison of *dSPE results with previously published procedures*

To gain a better overview of the dSPE recovery and to understand the main advantages of the presented methodology, Table 3 comparing the previously published studies involving ONs extraction from serum samples is presented. In this comparison, only SPE and dSPE methods for ONs are included, except for microextraction methods and methods with magnetic nanoparticles. It can be concluded that our method provides comparable recovery to other methods, maintaining simplicity, reproducibility, and speed. Moreover, in our method, no salt preparation for adsorption/desorption solutions is needed (only acid/base) and thanks to the desorption to a mixture of ACN/water, the sample is ready for direct injection and analysis by HILIC (only for very short ONs, additional 2x dilution by ACN is required). Regarding the evaluation of the greenness of our methodology, calculations using the AGREE and AGREEprep tools were performed. The calculated score is below 0.5 in both cases (see Fig. S21). In the case of AGREE, the score for the entire analytical procedure is 0.47, and in the case of the sample preparation method alone, the score is 0.38. These results clearly indicate that this method cannot be described as green. In the case of sample preparation, this is due to the use of LLE extraction with phenol, chloroform, isoamyl alcohol, and acetonitrile. However, it should be noted that this is the only method that allows proteins to be precipitated without co-precipitating oligonucleotides. Thus, in this case, the sensitivity of the assay is more important than the greenness of the method. Chromatographic separation in the HILIC system also lowers the AGREE score due to large volumes of ACN. In defense of the method, it should be noted that it is performed in a UHPLC system with reduced flow (reduced column diameter). An alternative was an ion-pair system using amine and HFIP, which seems even less green.

## 4. Conclusions

We developed a first hydrophilic interaction-based dSPE and LC-MS procedure for the extraction, separation, and determination of modified, therapeutic ONs. The method uses a newly synthesized amide-based adsorbent for dSPE and HILIC MS/MS analysis of the extracted ONs.

Based on the results obtained in the retention study, it can be concluded that the zwitterionic SP provides excellent selectivity and chromatographic performance (suppression of diastereomer separation) for short ONs. Longer ONs require a high salt concentration for elution, which disables using MS detection. Despite partial diastereomer separation, the amide column provides at least partial separation of all tested, modified ONs differing in length. Only slight changes in retention times and peak shapes were observed with MP pH, concentration or temperature changes, which proved to be an advantage. For further investigation, an amide column at 40 °C and 10mM ammonium acetate, pH 6.8 was chosen. Regarding the MS/MS analysis, the collision energy must be carefully optimized together with ion source parameters. Only this approach will ensure the highest possible sensitivity. However, our research has confirmed effects mentioned earlier by other scientists: HILIC MS/MS has lower sensitivity than IP RP LC. Still, no ion-pair reagents are used, and there is no contamination of MS. At the same time, we have also developed a simple and valuable method for extracting modified ONs without using ion-pair mode, but instead using hydrophilic interactions, adsorbents, and solvents analogous to those used in HILIC. The newly synthesized amide-based adsorbent containing amino-propyl ligands was used. It was shown that adsorption/desorption is based on a mixed mechanism (involving weak ion-exchange and hydrogen bonding). The developed and optimized procedure was successfully applied to enriched serum samples containing five modified ONs differing in length. The recoveries for all ONs were higher than 75% with RSD <5 %. To summarize, the whole procedure, hydrophilic interaction-based dSPE and HILIC MS/MS may be straightforwardly applied to the real patient samples. The only risk and weak point of the developed procedure is the sensitivity (which may prove insufficient for some applications involving the detection of ASO metabolites in serum samples). However, this may be overcome by the concentration of extracts after LLE/dSPE, before HILIC MS/MS. Nevertheless, the developed HILIC-based extraction, separation, and quantification method enables: the separation of modified ONs; detection and quantification; easy, repeatable, and efficient (high recovery) extraction with low biological matrix interference. Such comprehensive studies and their results have been presented for the first time for therapeutic ONs.

## 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.

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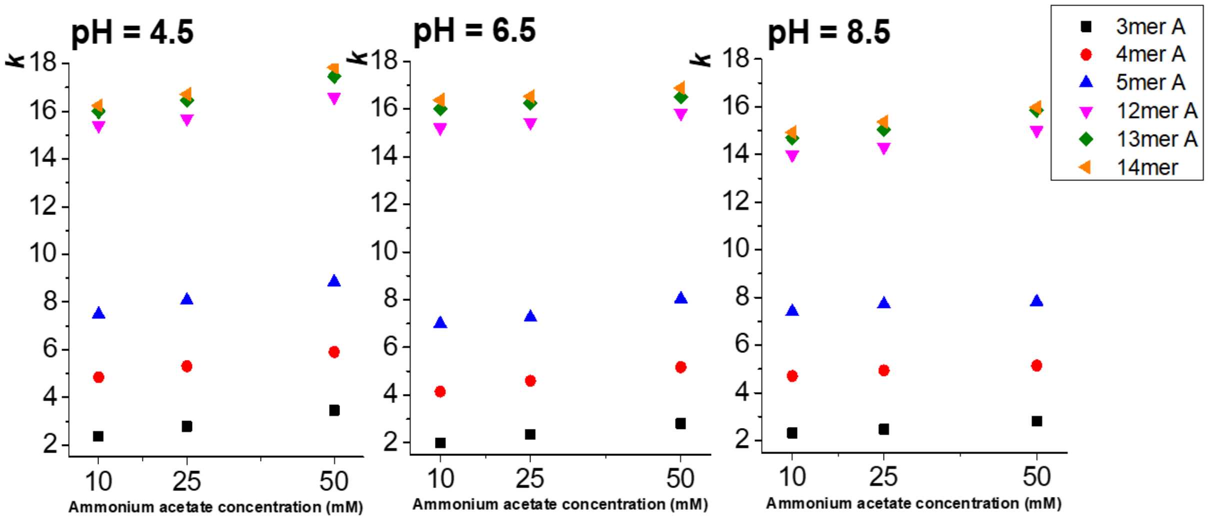
*A: Amide column*



*B: Zwitterionic column*

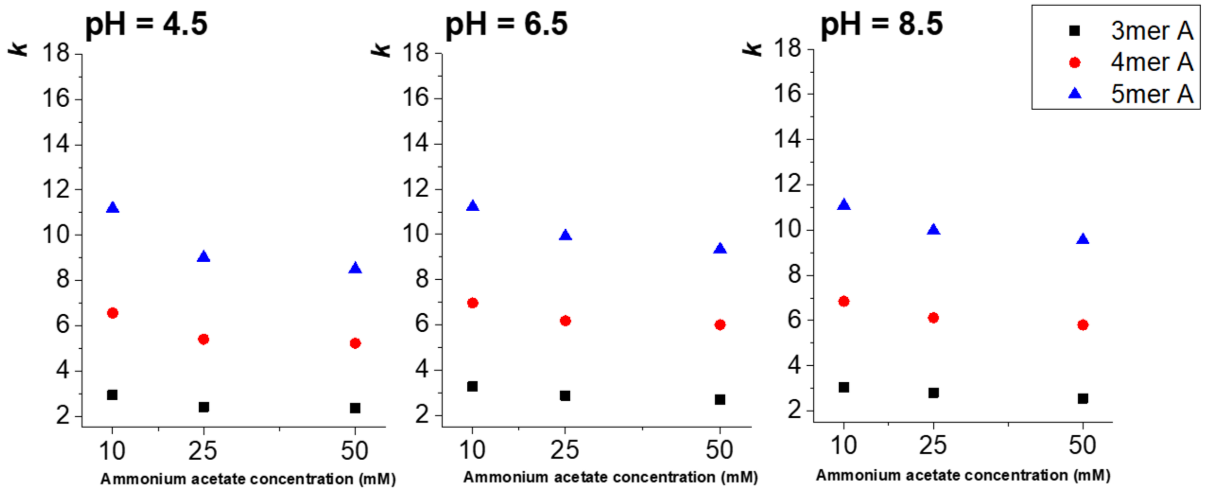


**Fig. 1:** The effect of mobile phase composition (pH and ammonium acetate concentration) on retention and resolution of ONs. Columns: Acquity Premier BEH Amide (A) and Atlantis Premier BEH Z-HILIC (B). Mobile phase composition: MP A: 10mM/25mM/50mM ammonium acetate; pH 4.5/pH 6.5/pH 8.5 in water; MP B: ACN/water (80/20; *v/v*) with the same amount of ammonium acetate as in MP A. Gradient elution: 0-10 (min), 100-64 (%B); column temperature: 40°C.

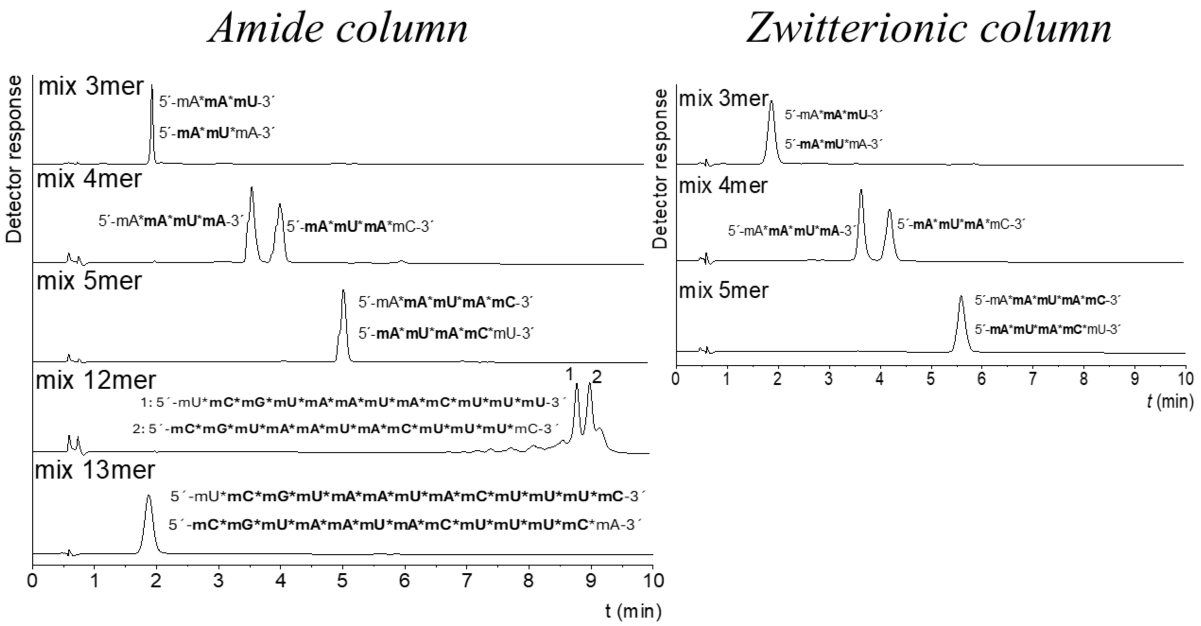


**Fig. 2**: Separation of mixtures of ONs with the same length. Mobile phase composition: MP A: 10mM ammonium acetate, pH 8.5 in water; MP B: ACN/water (80/20; *v/v*) with the same amount of ammonium acetate as in MP A. Gradient elution: 0-10 (min), 100-64 (%B); column temperature: 40°C. Columns: Acquity Premier BEH Amide and Atlantis Premier BEH Z-HILIC.

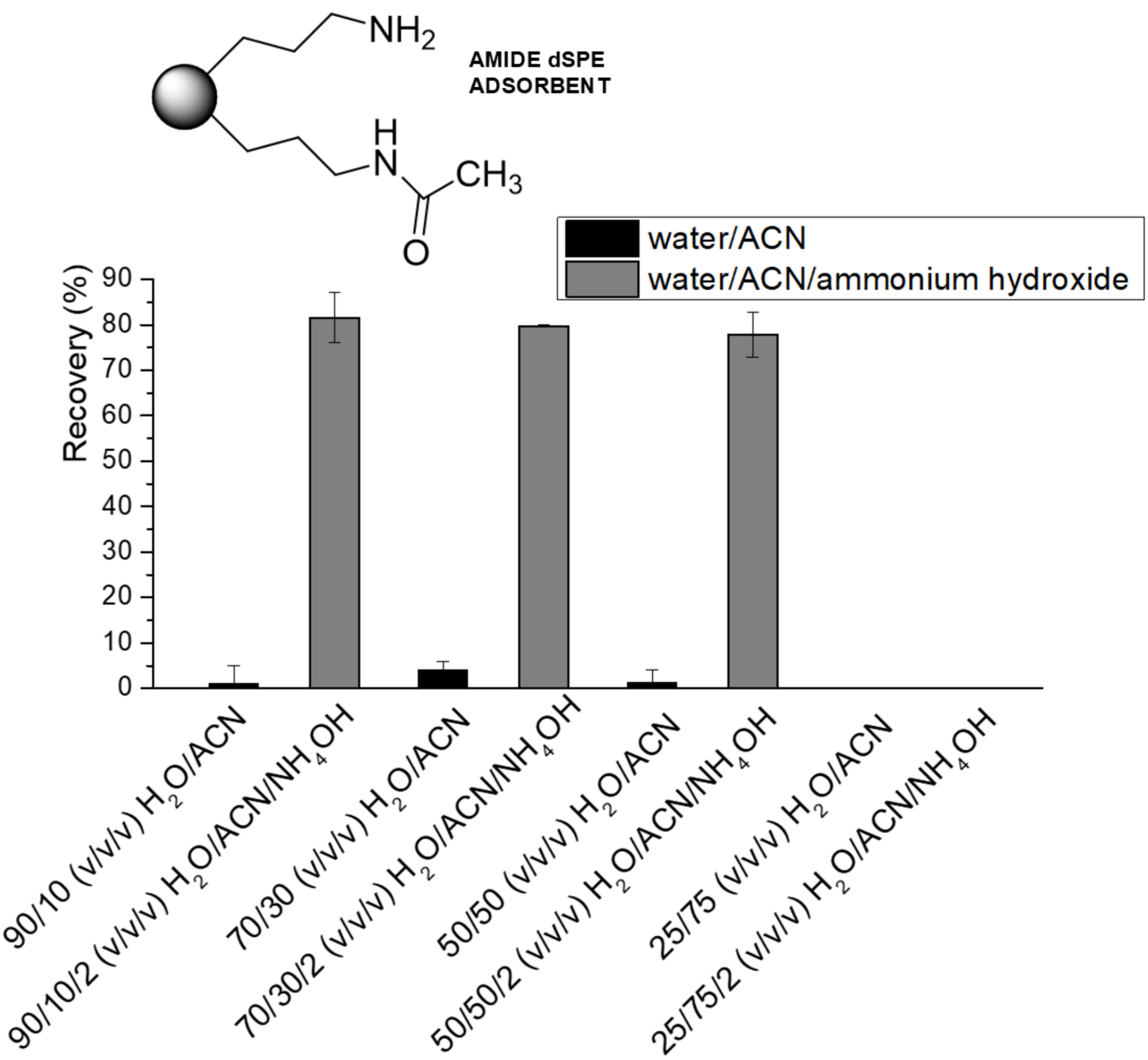


**Fig. 3**: The effect of desorption solution on ONs recovery. Peak areas measured by UHPLC. Mobile phase composition: MP A: 10mM ammonium acetate in water; pH 6.8; MP B: 10mM ammonium acetate in 80/20 ACN/water (v/v). Gradient elution: 0-6 (min), 80-37 (%B). Temperature: 40°C, column: Acquity Premier BEH Amide.

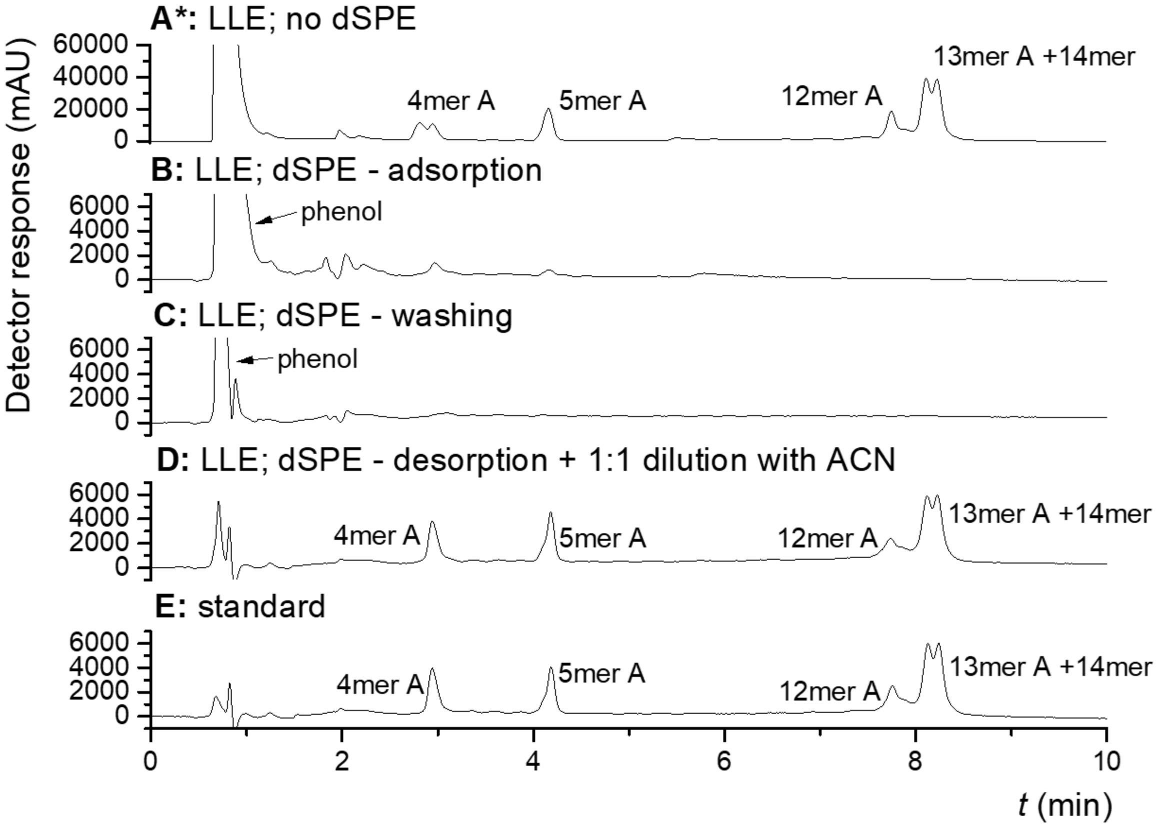


**Fig. 4:** The application of LLE and dSPE for the extraction of therapeutic, modified ONs from fortified serum samples using UV detection. Chromatograms obtained for supernatant solutions at different stages of the extraction procedures. Mobile phase composition: MP A: 10mM ammonium acetate in water; pH 6.8; MP B: 10mM ammonium acetate in 80/20 ACN/water (v/v). Gradient elution: 0-6 (min), 80-37 (%B). Temperature: 40°C, column: Acquity Premier BEH Amide. *Different detector response for (A) is caused by the fact that this sample was not diluted by ACN, which is also the reason for bad peak shape of 4mer.

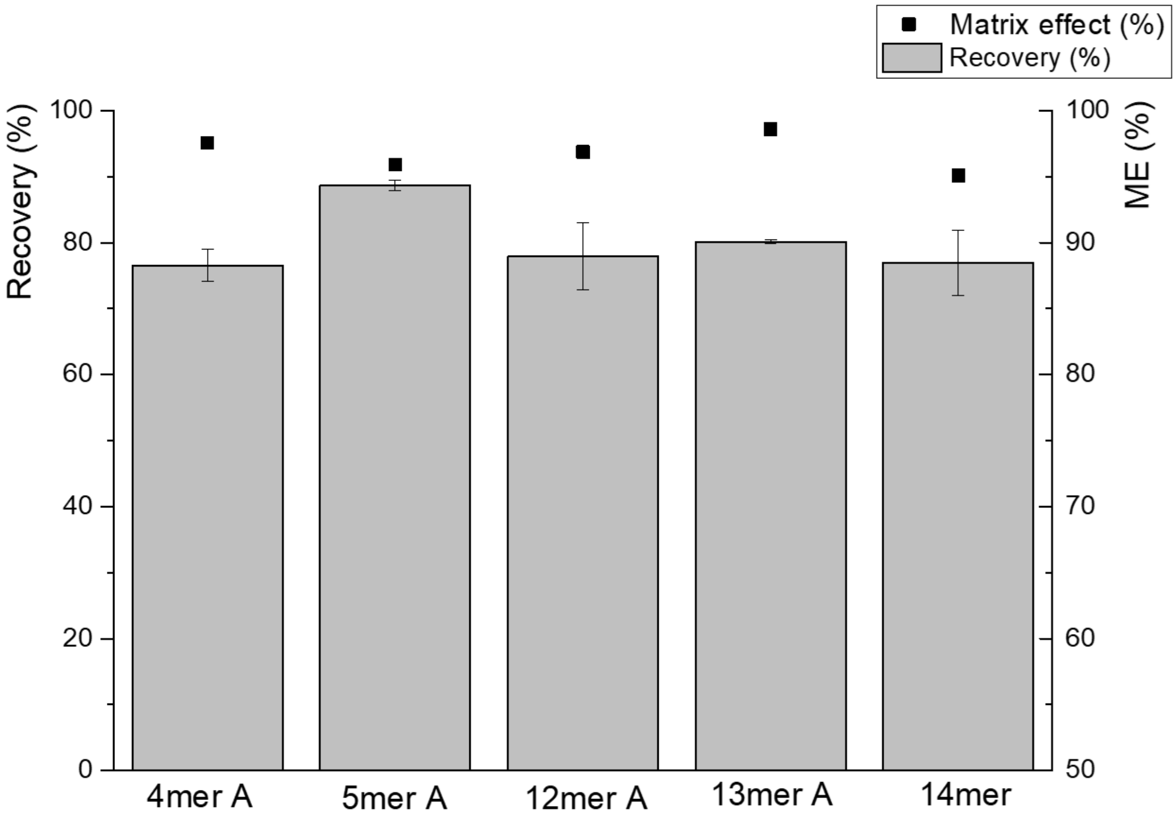


**Fig. 5:** Recoveries and matrix effects for dSPE procedure applied on enriched plasma. Peak areas measured by UHPLC. Mobile phase composition: MP A: 10mM ammonium acetate; pH 6.8 in water; MP B: 10mM ammonium acetate in ACN/water (80/20; *v/v*). Gradient elution: 0-10 (min), 100-64 (%B). Temperature: 40°C, column: Acquity Premier BEH Amide.

**Table 1**: List of tested ONs, their molecular masses, selected precursors, fragmentation ions, and optimized collision energies

| NAME | SEQUENCE | USED FOR | MOLECULAR MASS (Da) | PRECURSOR ION (m/z) | CHARGE STATE | PRODUCT ION (m/z) | COLLISION ENERGY (eV) |
|---|---|---|---|---|---|---|---|
| 3mer A | 5´-mA***mA*mU**-3´ | retention study, validation | 977 | 487.1 | -2 | 134 | 30 |
| 3mer B | 5´-**mA*mU***mA-3´ | retention study | 977 | | | | |
| 4mer A | 5´-mA***mA*mU*mA**-3´ | retention study, validation, dSPE from enriched serum | 1336 | 666.8 | -2 | 134 | 50 |
| 4mer B | 5´-**mA*mU*mA***mC-3´ | retention study | 1312 | | | | |
| 5mer A | 5´-mA***mA*mU*mA*mC**-3´ | retention study, validation, dSPE from enriched serum | 1671 | 834.3 | -2 | 134 | 60 |
| 5mer B | 5´-**mA*mU*mA*mC***mU-3´ | retention study | 1648 | | | | |
| 12mer A | 5´-mU***mC*mG*mU*mA*mA*mU*mA*mC*mU*mU*mU**-3´ | retention study, validation, dSPE from enriched serum | 4064 | 1353.4 | -3 | 111 | 90 |
| 12mer B | 5´-**mC*mG*mU*mA*mA*mU*mA*mC*mU*mU*mU***mC-3´ | retention study | 4063 | | | | |
| 13mer A | 5´-mU***mC*mG*mU*mA*mA*mU*mA*mC*mU*mU*mU*mC**-3´ | retention study validation, dSPE from enriched serum | 4399 | 1098.3 | -4 | 111 | 100 |
| 13mer B | 5´-**mC*mG*mU*mA*mA*mU*mA*mC*mU*mU*mU*mC***mA-3´ | retention study | 4423 | | | | |
| 14mer | 5´-mU*mC*mG*mU*mA*mA*mU*mA*mC*mU*mU*mU*mC*mA-3´ | retention study, validation, dSPE from enriched serum | 4759 | 1188.4 | -4 | 111 | 100 |
| 20mer | 5´-ACTGACTGACTGACTGACTT-3´ | dSPE procedure development – adsorption and desorption conditions | 6092 | | | | |
| 18mer | 5´-GGTCGTAATACTTTCACT-3´ | dSPE procedure development – sorption capacity, SPE, dSPE of enriched serum | 5465 | | | | |

0 * = phosphorothioation; m = 2´-O-methylribose; sequence in bold is the same part from A and B ON

**Table 2:** Calibration curve and validation parameters

| ON | Calibration curve equation | $R^2$ | LOD [µM] | LOQ [µM] | LOD [µg] | LOQ [µg] | Intraday 10 [µM] RSD % | Intraday 3.3 [µM] RSD % | Intraday 1.1 [µM] RSD % | Interday 5 [µM] RSD % | Interday 2.5 [µM] RSD % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4merA | y = 179293x - 15042 | 0.9991 | 0.005 | 0.015 | 0.013 | 0.05 | 1.34 | 3.36 | 4.33 | 10.21 | 10.40 |
| 5merA | y = 121085x - 7879.9 | 0.9992 | 0.005 | 0.015 | 0.016 | 0.07 | 1.58 | 3.70 | 5.27 | 0.61 | 1.94 |
| 12mer A | y = 721.14x - 60.462 | 0.9994 | 0.156 | 0.470 | 1.27 | 5.08 | 2.81 | 4.66 | 4.98 | 2.16 | 4.10 |
| 13mer A | y = 2920.9x - 567.15 | 0.9990 | 0.156 | 0.470 | 1.37 | 5.50 | 2.36 | 4.41 | 4.64 | 10.18 | 5.42 |
| 14mer | y = 2615.5x - 400.13 | 0.9990 | 0.156 | 0.470 | 1.49 | 5.95 | 2.08 | 4.18 | 4.87 | 5.55 | 8.94 |

injection volume 2µl; **intraday precision**: 7 injections at three concentration levels; **interday precision**: 1st, 3rd and 5th day, 5 injections for each, two concentration levels

**Table 3:** Comparison of various solid-phase extraction methods for ONs

| Sorbent type | Analytes | Recovery (%) | Reference and notes |
|---|---|---|---|
| **OUR METHOD**<br>silica +aminopropyl + amide | 3-14mer modified ONs (PS, ME) | 75-89% from enriched serum | + fast, reproducible<br>+ easy to operate (no salts preparation)<br>+ high recovery<br>+ sample after desorption ready to HILIC analysis |
| silica + hybridizable complementary oligonucleotides | umodified ONs; modified ONs (PS, ME, MOE) | 40-65% from enriched serum | [22,39]<br>- need of special adsorbent for individual ONs<br>- time consuming (long desorption at high temperature)<br>+ very selective |
| silica + amino + carboxyl groups | unmodified ON and PS ON | 80-94% from enriched serum | [22,32]<br>- need of additional dilution by organic solvent prior HILIC<br>+ fast, reproducible<br>+ easy to operate (preparation of salts for adsorption and desorption)<br>+ high recovery |
| unmodified silicagel | umodified ONs; modified ONs (PS, ME, MOE) | 45-50% from enriched serum | [33]<br>- low recovery<br>+ fast, reproducible<br>+ easy to operate (preparation of salts for adsorption and desorption) |
| silica + aminopropyl | 22mer RNA | 60% from enriched serum | [34]<br>- low recovery<br>+ fast, reproducible<br>+ easy to operate (no salts preparation) |
| Oasis HLB and Clarity OTX® | nusinersen | 66-76% from rabbit plasma | [40]<br>- using of IP agents (MS contamination, more complicated for preparation)<br>+ fast, reproducible |
| weak anion-exchange | PS ONs | 60-80% from enriched plasma | [41]<br>- lower recovery<br>+ fast, reproducible<br>+ easy to operate (preparation of salts for adsorption and desorption) |

phosphorothioate (PS), 2′-O-methyl (ME) and 2′-O-(2-methoxyethyl) (MOE)

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