Synthesis, characterization, and application of sorbents for the mixed-mode extraction of antisense oligonucleotides from cerebrospinal fluid samples

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Abstract

Background:

Antisense oligonucleotides are synthetic nucleic acid molecules capable of selectively modulating gene expression. As the number of approved antisense oligonucleotide therapies grows, reliable analytical procedures are needed to monitor their pharmacokinetics, metabolism, and safety. Current solid phase extraction strategies often suffer from limited recovery or poor reproducibility. Methods using non-polar, hydrophilic or anion-exchange sorbents, require application of ion-pair reagents, or high concentration of salts or long conditioning. On the other hand, the potential of sorbents with hydrophobic-hydrophilic properties used for mixed-mode extraction remains unexplored. Our study presents the first application of newly synthesized materials for isolation of therapeutic antisense oligonucleotides from cerebrospinal fluid samples.

Results:

Five silica-based sorbents modified with aminopropyl, aliphatic, aromatic, and dicarboxylic groups were designed, synthesized, and characterized. The functionalization of sorbents enables various interactions with oligonucleotides, namely electrostatic, hydrophobic, $\pi \ldots \pi$ interactions, and hydrogen bonding. A new, mixed-mode dispersive solid phase extraction procedure was developed using a central composite design, enabling a systematic evaluation of the factors governing antisense oligonucleotide desorption. The effectiveness of the procedure was assessed for oligonucleotides varying in chemical modification and length. Recoveries depended on both factors and were the highest shortmers with 2′-O-methyl modification. This effect is advantageous for the extraction of antisense oligonucleotide metabolites. Developed method was successfully used for the extraction of antisense oligonucleotides from cerebrospinal fluid enabling reproducible recoveries (40–93%). The matrix effects ranged from 89% to 100%. The highest recoveries were obtained for an oligonucleotide modified at two structural elements making the method advantageous for the extraction of oligonucleotides used in therapy.

Significance:

The proposed mixed-mode dispersive solid-phase extraction procedure provides a simple, fast, and reproducible approach for isolating of antisense oligonucleotides from cerebrospinal fluid. The method significantly simplifies sample preparation, since the procedure may be straightforwardly applied without additional purification steps. These results for the first time demonstrate the suitability and high analytical potential of hydrophobic-hydrophilic sorbents for the extraction of antisense oligonucleotides from biological samples.

Key words: antisense oligonucleotides; dispersive solid phase extraction; sorbents with mixed properties; central composite design; cerebrospinal fluid

1. Introduction

Antisense oligonucleotides (ASOs) are a group of therapeutic molecules that enable the modulation of gene expression by interacting with target RNA transcripts. They are chemically modified, short, synthetic nucleic acid sequences with specific binding to RNA sequences [] ASOs offer significant therapeutic potential for genetic diseases, including rare and difficult-to-treat neurological disorders, with several ASO-based therapies already approved for clinical use to date [1,2,3]. Since the FDA approval of the first ASO-based drug (fomivirsen, Vitravene), in 1998, the field has undergone continuous development, and ASOs are now active pharmaceutical ingredients in therapies of e.g. spinal muscular atrophy, and Duchenne muscular dystrophy [4,5,8-,11]. Although approved therapies are highly effective in treating a variety of genetic diseases, their introduction requires monitoring of pharmacokinetics, metabolism, and treatment safety. Consequently, the development of appropriate analytical strategies for ASO extraction and determination in biological samples is crucial [9,13,14].

Numerous ASO extraction methods have been used to date, including protein precipitation, enzymatic digestion of proteins, liquid-liquid extraction (LLE), hybridisation, and solid-phase extraction (SPE) [14,16–19]. The key factor in each of these attempts is the protein removal since ASOs have high affinity to these molecules, leading to low recovery and reproducibility [20]. The two most commonly used methods for this purpose are LLE and protein digestion. First one provides high (75-90%) recoveries and proteins denaturation [21–23]. Alternatively, application of proteinase K allows release of ON from protein complexes, yielding 80-95% recovery but with simultaneous introduction of amino acids and peptides impurities [24,25].

SPE is used to purify extracts after protein removal and for selective ASO extraction. Due to the different types of adsorbents used, extraction is carried out in different modes. SPE in ion pair (IP) mode, using adsorbents such as polymeric Oasis HLB, silica-based C18, C8, phenyl, yielded recoveries of ~70-90% for ASOs isolated from plasma or serum [17,18,26]. Nevertheless, this approach has certain disadvantages, namely, application of ion pair reagents, lengthy procedures because of time-consuming conditioning of sorbent and utilization of gravitational flow [17,18,26]. An alternative to SPE is microextraction by packed sorbent (MEPS), since it shortens the time, without losing recovery (~80-95%) and repeatability [17,27]. However, the limited sorbent amount restricts MEPS's applicability to small sample volumes [17,27]. SPE using strong (quaternary ammonium groups) and weak (N-alkylamine groups) anion exchangers proved to be advantageous in some ASO applications. They enable effective extraction of ASO from biological samples with recoveries of 80-90% and high reproducibility [28, 29]. However, application of strong anion exchangers (SAX) necessitates the need to use high concentrations of inorganic salts for elution, causing an additional desalting step before liquid chromatography with mass spectrometry (LC–MS) analysis [29]. Weak anion exchangers (WAX) offer a more MS-compatible alternative. ASOs are eluted using a low concentration of organic salts; however, careful selection of pH remains crucial [28]. The latest application to SPE of ASO is the hydrophilic interaction mode [30-32]. To date, silica, aminopropyl-modified silica, and amide phase have been used as sorbents. Elution was achieved using an acetonitrile/water mixture with 2% NH_4OH [31] or by changing the pH of ammonium acetate [30]. The method avoids the use of ion pair reagents and provide LC-MS-compatible extracts, but recoveries and reproducibility were lower compared to IP, SAX, WAX (~45-80%), especially for biological samples [30-32].

The other type of ASO extraction is hybridization, which relies on the selective binding of the target ASO to complementary strand immobilized on a support. The main advantages of this approach include low matrix effect, high selectivity, and satisfactory recoveries (~65-74%). However, this approach is associated with several limitations, high cost related to the design of individual probes and low efficiency in the case of short metabolites, which lose their ability to form stable duplexes with the complementary strand [33-35]. More recently, dispersive SPE (dSPE) has emerged as an alternative technique for ASO isolation. Using this approach, high recoveries of ASOs and their metabolites have been reported with weak anion exchangers, such as e.g. polymeric poly(ionic liquids), silica gel modified with amino and carboxyl groups, as well as with amino acid-based sorbents [16,36-38]. Importantly, dSPE doesn’t require the use of high concentrations of inorganic salts or IPR, and recoveries exceeding 80% have been achieved for serum samples [36-38]. Overall, dSPE-based methods offer a promising balance between simplicity and extraction efficiency for ASO isolation from biological matrices. This provide a strong foundation for further method development. Nevertheless, the potential of dSPE has not yet been full explored, especially in terms of the use of materials with mixed, hydrophobic-hydrophilic properties. For this reason, the main aim of our research was to explore the potential of these types of sorbents in ASO extraction. Consequently, our study aimed to design, synthesize, and characterize new sorbents with diverse properties to combine various types of interactions between the material and ONs. The idea was to increase the selectivity of ASO extraction in mixed mode and to simplify the procedure. The study used five sorbents functionalized with propylamine groups and a C12 aliphatic chain, benzoic acid, cholesterol, butanedioic acid, and pentanedioic acid. These materials and mixed-mode mechanism were used for the first time for the isolation of ASO.

2. Materials, reagents, and methods

2.1. Materials and reagents used during the study

Silica gel, used as the support for sorbent preparation, was purchased from Sigma-Aldrich (Poznań, Poland). This material had an average pore diameter of 60 Å, a pore volume of 0.7-0.85 mL/g, and a particle size range of 63-200 µm. The following reagents were used for chemical modification of the silica support: aminopropyltrimetoxysilane, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, ≥ 98%), 2-(N-morpholino), ethanesulfonic acid (MES, ≥ 99%), butanedioic acid, pentanedioic acid were obtained from Sigma-Aldrich (Poznań, Poland). Benzoyl chloride (≥ 99%), dodecanoyl chloride (≥ 99%), triethylamine (≥ 99%), cholesteryl chloroformate (≥ 99%) were purchased from Alfa Aesar (Haverhill, MA, USA). The organic solvents used during the synthesis, methanol and toluene, were purchased from J.T. Baker (Deventer, Netherlands).

Mobile phases and solutions used during dSPE were purchased from the following manufacturers: methanol (MeOH, Chromasolv®, gradient grade, ≥ 99.9%) from Honeywell (Charlotte, NC, USA); ammonium acetate (≥ 99%), acetic acid (≥ 99%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, ≥ 99.9%), N,N-dimethylbutylamine (DMBA, 99%) and hexamine (HA, 99%) from Sigma-Aldrich (St. Louis, Missouri, USA); 25% ammonium hydroxide solution (NH_4OH) and formic acid (FA, ≥ 99%) from Avantor (Gliwice, Poland). Deionised water was obtained from a Milli-Q system (Millipore, Burlington, MA, USA).

All tested ONs (Table 1) were supplied by Sigma-Aldrich (St. Louis, Missouri, USA) in lyophilized form. ON solutions of 100 mM were prepared by adding the proper volume of deionized water. Further dilutions of the solutions were carried out using a conditioning solution, as indicated in the manuscript.

Table 1. Oligonucleotides used during the study

Name	**Molecular mass [Da]**	**Type of modification**	**Sequence (5′→ 3′)**
Nu DNA	5465	Unmodified	GGTCGTAATACTTTCACT
NuPs18	5929	Phosphorothioate	GGUCGUAAUACUUUCACU
NuPs15	4783	Phosphorothioate	GGTCGTAATACTTTC
NuPs12	3837	Phosphorothioate	GGTCGTAATACT
NuPs9	2882	Phosphorothioate	GGTCGTAAT
NuPs6	1904	Phosphorothioate	GGTCGT
NuMe	5908	2′-O-methyl	GGUCGUAAUACUUUCACU
NuMePs	6181	2′-O-methyl and Phosphorothioate	GGUCGUAAUACUUUCACU

2.2. Instrumentation and chromatographic conditions

A Thermo Vanquish Flex (Karlsruhe, Germany) and Dionex UltiMate 3000 (Karlsruhe, Germany) ultra-performance liquid chromatography (UHPLC) systems were used to perform quantitative and qualitative analysis of ONs. Both chromatographs were equipped with diode array detectors (DAD). The detection wavelength was set to 260 nm. The injection volume was 1 µL. The autosampler temperature was set to 10°C. Data were collected using the Chromeleon 7 software. The study used a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Milford, MA, USA). The experiments were performed using IP PR UHPLC. The following mobile phase was used for the analyses of unmodified ASO: A1: an aqueous solution of 5 mM DMBA and 150 mM HFIP; B: methanol. The flow rate was equal to 0.3 mL/min, while the column temperature was 60°C. Analysis was performed at a gradient of 0-80% v/v of B in 6 min. For the analyses of modified ASO, the following mobile phase was used: A2: an aqueous solution of 5 mM HA and 150 mM HFIP; B: methanol. Flow rate was equal to 0.35 mL/min, while column temperature was 65°C. Analysis was performed at a gradient of 0-80% v/v of B in 6 min.

The pH of the prepared solutions was monitored using a CP-505 Elmetron pH meter (Zabrze, Poland). During extraction, samples were centrifuged using a Frontier Micro FC5515 centrifuge from OHAUS Europe GmbH (Nänikon, Switzerland).

Elemental analysis was performed using a Vario MACRO CHN analyser from ELEMENTAR Analysensysteme GmbH (Langenselbold, Germany). 13C NMR measurements were performed on a Bruker Avance III 700 MHz (Karlsruhe, Germany). FTIR spectra in the range $\tilde{\upsilon}$ = 4000 – 400 cm^{-1} were recorded on FT-IR Vertex 70V from Bruker (Billerica, MA, USA).

2.3. Synthesis and characterization of the sorbents

Sorbents with covalently bonded amino acids were prepared according to the following procedures. 10 g of the silica gel was dried at 180°C under vacuum for 6 hours. Then, a solution of aminopropyltrimethoxysilane (8 mL) in toluene (27 mL) was added to the dried silica gel. The reaction was carried out for 16 hours at 50°C. The excess of silane was removed by washing the sorbent with toluene, isopropanol, and methanol. The synthesized aminopropyl material (NH2) was dried at 60°C ± 5°C for 18 hours. In the second stage of the synthesis, NH2 material was used for the synthesis of five different sorbents.

An alkylamide sorbent (AP) was synthesized in the following way: NH2 material (1.5 g) was mixed with 1.2 mL of dodecanoyl chloride and 0.4 mL of triethylamine (in 10 mL of toluene). The reaction was carried out at 50°C for 20 hours. A benzoic acid (BA) material was prepared similarly, but 0.9 mL of benzoyl chloride and 0.4 mL of triethylamine in 10 mL of toluene were added. The cholesterol sorbent (CHOL) was synthesized using 1.5 g of NH2 and 1.3 g of cholesteryl chloroformate in 10 mL of toluene. Next, 0.4 mL of triethylamine was added. The prepared sorbents were washed with toluene, isopropanol, and methanol. They were then dried for 20 hours at 50°C.

Materials containing bonded butanedioic (C4) and pentanedioic acids (C5) were synthesized with different procedures. The 1.6 g of NH2, 0.24 g of butanedioic acid or 0.26g of pentanedioic acid, 0.40 g of EDC, and 7 mL of MES (0.1 M, pH 4.5) were mixed. The synthesis was then carried out at 40°C for 24 hours. The reaction products were washed with MES, water, and methanol. The prepared sorbents were dried at 50°C for 16 hours. The structures of the sorbents are shown in Figure 1.

The synthesized materials were subjected to elemental analysis to determine the percentage of carbon on their surface (Table S1). The coverage density was also determined based on equation (1) in the supplementary information (Table S1). The structure of the synthesized sorbents was confirmed by FT-IR spectroscopy and 13C CP/MAS NMR in the solid phase.

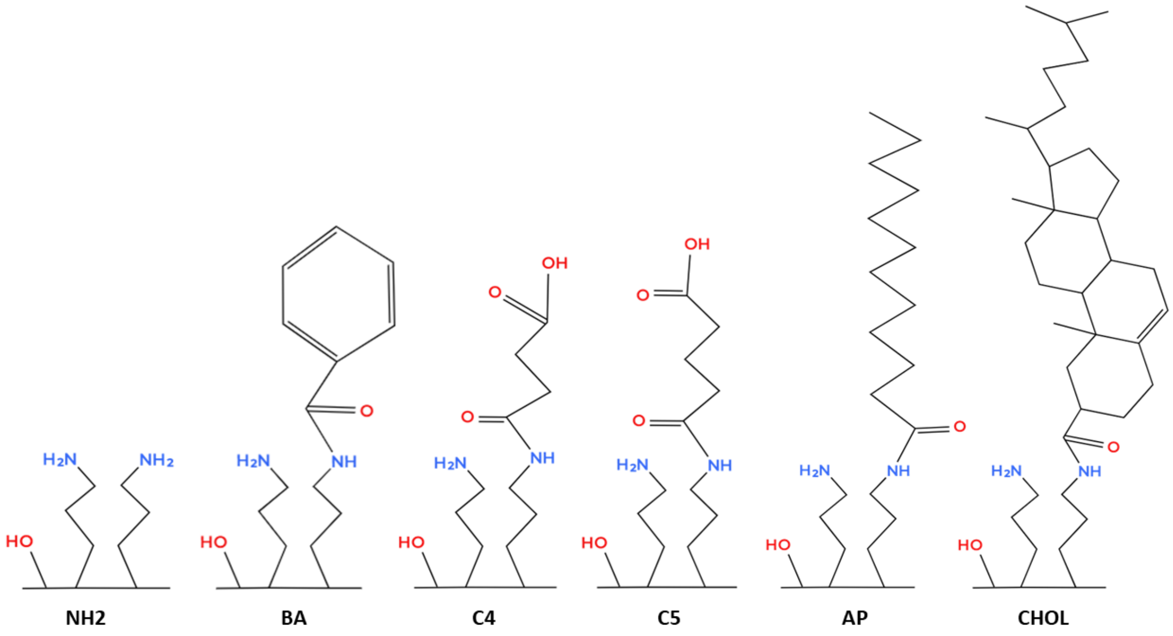

Fig. 1. Chemical structures of sorbents used in the study for the extraction of ONs.

2.4. Development of dSPE procedure for ONs

2.4.1. Conditioning

Two different approaches were tested during conditioning: 1. 100 µL MeOH, 100 µL H_2O, and 200 µL ammonium acetate (AA) solution (pH 4, 10 mM) (based on our previous experience and literature data) [16,38]; 2. 200 µL AA solution (pH 4, 10 mM) (simplified variant).

2.4.2. Sample load of ONs and washing

The adsorption tests were performed using 2 mg of sorbent and 100 µL of 5 µM Nu DNA dissolved in 10 mM AA (pH 4) (each experiment in two replicates). The washing was performed using 100 µL 9:1 (v/v) of 10 mM AA (pH4)/MeOH (Fig. 2). After each stage of the procedure - conditioning, sample load, and washing - the suspensions were shaken for 5 minutes and then centrifuged for 12 minutes at 14,000 rpm.

2.4.3. Optimization of the elution solvent composition using a central composite design (CCD)

The optimization of dSPE desorption was performed using the design of experiments (DoE) approach, employing CCD. The study analyzed the influence of three independent variables on the recovery, these were: pH of the salt solution (9-11), salt (ammonium acetate) concentration (0-50 mM), and organic solvent content in the elution solution (0-100 % v/v). The recovery of ON was the dependent variable. The CCD parameters were calculated using the online statistics calculator Numiqo e.U [39]. This tool allowed us to generate a complete experimental layout with central and axial points. In accordance with CCD principles, each factor was tested at three levels (-1, 0, 1). This resulted in 15 different eluent compositions. Response surface plots were generated in Statistica (StatSoft, Inc., Tulsa, OK, USA) from the experimental data. Detailed information on the parameters, levels, and statistical data is provided in paragraph 3.2.2.

2.4.4. Final dSPE procedure

The C5 sorbent was selected for the procedure. The optimized dSPE procedure included: 1. conditioning: 200 µl of AA (pH 4, 10 mM); 2. sample load: 100 µl of 5 µM ONs in AA (pH 4, 10mM); 3. washing: 100 µl AA (pH 4, 10 mM)/MeOH (9:1 v/v); 4. elution: 100 µL AA (pH 11, 20 mM)/MeOH (9:1 v/v). After each step, the samples were vortexed for 5 min and centrifuged for 12 min at 14,000 rpm.

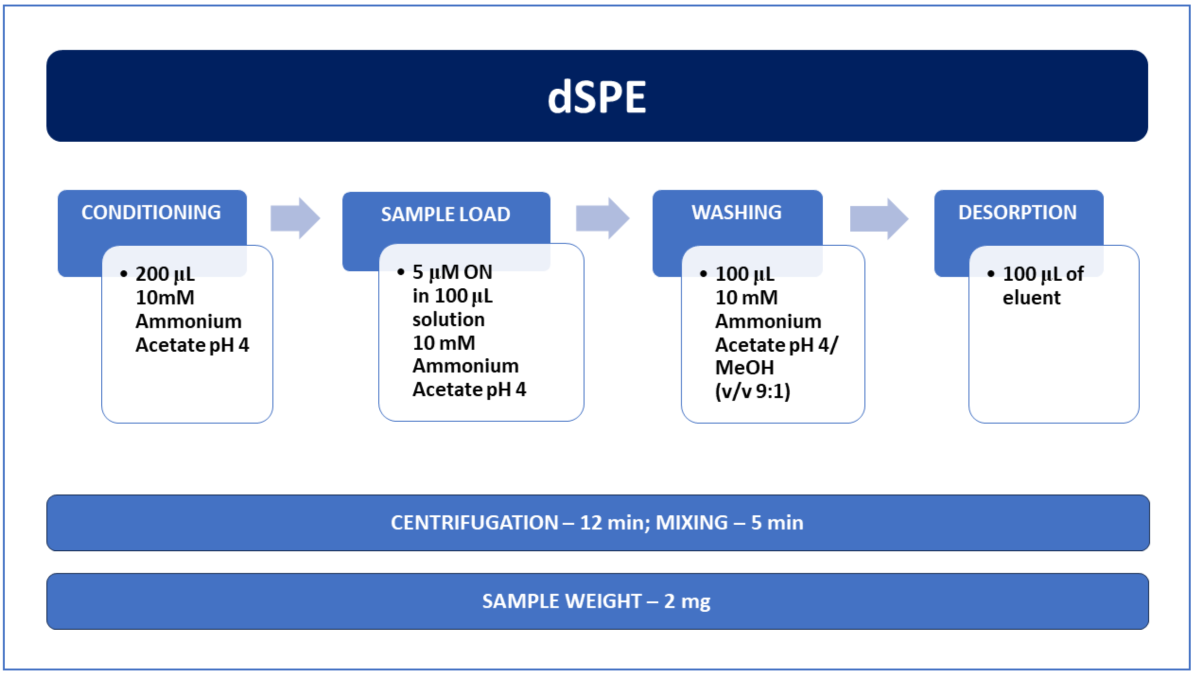

Fig. 2. Diagram showing the optimized dSPE procedure for C5 sorbent.

2.5. Adsorption and desorption kinetics and sorption capacity

The adsorption and desorption kinetics, as well as sorption capacity, were examined for C5 sorbent. A 2 mg of sorbent was conditioned and washed according to the procedure described in section 2.4.4. A 5 µM solution of Nu DNA was used. The samples were collected after 1, 2, 5, 10, and 15 minutes of sample load. The 1 mg of C5 was used during the determination of sorption capacity. Adsorption isotherm experiments were performed using nine experimental points with initial oligonucleotide concentrations ranging from 10 to 100 µM. The samples were shaken for 1 minute and then centrifuged for 12 minutes at 14,000 rpm. The supernatant was analyzed using UHPLC. The equilibrium concentration (C_e) was calculated based on the amount of oligonucleotide remaining in the solution, while the amount adsorbed was calculated from the difference between the initial and equilibrium amounts of oligonucleotide. The adsorption isotherm was constructed by plotting the amount of adsorbed oligonucleotide as a function of its equilibrium concentration.

2.6. Validation of the chromatographic method

Calibration curves were constructed based on the results obtained for standard solutions of three different ASOs (NuMe, NuPs, NuMePs) in the concentration range of 1.25-12 μM. The linearity of the method was assessed by determining the coefficient of determination (R^2). Inter-day repeatability was determined by performing five injections of three different sample concentrations (1.25, 5, and 10 μM) on days 1, 4, and 7. Intraday accuracy was assessed by performing seven injections for three concentrations (1.25, 5, and 10 μM) within one day. Precision was determined as the relative standard deviation (RSD). The limits of detection (LOD) and quantification (LOQ) were determined experimentally using the signal-to-noise ratio method. LOD was defined as the concentration at which the signal-to-noise ratio is at least 3:1, while LOQ was defined as the concentration at which the signal-to-noise ratio is at least 9:1. The matrix effect was determined based on the protocol presented in the literature[40]. First, the extraction procedure was performed for 100 μL of CSF sample (without fortification with ASO) and resulting in the blank sample extract. Next, blank extract was spiked with100 μM ASO to a final concentration of 10 μM. Chromatographic analysis was performed for both 10 μM ASO standard and the blank extract fortified with ASO to a similar concentration. The peak area of the analyte in a post-extraction spiked matrix sample was compared to the peak area obtained for a neat standard solution at an equivalent concentration. The following equation was applied:

$$ME = \frac{A1}{A2} * 100\%$$

Where: ME – matrix effect; A1 – peak area of ASO in post-extraction spiked matrix; A2 – peak area of ASO standard.

2.7. Cerebrospinal fluid samples preparation

The cerebrospinal fluid samples (CSF) were taken according to guidelines approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022). The samples were fortified with NuMe, NuPs, and NuMePs at three concentration levels corresponding to high (10 μM), medium (5 μM), and low (1.25 μM for NuMe and at 2.5 μM for NuPs and NuMePs) concentrations. Fortified samples were prepared by spiking appropriate volumes of ASO standard solutions into CSF.This solution was loaded directly to the C5 sorbent. The procedure was carried out in accordance with the method described in Section 2.4.4.

3. Results and discussion

3.1. Sorbents

3.1.1. Justification of the selection of sorbents for the extraction of therapeutic ONs

Due to the fact that our primary goal was to test the potential of sorbents with mixed properties for ASO extraction, it was necessary to properly design and synthesize these materials. We selected sorbents with different functional groups so that ONs could interact with the surface (both during adsorption and desorption) through different types of interactions. Since ONs can interact through electrostatic interactions, we decided that each sorbent surface should contain aminopropyl groups (as residual ones after the first stage of synthesis) (NH2). We chemically bonded aromatic, aliphatic, and polar groups to the surface of the sorbents. In this way, we ensured a variety of properties and interactions. It should be emphasized that such research for ASO was conducted for the first time, and these materials had not been previously used for ONs extraction.

Modification of the surface with benzoic acid (BA) enables π...π interactions with nitrogen bases in ONs. The introduction of an aliphatic chain (AP) or a cholesterol molecule (CHOL) made the sorbent more hydrophobic, providing the possibility of van der Waals, hydrophobic, and weak dipole–dipole interactions (CHOL). Modification of an aminopropyl surface with dicarboxylic acids (C4 and C5) introduces additional carboxyl groups capable of interacting with ONs by hydrogen bonds. Moreover, carboxyl groups can undergo ionization depending on the pH of the solvent (essential especially during desorption). In addition, each of the sorbents contains residual amino groups and carbonyl ones. The first ones enable electrostatic interactions with the negatively charged phosphate skeleton of ONs, while carbonyl may interact by hydrogen bonding [16,38]. The development of new sorption materials for ON purification is key to improving the efficiency and selectivity of extraction processes. The mixed-mode approach seems particularly promising.

3.1.2. Characterization of sorbents

An elemental analysis was performed to determine the nitrogen, hydrogen, and carbon content in the synthesized materials. The increase in carbon and hydrogen content was noticed for AP, BA, C4, C5, and CHOL sorbents compared to NH2 one (Table S1), proving that the ligands were bonded in the second step of the synthesis. Moreover, results showed that the nitrogen content remained unchanged after the second step of the synthesis compared to the first one. The coverage density (Table S1) after the first step of the synthesis (modification of silica gel with aminopropyl ligands) was greater than that of the second step. Comparable values were observed for BA, C4, and C5 sorbents, but lower for AP and CHOL sorbents. It is probably due to steric effects and spatial constraints of large, hydrophobic ligands, which hinder their dense packing.

The FT-IR spectra for NH2 sorbent (Fig. S1 A) show intense bands in the range of 1080-800 cm^{-1} corresponding to Si-O-Si vibrations. Additional signals appear in the vicinity of 1560-1650 cm^{-1} (deformation vibrations of the $-NH_2$ group). The presence of these bands confirms the effective modification of the silica surface with aminopropyl groups. In the case of surface modification with benzoic acid, bands in the range of approximately 1700 cm^{-1} and 1450-1600 cm^{-1} are observed, corresponding to C=O and aromatic ring vibrations (Fig. S1 B). The FTIR spectrum of AP surface shows characteristic bands in the range of 2920-2850 cm^{-1} corresponding to C-H vibrations of the aliphatic chain. Additional signals around 1450-1600 cm^{-1} indicate the occurrence C=O vibrations (Fig. S1 C). In the FTIR spectrum for the surface functionalized with dicarboxylic acids, the band in the region of ~1700 cm^{-1} corresponds to C=O bond vibrations, which indicates the presence of acids on the surface of the material (Fig. S1 D, E). The FTIR spectrum of a surface modified with cholesterol exhibits characteristic bands in the region of 2800–3000 cm^{-1} corresponding to C–H stretching vibrations of the steroid and aliphatic moieties, while additional bands around 1450 cm^{-1} and 1380 cm^{-1} correspond to CH_2 and CH_3 bending vibrations (Fig. S1 F).

The final confirmation of the structure was based on 13C NMR spectra. The two exemplary 13C NMR spectra for BA and C5 were presented in the supplementary information (Fig. S2). For the remaining materials, the signals recorded on the spectra were described in Section 2 of the Supplementary Information. For NH2 only signals from aliphatic groups in the range of 10-55 ppm were observed. The chemical shift $\delta = 50$ ppm characterizes a carbon atom bonded to a silicon atom. In the case of AP, the spectrum showed characteristic signals originating from carbon atoms in a long aliphatic chain (10-35 ppm). For the BA, there are signals at 125-140 ppm originating from carbon atoms in the aromatic ring, and a signal in the region of 165-170 ppm, attributed to the carbon atom of the carbonyl group (C=O). In the spectrum obtained for C4 and C5, a signal of the carbonyl group at 175-178 ppm and resonances in the range of 30-55 ppm, corresponding to the aliphatic groups of both aminopropyl and dicarboxylic acid, are observed. Signals in the range of 10-55 ppm correspond to aliphatic carbons originating from cholesterol fragments were noticed at the CHOL spectrum. Signals at 120-140 ppm originate from double bonds (C=C) in the CHOL molecule, while signal in the 155-165 ppm range confirms the presence of carbonyl groups (C=O) in the structure. These results confirm the successful completion of the second synthesis step, with the ligands effectively attached to the silica gel surface. Additional information on FT-IR and 13C NMR spectra can be found in the supplement (Figure S2 and section 2 description).

3.2. Development of dSPE procedure

The next stage of research aimed to develop a dSPE procedure enabling effective extraction of ONs using sorbents with mixed hydrophobic-hydrophilic properties. Nu DNA, an unmodified nusinersen analogue was used in the preliminary studies, as the method should find application for the isolation of active ingredients of ASO-based drugs. Consequently, these studies are very important. Furthermore, they are novel due to the application of new materials and a mixed mode of extraction.

3.2.1. The conditioning, adsorption, and washing in dSPE procedure

Two different approaches of conditioning were tested in the first step of the experiment. They differed in the number of steps. The first one involved the application of three different solvents, while the second one - just one of them (simplified approach). Both approaches showed comparable results, and complete ON adsorption occurred. Therefore, we used the simplified conditioning with 200 µL AA (pH 4, 10 mM), as it was less time-consuming.

The Nu DNA adsorption process was evaluated for all synthesized sorbents: AP, BA, C5, C4, and CHOL. After the conditioning stage, 100 µl of a 5 µM Nu DNA solution prepared by diluting the stock solution in AA (pH 4, 10 mM) was loaded onto the surface of each sorbent. Adsorption occurred because of electrostatic interactions between the positively charged residual aminopropyl groups and the negatively charged ONs. For NH2, AP, BA, C5, and C4 sorbents, complete adsorption of 5 µM Nu DNA was achieved. In the case of the CHOL, the adsorption was equal to 51 ± 1%. The situation has not changed when we have reduced ON concentration to 2.5 µM or when we have increased the amount of sorbent. Due to the presence of large cholesterol molecules, spatial interactions may limit effective adsorption of ONs at the CHOL surface. The steric hindrances probably prevent electrostatic attraction, which is the basis for ONs adsorption at the synthesized materials. Due to the lack of complete ON adsorption, CHOL was excluded from further study. For comparison purposes, the developed procedure was also used with an NH_2 sorbent, and complete adsorption of the Nu DNA onto the sorbent was observed, which, as expected, was based primarily on electrostatic interactions.

The washing step involved 100 µL AA (pH 4, 10 mM)/MeOH (9:1 v/v), and it was selected based on our earlier experience [38]. No ON leaching was observed under these conditions.

The results obtained clearly indicate that the selected conditioning, loading, and washing conditions ensure effective adsorption of ON; therefore, we decided to use them in the further dSPE procedure.

3.2.2. Optimization of solvent composition for ONs elution during dSPE

The composition of the eluent used during the elution was optimized using CCD for each of the sorbents. This approach allows study the impact of different experimental factors on the recovery using a minimum number of experiments. Moreover, CCD facilitates investigation beyond the predefined values of the experiment.

Three independent variables were tested, namely pH of the salt solution, salt concentration, and organic solvent content. They were selected based on the predicted interactions responsible for adsorption, namely, electrostatic, hydrophobic, $\pi \ldots \pi$, and hydrogen bonding. Changing salt concentration, pH, and organic solvent should disrupt them. Fifteen dSPE extractions were performed in replicates according to the conditions established within the CCD framework (Table S2). The results of these extractions were then used to construct three-dimensional contour plots (Fig. 3). Fully encoded CCDs and ONs recovery are listed in Table S2. The use of CCD allowed for estimation of nonlinear relationships between independent factors (pH of the salt solution, salt concentration, organic solvent content) and ONs recovery.

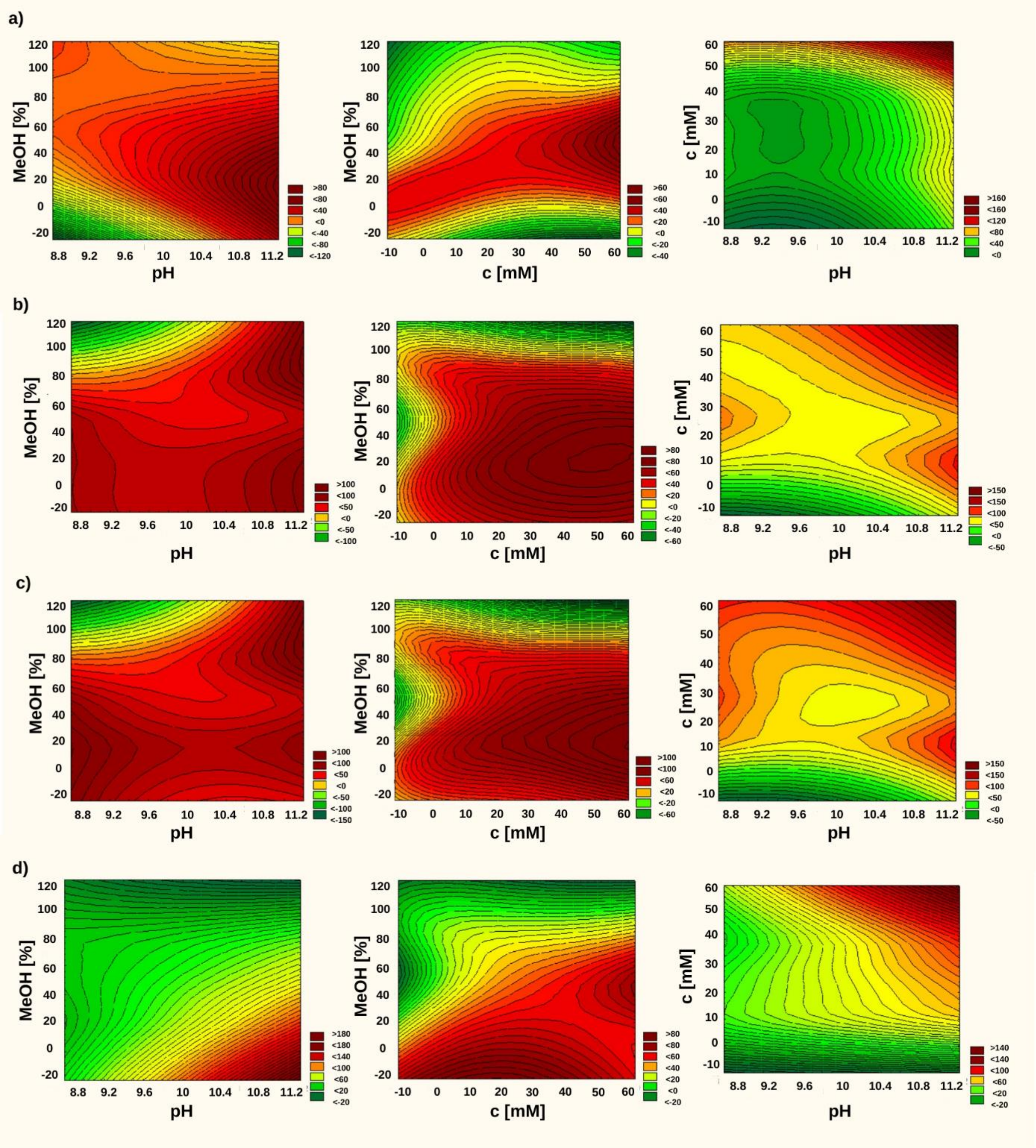

Fig. 3. Three-dimensional contour plots with CCD results for: a) AP, b) C4, c) C5, d) BA.

In the case of sorbents AP and BA, clear trends were observed, reflecting differences in their structure and the resulting interaction mechanisms (Fig. 3 A, D). AP, containing an alkyl chain, showed a strong dependence on the methanol content in the elution solution. Increasing the content of MeOH at high pH improved recovery by up to 30%, indicating the presence of hydrophobic interactions between the sorbent and ON (Fig. 3 A). High recovery is observed at pH 11 with 20-80% v/v of MeOH, while lower MeOH content reduces desorption efficiency (Fig. 3. A). In contrast, salt concentration had a moderate effect. The highest recoveries were achieved using AA at a concentration of 40-60 mM. This suggests that elution from AP requires weakening of electrostatic attraction (high pH) and disruption of hydrophobic interactions (addition of MeOH).

For BA sorbent, the range of parameter values for which high recovery was achieved is very narrow (Fig. 3 D). The recovery increased significantly with changes in pH and salt concentration, while the effect of methanol was less pronounced compared to AP. High recovery (>85%) was achieved at pH above 10 and MeOH content below 20 v/v. Desorption most likely results from the weakening of electrostatic interactions between the sorbent and ONs (high pH). Furthermore, increasing the AA concentration improved recovery, with values above 90% achieved for AA concentrations above 40 mM.

For C4 and C5 sorbents, analogous tendencies were noticed at 3D contour plots, probably due to the similar structure (functional groups) (Fig. 3 B,C). This indicates that the desorption mechanism for these sorbents will be the same. The addition of an organic solvent to an elution solution at high pH improved recovery by 20%. The recovery will remain high for pH equal 11 and MeOH content of 20 or 80 %v/v (Fig. 3 B,C). In contrast, the salt concentration could remain low (10 mM) without reduction of ON recovery. On the other hand, increasing AA concentration also provides recoveries greater than 90% (Fig. 3 B,C). This increases the applicability of C4 and C5 because the user can change several parameters without losing recovery (e.g., use high or low salt concentration, 20 or 80% v/v MeOH) or even to increase recovery.

As a control, oligonucleotide desorption from NH2 was determined. The AA solutions (pH 11), first at 10 mM and then at 60 mM were applied. Despite the high pH and increased buffer concentration, analyte recovery remained low (~10%). These results indicate that elevated pH and increased ionic strength are insufficient to efficiently disrupt the interactions responsible for ON retention on the NH2 sorbent.

CCD enabled the effective determination of the optimal eluent composition. The maximum recoveries obtained for individual sorbents in the CCD plan were in the range of 58-88% (the lowest for AP, the highest for C5) (Table S2). Based on the 3D contour plots (Fig. 3) the following eluent compositions were selected for elution of ONs from synthesized sorbents: AP: 80/20 % (v/v) AA (pH 11, 60 mM)/MeOH; BA: 100 % AA (pH 10.5, 60 mM); C4 and C5: 90/10 % (v/v) AA (pH 11, 20 mM)/MeOH. The next stage of the research was to verify whether the selected composition of the elution solution, used in the optimized dSPE procedure, ensures high and repeatable recoveries of ON. The highest Nu DNA recoveries were obtained for BA (95 ± 2%) and C5 (84 ± 2%) materials (Fig. 4). Lower values were observed for C4 (80 ± 2%), while the lowest for AP (70 ± 1%). The use of CCD enabled rapid optimization of dSPE procedures and selection of conditions leading to high recoveries. Based on the obtained results, C5 and BA sorbents were selected for further studies, as they were characterized by both high recoveries and good repeatability of results, confirming their application potential in the dSPE procedure for ASO.

3.2.3. Concise considerations on the interactions occurring during ON adsorption and desorption

On the surface of tested materials, ON adsorption occurs, probably mainly based on electrostatic interactions and hydrogen bonds. This mechanism is consistent with observations described in the existing literature for previously tested sorbents [16,38].

Other types of interactions are also possible and they depend on the type of functional groups at the surface of individual sorbents. Residual amino groups ($-NH_2$), present on the surface of all tested sorbents, can participate in the formation of hydrogen bonds and, after protonation, in electrostatic interactions. In the case of BA, $\pi \ldots \pi$ interactions between the aromatic group and the nitrogen bases of ON are possible. In addition, hydrogen bonds may occur with the carbonyl group or with deprotonated aminopropyl groups. Carboxyl groups (C4 and C5) can participate in hydrogen bonds, but depending on the pH, they can also participate in electrostatic interactions (e.g. electrostatic repulsion). In turn, the C12 aliphatic chain (AP) imparts the surface a partially hydrophobic character, which promotes the formation of hydrophobic interactions.

ONs are negatively charged due to the presence of phosphate groups in the sugar-phosphate backbone. Residual amino groups present on the surface of sorbents have a specific pK_a value, and their protonation depends on the pH of the solution. Below the pK_a value, they occur in protonated form ($-NH_3^+$), which promotes electrostatic interactions with negatively charged ON. Therefore, low pH was used during the adsorption, and this step of extraction relied entirely on electrostatic attraction. Above pK_a amino groups undergo deprotonation ($-NH_2$), thus weakening the interaction with the sorbent surface and facilitating desorption. The change in pH causes ionization of carboxyl groups present in the structure of C4 and C5 and consequently provides electrostatic repulsion between these groups and phosphate ones in ON. This causes rapid desorption of ONs from both materials, and it is probably the main reason for the highest recoveries observed for C5 and C4 (Fig. 4). The above indicates that electrostatic interactions are significant, especially during adsorption.

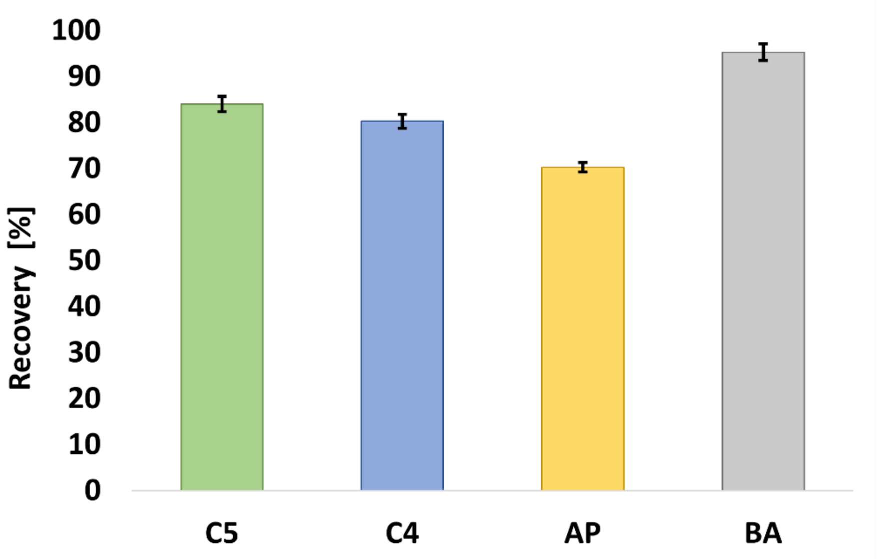

Fig. 4. A comparison of recoveries of NU DNA for tested sorbents.

The salt concentration in the eluent also affects ON desorption from the surface of the tested sorbents. An increase in salt concentration leads to a weakening of hydrogen bonds. At the same time, a decrease in the dielectric constant of the solution affects weak intermolecular interactions, such as van der Waals or π...π interactions (in the case of e.g. AP). As a result, at higher salt concentrations, the interactions between ON and the surface become less stable, which promotes desorption and leads to increased recovery. This phenomenon appears to be significant in the case of desorption from AP and BA surfaces.

The organic solvent in the elution solution impacts the strength of electrostatic and hydrophobic interactions between ON and the sorbent surface. With an increase in MeOH content, a weakening of ionic interactions (resulting from a decrease in the solvent's dielectric constant) is observed, which may promote ON desorption. At the same time, MeOH disrupts hydrophobic interactions. In the case of the AP, an increase in recovery was observed with an increase in MeOH content in the range of 10-30% (v/v), especially at high pH of the eluent. This may indicate the occurrence of hydrophobic interactions between the nonpolar fragment of the ON and the alkyl chains on the sorbent surface. In the case of the BA sorbent, the increase of MeOH content had no significant effect on recovery. This suggests that the desorption process occurs mainly due to the disruption of π...π interactions and weakening of hydrogen bonds. For C4 and C5 sorbents, MeOH addition has no observed effect on recovery. Hydrophobic bonds do not directly influence the desorption mechanism in these sorbents.

3.3. Sorption and desorption kinetics and sorption capacity

The adsorption isotherm for Nu DNA on the C5 adsorbent is presented in Figure S3. The sorption capacity was calculated based on the adsorption isotherm for (described in 2.5 section), and it was equal to 16.15 ± 0.2 μg/mg. This parameter indicates that a large amount of ON can be adsorbed on the surface of C5, which is advantageous during the application of the developed procedure for real samples. The adsorption of Nu DNA over time was studied for C5. After the first minute, 100% of Nu DNA was adsorbed. This effect may be due to electrostatic attraction between the negatively charged ON and the positively charged surface of the sorbent. Desorption of Nu DNA from C5 was complete within 5 minutes, with 81 ± 1% of the previously adsorbed Nu DNA being recovered. Rapid adsorption and desorption are advantageous because it shorten the extraction procedure.

3.4. Application of mixed-mode dSPE procedure to ASO

3.4.1. ASO with various modification types

Due to the planned use of the developed procedure for ASO extraction from biological samples, an important stage of the research was to determine the impact of chemical modifications of ASO on the recovery. ASOs contain various types of modifications, such as the substitution in sugar residues (e.g., 2'-O-methyl or 2′-O-(2-methoxyethyl)) or the replacement of phosphodiester bonds with phosphorothioate ones, significantly increasing the ASOs' stability and affecting their binding properties. Therefore, it was crucial to investigate the extent to which these changes in the ON structure impact their recovery. Present research has been conducted for ASOs that are analogues of nusinersen (similar sequence), modified with a 2'-O-methyl group (NuMe), a phosphorothioate group (NuPs), and also containing both modifications in their structure (NuMePs) (Table 1). For C5 the highest recoveries were observed for NuMe (92 ± 2%), followed by Nu DNA (84 ± 2%), NuMePs (85 ± 1%), and NuPs (44 ± 1%) (Fig. 5A). Replacement of phosphodiester bonds with phosphorothioate ones leads to increased hydrophobicity of the ON, due to the presence of sulfur. It is a larger and less electronegative atom compared to oxygen; consequently, changes occur in the conformation of the sugar phosphate backbone [41,42]. Moreover, sulfur affects the charge distribution in the backbone [41]. This may promote stronger interaction with protonated amino groups on the sorbent surface and reduce desorption efficiency. Introducing a 2'-O-methyl group to each of the ribose also increases the hydrophobicity of ON, but does not significantly disturb electrostatic interactions. Greater recoveries were determined for ON modified in two structural elements

NuMePs. The two effects described above partially offset each other and as a result, the recovery is lower than for NuMe, but higher than for NuPS.

In the case of BA the highest recoveries were obtained for the Nu DNA (95 ± 2%), while lower for NuMe (84 ± 3%), NuMePs (70 ± 2%), and NuPs (37 ± 1%) (Fig. 5B). Nu DNA is unmodified and the most hydrophilic. Nu DNA primarily interacts with the sorbent surface through electrostatic interaction, easily disrupted under elution conditions. The introduction of phosphorothioate bonds in NuPs increases their hydrophobicity compared to Nu DNA. According to literature data, the sulfur atom can participate in π–π interactions with aromatic groups. In this case, the presence of sulfur affects both the adsorption and desorption of the ASO. As a result, the recovery for NuPs is lower compared to Nu DNA [43]. Sulphur interacts more strongly with the aromatic fragments of BA, which may reduce the recovery compared to NuMe.

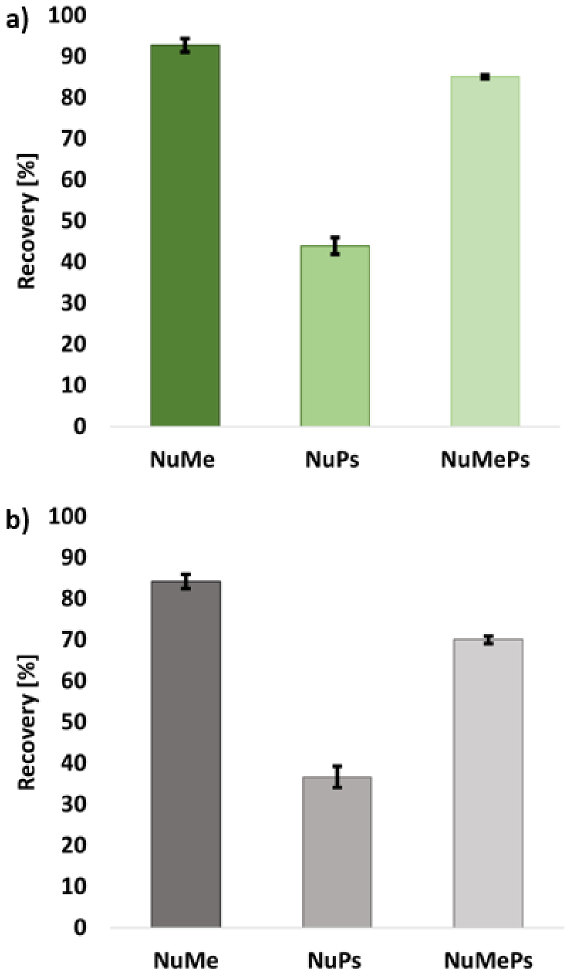

Fig. 5. A comparison of the impact of ASO modifications on recovery depending on the sorbent: a) C5; b) BA.

3.4.2. ASO with various lengths

Next, the impact of ASO length on the recovery was investigated. This approach is important for the analysis of biological samples, since ASOs metabolites are shorter fragments (shortmers) of the main sequence resulting from enzymatic degradation. The 18-, 15-, 12-, 9-, and 6-mer NuPs ONs were selected for the study. The results are presented in Fig. 6.

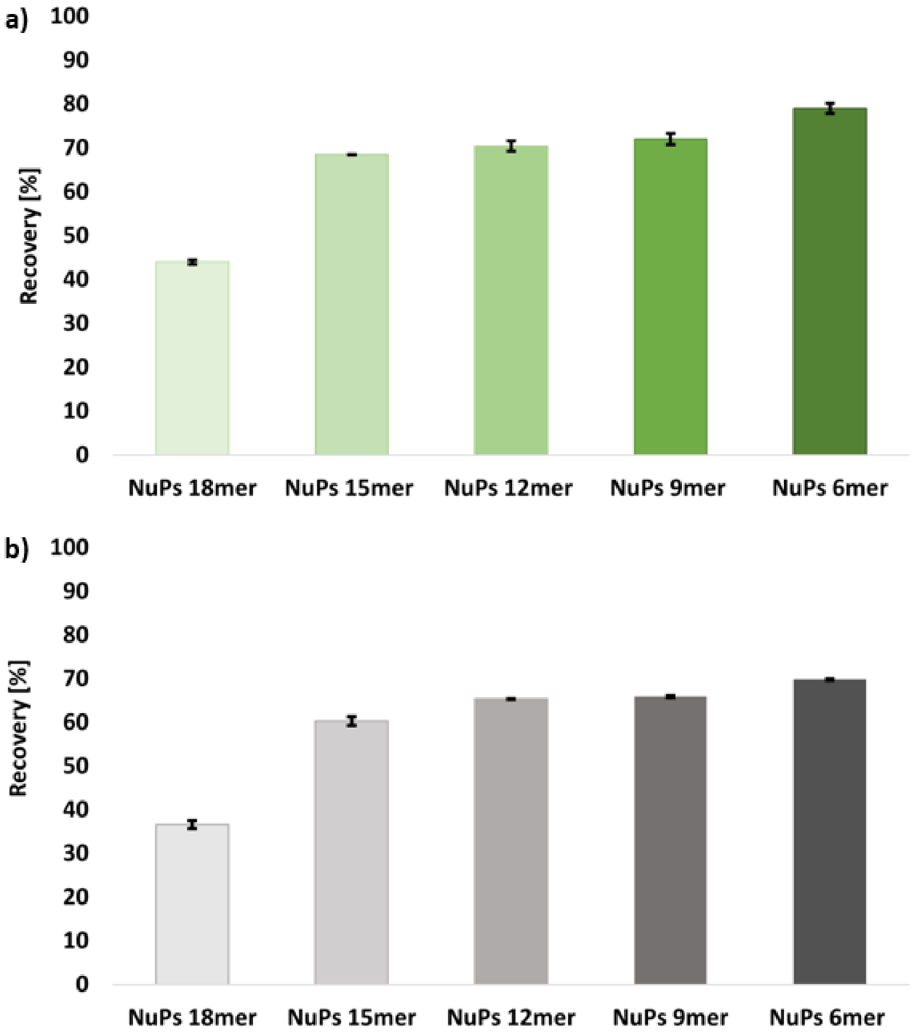

Fig. 6. A comparison of the impact of ASO length on recovery for: a) C5; b) BA.

For C5 the highest recoveries were obtained for NuPs 6-mer (79 ± 1%), and with increasing sequence length, a gradual decrease in recovery was observed (from 72 ± 1% for 9-mer to 36 ± 1%for 18-mer) (Fig. 6A). The lower recovery observed for longer ASOs is due to the greater number of charged phosphate and nucleobase functional groups they possess, which are capable of interacting with the surface. These additional interaction sites increase the ASO's overall affinity to the surface. This probably hinders desorption and reduces recovery. In contrast, shorter ONs have fewer phosphate/phosphorothioate groups, interact more weakly and are therefore recovered more efficiently. A similar trend was noticed for BA material (Fig. 6B). Nevertheless, shorter ASOs show higher recoveries than longer ones. The reduced surface contact of shorter ASOs with the sorbent likely diminishes the strength of these interactions. These results are particularly important in the context of monitoring ASO therapy.

3.5. Selection of the sorbent for the next stage of the research

The C5 sorbent was selected for further research due to the high and repeatable recoveries obtained for modified ONs of various lengths, which is crucial in monitoring ASO therapy. Although favourable sorption properties can be partially attributed to both C5 and BA, the decisive criterion was the composition of the eluent. In the case of C5, elution can be carried out under conditions fully compatible with MS. This significantly simplifies the analytical procedure. In contrast, the use of the BA sorbent requires the application of high salt concentration for ASO desorption. This leads to signal attenuation in the MS ionisation source and requires additional desalination steps, limiting the practical application of this material in LC-MS.

3.6. Chromatographic method validation

The IP RP UHPLC technique was used for the analysis of ONs. It enabled effective separation and quantitative determination of ASO. The method was validated to confirm its accuracy, precision, and repeatability. The calibration curve was developed based on the results obtained for standard solutions in the concentration range of 0.63-12 μM for NuMe; 1.88-12 μM for NuPs; 1.88-12 μM for NuMePs. The linearity of the method was confirmed by a high coefficient of determination (Table 2). For NuMe, the LOQ was 0.63 μM, and the LOD was 0.21 μM; for NuPs, the LOQ was 1.88 μM, and the LOD was 0.63 μM; for NuMePs, the LOQ was 1.88 μM and the LOD was 0.63 μM (Table 2). Intra-day and inter-day variability were assessed for three concentrations, with RSD values not exceeding 7 % and 5% for NuMe, 5%, 7% for NuPs, and 2 %, 1% for NuMePs, respectively (Table 2). It may be concluded that the chromatographic method enables reliable quantification of the oligonucleotide.

Table 2. Validation and calibration curve parameters for determined NuMe, NuPs and NuMePs

ON	Calibration curve equation	Concentration range [µM]	R^2	LOD [µM]	LOQ [µM]	Intra-day for 1.25 µM RSD [%]	Intra-day for 5 µM RSD [%]	Intra-day for 10 µM RSD [%]	Inter-day for 1.25 µM RSD [%]	Inter-day 5 for µM RSD [%]	Inter-day for 10 µM RSD [%]
NuMe	y = 0.4034x – 0.0482	0.625-12	0.9992	0.200	0.625	7.00	0.63	0.51	3.41	3.09	4.88
NuPs	y = 0.3094x – 0.1964	1.875-12	0.9993	0.625	1.875	1.95	5.00	1.00	7.12	1.41	6.00
NuMePs	y = 0.4099x – 0.2515	1.875-12	0.9996	0.625	1.875	0.14	0.49	1.16	0.51	1.00	0.52

3.7. Application of the developed dSPE method to the extraction of ASO from cerebrospinal fluid

The developed dSPE procedure was used to extract NuMe, NuPs, and NuMePs from CSF samples. During the conditioning, sample load, and washing steps, the samples were acidified with formic acid to a final concentration of 0.2%. CSF was fortified with ASOs to a concentration of: 1.25, 2.5, 5 and 10 μM and loaded directly on the conditioned C5 surface. In the case of CSF, no preliminary sample purification was necessary due to its relatively low protein and interfering substance content. The lack of additional purification steps, such as LLE or protein precipitation, significantly reduced sample preparation time and solvent consumption. Our method allowed for the effective and selective extraction of all three ASOs.

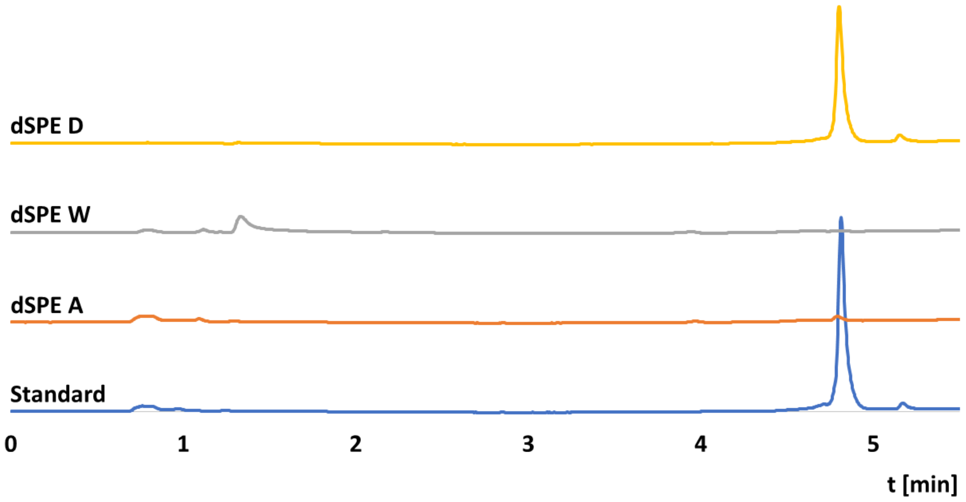

Fig. 7. Exemplary chromatograms for samples obtained at various dSPE stages for the extraction of NuMePs from CSF using C5 sorbent. Notation: A- sampleload, W – washing, D – desorption/elution.

The results for NuMePs (Fig. 7) indicate that both matrix impurities and ASO are adsorbed onto the sorbent. No additional peaks corresponding to matrix components are observed in the resulting supernatant during the washing step, confirming that impurities remain irreversibly adsorbed to the sorbent. Moreover, NuMePs is eluted under the desorption conditions used, contrary to components originating from the matrix. The recoveries for ASO varied depending on the type of modification. The highest recoveries in the range of 1.25–10 μM (for NuMe) and 2.5–10 μM (for NuPs and NuMePs) were obtained for NuMePs (90-94%), lower for NuMe (58-65%), and the lowest for NuPs (37-42%) (Fig. S4). The recoveries for NuMe and NuPs were lower than those of their respective standards, indicating the possible matrix effect. Its determination revealed the following values: 91% for NuMe, 89% for NuPs, and 100% for NuMePs. The obtained extracts were pure, as confirmed by chromatograms without the interfering signals from the CSF matrix (Fig. 7). The use of the hydrophobic-hydrophilic C5 enabled selective extraction of ASOs without the need for additional purification. This highlights the effectiveness and potential of these materials in ASO extraction.

3.8. A comparison of our method with the previously applied dSPE

Table 3 summarises the existing literature on the use of dSPE for ASO isolation and compares these methods with our newly developed mixed-mode procedure. The literature describes the use of several sorbents with hydrophobic-hydrophilic properties for ONs extraction, typically achieving recoveries of 60-90% depending on the matrix and the type of ON modification. These methods are characterized by simplicity of protocols and good reproducibility. However, all previously described methods have limitations, such as the need to use lysis buffers or additional purification steps or the risk of carry-over effect. On the other hand, most of them were applied to serum samples, which have a more complex composition than CSF. CSF contains a relatively low protein concentration, making it easier to extract ASO. This may be one of the reasons why it was possible to directly extract ASO from CSF using our method. Nevertheless, we believe that the nature of the C5 sorbent surface also played a significant role here. Moreover, our mixed-mode dSPE procedure provides high analyte recoveries, and demonstrates good reproducibility. Compared with the approaches summarized in Table 3, the proposed method provides comparable or improved recoveries. Table 3. Overview of previously reported SPE methods used for ASO extraction

Table 3. Overview of previously reported SPE methods used for ASO extraction

Sorbent	Type of ONs	Extraction mode	Recovery [%]	LOD	LOQ	Matrix	Advantages	Disadvantages	References
Poly(ionic liquids	ONs with various modification types (Ps, Me, LNA*); different lengths	WAX	80-84	0.27-0.37 µM	0.90-1.24 µM	Serum	Efficient isolation; fast; no additional purification steps required	Relatively long desorption time (30 min)	[36]
Clarity OTX™	ONs with various lengths and modification types (Ps, Me, MOE, LNA, 5′-vinyl, GalNAc (5′ i 3′))	Mixed-mode	~60-97	not reported	10 ng/mL	Plasma/serum, tissue homogenates, rat plasma	Simplicity; preferred when assessing multiple compounds simultaneously	Time-consuming, multistep sample preparation, irreversible binding reported; additional purification step before extraction (lysis)	[44] [45]
Dynabeads SAX / BcMag SAX	siRNAs (chemically modified)	SAX	75-90	not reported	50 ng/mL	Serum	Fast; effective removal of interferents	Requires high salt concentrations; additional sample pretreatment needed	[29]
Oasis® WAX, Clarity OTX	Unmodified ssDNA (15-mer); PS ODN (24-mer) and 3′ n-1 metabolite	WAX	58-80	not reported	10 pM-10 ng/mL	Human plasma; rat plasma	Fast; high selectivity;	Time-consuming, multistep sample preparation	[28] [46]

Silica-based sorbents functionalized with asparagine	Unmodified DNA ONs and ONs with PS modification	Mixed-mode	80-92	0.029-0.036 µM	0.10-0.12 µM	Serum	No organic solvents; good reproducibility; simplicity	Time-consuming, multistep sample preparation; additional purification step before extraction (protein removal)	[16]
Amino acid-based sorbents	Unmodified ON; ONs with various modification types (Ps, NuMePs, Me, MOE)	Mixed-mode	91-95	0.005 µM	0.016 µM	Serum	Easy to conduct; high recovery; good reproducibility; no organic solvents used	Time-consuming, multistep sample preparation; additional purification step before extraction (protein removal)	[38]
Silica sorbent modified with aminopropyl and carboxylic groups	Unmodified ON; modified ONs (NuPs; NuMe, NuMePs)	Mix-mode	>90	0.21-0.63 µM	0.63-1.88 µM	CSF	Easy to perform; high recovery; very good reproducibility; efficient isolation; fast; selective	Multistep sample preparation; requires the use of organic solvents	

5. Conclusion

In this work, the first dSPE procedure based on mixed-mode extraction was developed. Newly synthesized sorbents with mixed properties were successfully used for the isolation of modified therapeutic ONs. Four synthesised sorbents (AP, BA, C4, C5) demonstrated the ability to interact with ON in various ways, combining electrostatic, hydrophobic, $\pi...\pi$ and hydrogen bonding mechanisms, which significantly increased the efficiency of extraction. Among the tested sorbents, CHOL showed incomplete adsorption (~50%) and was excluded from studies. Comprehensive optimization of the extraction procedure showed that the highest, repeatable recoveries were achieved for the C5 and BA sorbents (>85%). Finally, C5 sorbent was selected for further research based on recoveries and reproducibility.

The optimised method enabled the effective extraction of ONs with different chemical modifications (2′-O-methyl, phosphorothioate) and different lengths, which is important in monitoring of ASO therapy. A decrease in recovery was observed with increasing ON length, confirming that shorter ONs are more effectively extracted. This effect may be useful during the application of a newly developed procedure to study ASO metabolism in biological samples. Chemical modifications further reduce their recoveries, which is important in the context of the analysis of therapeutic ASOs that are modified to increase stability and biological activity.

The application of the developed dSPE procedure to CSF samples enabled direct ASO extraction without the need for additional purification steps. ASOs were successfully adsorbed and efficiently eluted from C5 with low matrix effect. The results confirmed the effectiveness and selectivity of the developed mixed-mode dSPE procedure. The highest recoveries were obtained for NuMePs (~90%). This is particularly encouraging, as the ON contains two structural modifications, and therapeutic ASOs typically incorporate multiple chemical modifications as well. Such a high recovery, therefore, represents a meaningful result and indicates that the method is well-suited for the extraction of ASOs from CSF.

Nevertheless, the developed extraction method based on sorbents with mixed properties enables easy, repeatable, and efficient isolation of modified ONs, ensuring high recoveries and low biological matrix interference. The results obtained in this study highlight the advantages of mixed-mode extraction in the isolation of ASOs. Sorbents with hydrophobic-hydrophilic properties offer the potential for selective extraction of therapeutic ONs from CSF, without additional purification steps. This work therefore, expands the current knowledge on sorbents used for ASO extraction. Importantly, this study presents for the first time the potential of so far unexplored sorbents with a hydrophobic-hydrophilic sorbents for ASOs isolation.

The data that support the findings of this study are openly available in repository name ….

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Author contribution statement:

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Sylwia Studzińska: Conceptualization, methodology, resources, data curation, writing – original draft, project administration, funding acquisition

Maria Mazurkiewicz-Bełdzińska: Writing – review and editing

Jakub Szymarek: writing – review and editing

Declarations

Samples were taken in accordance with the relevant guidelines of the Medical University of Gdańsk and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022).

References

[1] R.M. Orr, Technology evaluation: fomivirsen, Isis Pharmaceuticals Inc/CIBA vision., Curr Opin Mol Ther 3 (2001) 288–94.

[2] M.C. Lauffer, W. van Roon-Mom, A. Aartsma-Rus, Possibilities and limitations of antisense oligonucleotide therapies for the treatment of monogenic disorders, Communications Medicine 4 (2024) 6. https://doi.org/10.1038/s43856-023-00419-1.

[3] M. Bege, A. Borbás, Rise and fall of fomivirsen, the first approved gene silencing medicine : A historical review, Acta Pharm Hung 92 (2022) 38–44. https://doi.org/10.33892/aph.2022.92.38-44.

[4] T. Gidaro, L. Servais, Nusinersen treatment of spinal muscular atrophy: current knowledge and existing gaps, Dev Med Child Neurol 61 (2019) 19–24. https://doi.org/10.1111/dmcn.14027.

[5] D.M. Paton, Nusinersen: antisense oligonucleotide to increase SMN protein production in spinal muscular atrophy., Drugs Today (Barc) 53 (2017) 327–337. https://doi.org/10.1358/dot.2017.53.6.2652413.

[6] R.N. Singh, N.N. Singh, Mechanism of splicing regulation of spinal muscular atrophy genes, in: Adv Neurobiol, Springer New York LLC, 2018: pp. 31–61. https://doi.org/10.1007/978-3-319-89689-2_2.

[7] H.-W. Son, T. Yokota, Recent Advances and Clinical Applications of Exon Inclusion for Spinal Muscular Atrophy., Methods Mol Biol 1828 (2018) 57–68. https://doi.org/10.1007/978-1-4939-8651-4_3.

[8] A. Kilanowska, S. Studzińska, In vivoandin vitrostudies of antisense oligonucleotides - a review, RSC Adv 10 (2020) 34501–34516. https://doi.org/10.1039/d0ra04978f.

[9] K. Ostrowska, N. Balińska, S. Studzińska, Short review on determination of nusinersen for spinal muscular atrophy treatment, Microchemical Journal 213 (2025) 113559. https://doi.org/10.1016/j.microc.2025.113559.

[10] D.E. Frank, F.J. Schnell, C. Akana, S.H. El-Husayni, C.A. Desjardins, J. Morgan, J.S. Charleston, V. Sardone, J. Domingos, G. Dickson, V. Straub, M. Guglieri, E. Mercuri, L. Servais, F. Muntoni, Increased dystrophin production with golodirsen in patients with Duchenne muscular dystrophy, Neurology 94 (2020). https://doi.org/10.1212/WNL.0000000000009233.

[11] C.M. McDonald, P.B. Shieh, H.Z. Abdel-Hamid, A.M. Connolly, E. Ciafaloni, K.R. Wagner, N. Goemans, E. Mercuri, N. Khan, E. Koenig, J. Malhotra, W. Zhang, B. Han, J.R. Mendell, Open-Label Evaluation of Eteplirsen in Patients with Duchenne Muscular Dystrophy Amenable to Exon 51 Skipping: PROMOVI Trial, J Neuromuscul Dis 8 (2021) 989–1001. https://doi.org/10.3233/JND-210643.

[12] S.T. Crooke, R.S. Geary, Clinical pharmacological properties of mipomersen (<scp>K</scp> ynamro), a second generation antisense inhibitor of apolipoprotein <scp>B</scp>, Br J Clin Pharmacol 76 (2013) 269–276. https://doi.org/10.1111/j.1365-2125.2012.04469.x.

[13] W. Li, Q. Zhang, H. Miao, J. Xu, Real-world analysis of the efficacy and safety of nusinersen in pediatric patients with spinal muscular atrophy., Orphanet J Rare Dis 20 (2025) 87. https://doi.org/10.1186/s13023-025-03603-9.

[14] S. Studzińska, Ł. Nuckowski, B. Buszewski, Oligonucleotides Isolation and Separation—A Review on Adsorbent Selection, Int J Mol Sci 23 (2022). https://doi.org/10.3390/ijms23179546.

[15] D. Cipolla, H.-K. Chan, Current and Emerging Inhaled Therapies of Repositioned Drugs, Adv Drug Deliv Rev 133 (2018) 1–4. https://doi.org/10.1016/j.addr.2018.09.008.

[16] S. Studzińska, A simple and green solid phase extraction method for oligonucleotides using adsorbent with amino and carboxylic moieties, Talanta 255 (2023) 124224. https://doi.org/10.1016/j.talanta.2022.124224.

[17] S. Studzińska, O. Błachowicz, S. Bocian, O. Kalisz, A. Jaworska, J. Szymarek, M. Mazurkiewicz-Bełdzińska, Study of nusinersen metabolites in the cerebrospinal fluid of children with spinal muscular atrophy using ultra-high-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry, Analyst 149 (2024) 3739–3746. https://doi.org/10.1039/d4an00436a.

[18] X. Zhang, C. Sha, W. Zhang, F. Zhao, M. Zhu, G. Leng, W. Liu, Development and validation of an HILIC/MS/MS method for determination of nusinersen in rabbit plasma, Heliyon 10 (2024). https://doi.org/10.1016/j.heliyon.2024.e31213.

[19] G. Liu, A.-F. Aubry, Best practices in biological sample preparation for LC-MS bioanalysis, in: W. Li, J. Zhang, F.L.S. Tse (Eds.), Handbook of LC-MS Bioanalysis, 2013. https://doi.org/10.1002/9781118671276.ch14

[20] M. Ewles, A.R. Ledvina, B. Powers, C.E. Thomas, Observations from a decade of oligonucleotide bioanalysis by LC-MS, Bioanalysis 16 (2024) 615–629. https://doi.org/10.4155/bio-2024-0007.

[21] P. Turnpenny, J. Rawal, T. Schardt, S. Lamoratta, H. Mueller, M. Weber, K. Brady, Quantitation of Locked Nucleic Acid Antisense Oligonucleotides in Mouse Tissue Using a Liquid–Liquid Extraction LC–MS/MS Analytical Approach, Bioanalysis 3 (2011) 1911–1921. https://doi.org/10.4155/bio.11.100.

[22] S. Franzoni, A. Vezzelli, A. Turtoro, L. Solazzo, A. Greco, P. Tassone, M.T. Di Martino, M. Breda, Development and validation of a bioanalytical method for quantification of LNA-i-miR-221, a 13-mer oligonucleotide, in rat plasma using LC–MS/MS, J Pharm Biomed Anal 150 (2018) 300–307. https://doi.org/10.1016/j.jpba.2017.12.027.

[23] S. Studzińska, R. Rola, B. Buszewski, Development of a method based on ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry for studying the in vitro metabolism of phosphorothioate oligonucleotides, Anal Bioanal Chem 408 (2016) 1585–1595. https://doi.org/10.1007/s00216-015-9266-1.

[24] J. Kim, B. Basiri, C. Hassan, C. Punt, E. van der Hage, C. den Besten, M.G. Bartlett, Metabolite Profiling of the Antisense Oligonucleotide Eluforsen Using Liquid Chromatography-Mass Spectrometry, Mol Ther Nucleic Acids 17 (2019) 714–725. https://doi.org/10.1016/j.omtn.2019.07.006.

[25] M. Herkt, A. Foinquinos, S. Batkai, T. Thum, A. Pich, Pharmacokinetic Studies of Antisense Oligonucleotides Using MALDI-TOF Mass Spectrometry, Front Pharmacol 11 (2020). https://doi.org/10.3389/fphar.2020.00220.

[26] E.M. Staley, Commentary on Hemolytic Anemia and Thrombocytopenia: How to Treat?, Clin Chem 66 (2020) 768–768. https://doi.org/10.1093/clinchem/hvaa039.

[27] S. Studzińska, M. Mazurkiewicz-Bełdzińska, B. Buszewski, Development of the Method for Nusinersen and Its Metabolites Identification in the Serum Samples of Children Treated with Spinraza for Spinal Muscular Atrophy, Int J Mol Sci 23 (2022). https://doi.org/10.3390/ijms231710166.

[28] B. Chen, M. Bartlett, A One-Step Solid Phase Extraction Method for Bioanalysis of a Phosphorothioate Oligonucleotide and Its 3′ n-1 Metabolite from Rat Plasma by uHPLC–MS/MS, AAPS J 14 (2012) 772–780. https://doi.org/10.1208/s12248-012-9394-x.

[29] G. Ye, M. Beverly, The use of strong anion-exchange (SAX) magnetic particles for the extraction of therapeutic siRNA and their analysis by liquid chromatography/mass spectrometry, Rapid Communications in Mass Spectrometry 25 (2011) 3207–3215. https://doi.org/10.1002/rcm.5221.

[30] R. MacNeill, Hydrophilic-Phase Extraction: A New Avenue of Solid-Phase Extraction for Oligonucleotide Bioanalysis, LCGC Europe (2022) 46–51. https://doi.org/10.56530/lcgc.eu.kt3381t8.

[31] P. Anand, M. Koleto, D.R. Kandula, L. Xiong, R. MacNeill, Novel Hydrophilic-Phase Extraction, HILIC and High-Resolution MS Quantification of an RNA Oligonucleotide in Plasma, Bioanalysis 14 (2022) 47–62. https://doi.org/10.4155/bio-2021-0216.

[32] Ł. Nuckowski, A. Kilanowska, S. Studzińska, Hydrophilic interaction in solid-phase extraction of antisense oligonucleotides, J Chromatogr Sci 58 (2020) 383–387. https://doi.org/10.1093/chromsci/bmz114.

[33] L. Dillen, L. Sips, T. Greway, T. Verhaeghe, Quantitative Analysis of Imetelstat in Plasma with LC–MS/MS Using Solid-Phase or Hybridization Extraction, Bioanalysis 9 (2017) 1859–1872. https://doi.org/10.4155/bio-2017-0145.

[34] S. Studzińska, M. Skoczylas, S. Bocian, A. Dembska, B. Buszewski, Attachment of hybridizable oligonucleotides to a silica support and its application for selective extraction of unmodified and antisense oligonucleotides from serum samples, RSC Adv 10 (2020) 16221–16230. https://doi.org/10.1039/D0RA01620A.

[35] P. Li, Y. Gong, J. Kim, X. Liu, J. Gilbert, H.M. Kerns, R. Groth, M. Rooney, Hybridization Liquid Chromatography–Tandem Mass Spectrometry: An Alternative Bioanalytical Method for Antisense Oligonucleotide Quantitation in Plasma and Tissue Samples, Anal Chem 92 (2020) 10548–10559. https://doi.org/10.1021/acs.analchem.0c01382.

[36] Ł. Nuckowski, E. Zalesińska, K. Dzieszkowski, Z. Rafiński, S. Studzińska, Poly(ionic liquid)s as new adsorbents in dispersive micro-solid-phase extraction of unmodified and modified oligonucleotides, Talanta 221 (2021) 121662. https://doi.org/10.1016/j.talanta.2020.121662.

[37] Ł. Nuckowski, K. Dzieszkowski, Z. Rafiński, S. Studzińska, Application of Magnetic Nanoparticles Coated with Crosslinked Zwitterionic Poly(ionic liquid)s for the Extraction of Oligonucleotides, Materials 14 (2021) 3146. https://doi.org/10.3390/ma14123146.

[38] S. Studzińska, K. Ostrowska, Z. Vosáhlová, K. Sasim, S. Bocian, Application of amino acid-based adsorbents for the extraction of antisense oligonucleotides from serum samples, Talanta 298 (2026) 128973. https://doi.org/10.1016/j.talanta.2025.128973.

[39] https://numiqo.com/statistics-calculator/design-of-experiments, (n.d.).

[40] W. Zhou, S. Yang, P.G. Wang, Matrix Effects and Application of Matrix Effect Factor, Bioanalysis 9 (2017) 1839–1844. https://doi.org/10.4155/bio-2017-0214.

[41] U. Flierl, T.L. Nero, B. Lim, J.F. Arthur, Y. Yao, S.M. Jung, E. Gitz, A.Y. Pollitt, M.T.K. Zaldivia, M. Jandrot-Perrus, A. Schäfer, B. Nieswandt, R.K. Andrews, M.W. Parker, E.E. Gardiner, K. Peter, Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators, Journal of Experimental Medicine 212 (2015) 129–137. https://doi.org/10.1084/jem.20140391.

[42] G. Liu, W. Fu, Z. Zhang, Y. He, H. Yu, Y. Wang, X. Wang, Y.-L. Zhao, Z. Deng, G. Wu, X. He, Structural basis for the recognition of sulfur in phosphorothioated DNA, Nat Commun 9 (2018) 4689. https://doi.org/10.1038/s41467-018-07093-1.

[43] V. Kojasoy, D.J. Tantillo, Impacts of noncovalent interactions involving sulfur atoms on protein stability, structure, folding, and bioactivity, Org. Biomol. Chem. 21 (2023), 11–23

[44] L. Sips, E.N. Ediage, B. Ingelse, T. Verhaeghe, L. Dillen, LC–MS quantification of oligonucleotides in biological matrices with SPE or hybridization extraction, Bioanalysis 11 (2019) 2445–2460. https://doi.org/10.4155/bio-2019-0117

[45] L. Ramanathan, H. Shen, LC-TOF-MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues, Bioanalysis 11 (2019) 1983–1992. https://doi.org/10.4155/bio-2019-0134

[46] M. Hemsley, M. Ewles, L. Goodwin, Development of a bioanalytical method for quantification of a 15-mer oligonucleotide at sub-ng/ml concentrations using LC–MS/MS, Bioanalysis 4 (2012) 1457–1469. https://doi.org/10.4155/bio.12.117