Application of amino acid-based adsorbents for the extraction of antisense oligonucleotides from serum samples

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Abstract

Antisense oligonucleotides are synthetic, short, single-stranded nucleic acid analogues used for several years as an active ingredient of drugs for treating genetic diseases. Their effective sample preparation is a topic that still requires in-depth research. Consequently, amino acid-modified silica adsorbents were utilized for the first time in the dispersive solid-phase extraction of antisense oligonucleotides. The impact of the composition of solvents used for adsorption and desorption was tested (pH, salt type, salt concentration, organic solvent type, and content in the solution). Adsorption of oligonucleotides occurred from water or salt solutions with low pH. Increasing the pH cause increase of recovery contrary to increase salt concentration. Moreover addition of methanol or acetonitrile to elution solvent reduce recovery. The developed method is based on weak ion exchange, with pH playing a key role in the extraction procedure. Thus, the adsorption and desorption are driven solely by the change in pH of the ammonium acetate solution. All tested adsorbents allowed for the efficient recovery of oligonucleotides. Nevertheless, adsorbent with bonded methionine showed the highest efficiency. The recovery depended on the structure of the antisense oligonucleotide (the highest for short and modified ones). The recovery also increases with increasing hydrophobicity of the modification. The developed method was successfully applied to oligonucleotide extraction from human serum samples resulting in a recovery of 91±1%. Only a 10 mM ammonium acetate with pH 4 (adsorption) or 11 (desorpion) is used throughout the whole procedure. The developed procedure is simple, efficient, selective, and environmentally friendly.

Keywords: antisense oligonucleotides; dispersive solid phase extraction; amino-acid based adsorbents, weak ion-exchange; recovery; serum

1. Introduction

Antisense oligonucleotides (ASOs) are a group of synthetic single-stranded nucleic acid analogues whose chemical structure has been modified to enhance stability and affinity for target RNA [1–3]. Via specific binding to mRNA sequences, ASOs enable modulation of gene expression by transcript degradation, translation inhibition, or modulation of alternative splicing [1,4]. Advanced oligonucleotide (ON) synthesis techniques have created new therapeutic opportunities, such as nusinersen (Spinraza) therapy for spinal muscular atrophy (SMA) or eteplirsen (Exondys 51) in the treatment of Duchenne muscular dystrophy (DMD) [1,5–7]. The two drugs listed above are merely illustrative examples. By March 2024, 20 products containing ONs had been released [8].

The emergence of gene therapy has underscored the importance of monitoring processes to maintain quality and control throughout production. ASOs have been detected within the complex biological matrices (e.g., plasma or cerebrospinal fluid) of patients undergoing treatment. They contain various compounds (e.g., proteins, lipids), and thus proper sample preparation methods are critical [5,9–11]. ONs have a very high affinity to proteins, making their recovery low because proteins have to be removed before further extraction and analysis. Traditional extraction methods, such as liquid-liquid extraction and proteinase K digestion, allow effective protein removal without losing ASO, but unfortunately, require additional purification steps [2,5,9,12]. Solid-phase extraction (SPE), micro-extraction by packed sorbent (MEPS), and hybridization are employed as the second step [9,11,13–15]. However, these methods necessitate larger amounts of solvents and are time-consuming [5]. Hybridization shows lower selectivity and efficiency for short metabolites, and it is also an expensive method [9]. SPE provides high recoveries and good purification; however, the disadvantage of SPE is the use of ion-pair reagents. MEPS provides high purification and reduces solvent consumption but provides lower recoveries than SPE [5]. Therefore, dispersive solid phase extraction (dSPE) is increasingly used, minimizing solvent usage, shortening the procedure time, and permitting the use of new adsorbents that can enhance selectivity to ASOs by introducing new functional groups at the surface of the support [5,9,16]. The use of adsorbents containing amine and carboxyl groups for dSPE extraction of ASO provided high recovery for both standards and real samples (80-92%) [16]. In another case, the use of magnetic nanoparticles coated with zwitterionic poly(ionic liquids) for dSPE allowed for a high recovery of ON (>80%) from enriched human serum [17]. The recent advances in sample preparation across diverse fields have introduced hybrid solid-phase extraction combined with dispersive liquid–liquid microextraction (SPE–DLLME) approaches, deep eutectic solvent-based extractions, and

solvent-free DLLME strategies, reflecting a general trend toward more versatile and sustainable extraction methods [18–20]. Our study extends this development to the extraction of antisense oligonucleotides from serum using amino acid–based adsorbents.

Our attention was drawn to amino-acid-based adsorbents because of the diverse structure of amino acid side chains. They enables various interactions with analytes, including electrostatic, hydrogen bonding, π–π interactions, and hydrophobic interactions, and thus exhibitis complex retention mechanism [21,22]. [22]. These materials served as stationary phases in liquid chromatography and were previously used for solid phase extraction in numerous studies [23–28] . Hovewer, amino-acid-based adsorbents have not previously been used in dispersive solid-phase extraction of ONs, even though the properties resulting from their structure are promising. Therefore, their potential for dispersive solid phase extraction of Ons was determined.

The objective of the present study was to develop a dSPE method employing amino-acid-based adsorbents for ASO, including nusinersen analogues, from biological samples. Five synthetic sorbents, functionalised with different amino acids, were utilised in the study. These materials were used for the first time in the extraction of ASO. ASO's adsorption and desorption process was optimized concerning the solvents used during sample loading and elution. Moreover, the impact of chemical modifications and ON length on extraction efficiency was tested, as it is an essential issue for ASO metabolites. The developed method was successfully used to extract ONs from enriched serum samples.

2. Materials and methods

2.1.Materials and reagents

Acetonitrile (ACN, Chromasolv®, gradient grade, ≥ 99.9%) and methanol (MeOH, Chromasolv®, gradient grade, ≥ 99.9%) were purchased from Honeywell (Charlotte, NC, USA). Ammonium acetate (purity ≥ 99%), acetic acid (purity ≥ 99%), 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP, ≥ 99.9%), phenol-chloroform-isoamyl alcohol mixture (BioUltra purity), and *N,N*-dimethylbutylamine (DMBA, 99%) 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). Deionized water was purified with the Milli-Q system from Millipore (Burlington, MA, USA).

The solid support of the adsorbents, Kromasil 100, with a particle diameter of 5 μm, pore diameter of 100 Å, pore volume of 0.9 ml/g, and surface area of 310 m^2/g was purchased from Akzo Nobel (Bohus, Sweden). The following reagents supplied by Sigma-Aldrich (St. Louis, MO, USA) were used for chemical modification of the silica support: aminopropyltrimetoxysilane, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Tyr-OH, Fmoc-Met-OH, N,N`-dicyclohexylcarbodiimide (DCC), piperidine, anhydrous dichloromethane (DCM), anhydrous N,N-dimethylformamide (DMF). Other organic solvents used during synthesis, methanol, and toluene were purchased from J.T. Baker (Deventer, Netherlands).

2.2. Apparatus and chromatographic conditions

Solid state FTIR spectra in the range $\tilde{\upsilon}$ = 4000 – 400 cm^{-1} were recorded on a Spectrum 2000 instrument from Perkin-Elmer (Shelton, CT, USA). 13C NMR measurements were performed on a Bruker Avance III 700 MHz (Karlsruhe, Germany) after placing ~300 mg samples in the double-bearing rotors of zirconia. The 13C cross-polarization magic-angle spinning (CP/MAS) NMR spectra were received with rotation frequency 8 kHz, pulse time 2 ms, acquisition time 0.01643 s, and relaxation time 6 s. Elemental analysis was done using a Perkin-Elmer CHN 240 analyzer (Shelton, CT, USA).

The quantitative and qualitative analysis of ONs was performed using UHPLC in IP RP mode. Thermo UHPLC Vanquish Flex (Karlsruhe, Germany) and Shimadzu Nexera-i LC-2040C 3D (Kyoto, Japan) liquid chromatographs equipped with PDA detectors were used. The data were collected using Chromeleon 7 and Lab Solution chromatography data systems. For those purposes, ACE C18-Ar column from Advanced Chromatography Technologies Ltd (Aberdeen, Scotland; 1.7 μm, 2.1×100mm), and Acquity UPLC BEH C18 column from Waters (Milford, MA, USA; 1.7 μm, 2.1×100mm) were used. Mobile phase (MP) composition was the following: MP A – 5mM DMBA/150mM HFIP in water; MP B – MeOH; gradient elution – 0min. 15% MP B; 6min. 45% MP B; 7min. 15% MP B; 12min. 15% MP B. UV-detection detection wavelength was equal to 260 nm. The autosampler temperature was set to 10°C, and the flow rate was 0.3 mL min^{-1}. The Acquity UPLC BEH C18 column temperature was equal to 30°C, while for ACE C18-Ar, it was 40°C. The pH meter CP-505 Elmetron (Zabrze, Poland) was used to prepare a solution for extraction. The suspensions during the extraction were centrifuged using a Frontier Micro FC5515 centrifuge from OHAUS Europe GmbH (Nänikon, Switzerland).

2.3. Synthesis and detailed characterization of selected adsorbent

The phases with covalently bonded amino acids were prepared according to the procedure described in the previous works [21,29,30]. Briefly, the silica gel was dried at 180 degrees Celsius under vacuum for 24 h, then, aminopropyltrimethoxysilane in toluene solution was added to the silica gel sample. After 12 hours of reaction at 50 degrees Celsius , excess silane was removed by washing the sorbent with toluene, methanol, and hexane then the gel dried at a temperature of 80°C ± 5 °C under reduced pressure. Further, the prepared support was bonded with the amino acid (with a protected amino group) using DCC. The solutions of Fmoc-blocked amino acid and DCC were prepared separately. Reaction were carried out at 40 degrees Celsius. The reaction products were washed with dichloromethane, toluene, and methanol on a Schott funnel to eliminate the excess reagents. The next synthesis stage was the deprotection of the bonded amino acids. The Fmoc groups were removed using 50 mL of a 20% solution of piperidine in anhydrous DMF. The resulting suspension was stirred for 15 min, and then the product was filtered and washed with toluene and methanol consecutively. The prepared adsorbent was dried under a vacuum at 60 °C overnight.

The structures of synthesized adsorbents were confirmed by FT-IR spectroscopy and 13C solid-state CP/MAS NMR. Measurements of the zeta potential of the adsorbent in solutions with different pH values and solvent compositions were also performed. The synthesized materials were also subjected to elemental analysis to determine the percentage of nitrogen and carbon at the surface.

Table 2 presents the carbon and nitrogen content at the surface of silica after its modification with aminopropyl groups and amino acid molecules. Moreover, coverage density was determined (according to the procedure described in the Supplementary materials).

2.4. Development of dSPE procedure

DNA ON was used to optimize adsorption and desorption conditions. The 2 mg of sorbent was used during each experiment, and it was performed twice. The dSPE procedure consisted of the following steps: i) sorbent conditioning, ii) sample loading, iii) washing, iv) elution. During sorbent conditioning, 100 µL of MeOH, 100 µL of water, and finally 200 µL of the same solution, in which the ON is dissolved during sample loading were used. The following solvents for adsorption were tested: water (pH 6.3, not adjusted), water (pH 4.0), 10 mM ammonium acetate (AA) (pH 4.0), 10 mM AA (pH 5.0), and 5 mM AA (pH 4.0). The pH was adjusted by adding acetic acid to reach the required pH value. After sorbent conditioning, 100 µL of 10 µM ON dissolved in solvent for adsorption was loaded and mixed (with vortex) for 5 minutes. The washing step was performed using 100 µL of MeOH/10mM AA, pH 4.0, 10/90 (v/v). For ON

elution, several desorption solutions were tested: 10 mM AA at pH 8.5; pH 9.5; pH 10.5 and pH 11.0; for pH 11.0, additionally with the following concentrations: 20 mM AA; 30 mM AA, and 50 mM AA. The pH was adjusted by adding 25% ammonium hydroxide solution to reach the required pH value. Additionally, the mixtures of 10 mM AA (pH 11.0) with ACN or MeOH in various volumetric ratios (75/25; 50/50; 25/75) have been tested for elution. Between each step, 10 minutes of centrifugation was performed to ensure proper distribution of sorbent and supernatant. 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 ONs standard was dissolved in the same solution as the eluent.
The effect of adsorption time was investigated. The study was performed for Met and NU ME. The adsorbent was conditioned according to the developed and optimized procedure (Figure S1). Five different adsorption times were investigated (1, 2, 5, 10, 20 min). Sorption capacity was also determined during the study. The 1 mg of adsorbent was conditioned according to the methodology presented in Figure S1. The next three attempts of sample load were performed: 150 µL of 100 µM NU ME, 180 µL of 100 µM NU ME, and 200 µL of 100 µM NU ME. The suspension was mixed for 2 minutes, then centrifuged for 15 minutes (14,000 rpm), and next the supernatant was analysed using UHPLC to determine the amount of NU ME remaining in the solution (not adsorbed). The amount of adsorbed ON was calculated from the difference between the amount used in the attempt and the amount remaining in the solution.
The finally optimized procedure uses 2 mg of Met adsorbent. Extraction steps were following: 1. conditioning: 100 µL MeOH, 100 µL H_2O, 100 µL10 mM AA pH 4 (2 min. shaking with vortex and 10 min. centrifuging with 14,000 rpm between each solvent); 2. sample load: ON dilluted with 10 mM AA pH 4 to final volume of 100 µL, 2 min. shaking with vortex and 10 min. centrifuging with 14,000 rpm; 3. washing: 100 µL 10 mM AA pH 4/MeOH (9:1), 2 min. shaking with vortex and 10 min. centrifuging with 14,000 rpm; 4. elution: 100 µL 10 mM AA pH 11, 2 min. shaking with vortex and 10 min. centrifuging with 14,000 rpm.

2.5 Serum samples preparation

Firstly, human serum samples were then spiked with NU ME to a concentration of 5 µM (25 µL of 100 u µM stock solution to 500 µL). Next, serum was diluted with water in the ratio of 1:3 (500 µL of serum and 1500 µL of water). The LLE was used to remove proteins in the first sample preparation step. For this purpose, a mixture of phenol/chloroform/isoamyl alcohol was added to previously diluted serum (1:1 v/v). The sample was shaken (using vortex for 30s) and then centrifuged for 45 minutes (14,000 rpm). Next, two approaches were used to extract

NU ME from serum samples after LLE: 1. direct dSPE using developed procedure; 2. removing residual phenol using second LLE with chloroform (1:4 v/v) (repeated three times) and than application of dSPE. The serum samples 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).

2.6 Validation of the chromatographic method

Ion-pair reversed-phase ultra-high-performance liquid chromatography (IP RP UHPLC) was used to determine ONs at each step of the development of sample preparation. A calibration curve was constructed based on the results obtained with standard samples of known NU ME concentration (0.16 – 5 µM). The linearity was determined by calculating the determination coefficient (R^2). The degree of repeatability was determined by performing five injections of two different concentrations (5 µM, 1.25 µM) on the first, third, and seventh days. The precision achieved was determined by performing seven injections daily, for three concentrations (5 µM, 1.25 µM, 0.01 µM). The limits of detection (LOD) and quantification (LOQ) resulted from experimental determination. The signal-to-noise ratio method was used. 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.

3. Results and discussion

3.1. Selected adsorbents

Synthesized adsorbents were characterized by elemental analysis. The data presented in Table 2 confirm the bonding of individual amino acids due to increased carbon and nitrogen content in the synthesized adsorbents. Moreover, coverage density was determined. Due to the steric hindrance, the bonded amino acid ligands' coverage is lower than the amino groups' primary coverage density (Table 2). In the case of smaller amino acids (e.g., Ala), achieving a higher coverage density was possible than in the case of amino acids of greater size (e.g., Met). In the FT-IR spectra, the signal corresponding to amino groups derived from aminopropyl ligands may be found at around $\tilde{\upsilon}$=1600 cm^{-1}. The characteristic absorption band of peptide-bonded material is observed at $\tilde{\upsilon}$=1550 cm^{-1} (Figure S2). However, the final confirmation of the structure is based on ^{13}C NMR spectra. The largest value of chemical shifts (δ=174 ppm) corresponds to carbonyl carbon, confirming the creation of peptide bonds (Figure S3). The smallest chemical shift δ=11 ppm is characterized by a carbon atom linked to a silicon atom.

Signals in the range δ=47-50 ppm were attributed to carbon atoms bonded with nitrogen atoms in unmodified amine or peptide bonds.

Regardless of the degree of modification, unreacted aminopropyl groups are present on the surface of each sorbent obtained. This creates unique acid-base properties with different pKa values. In the case of unmodified aminopropyl groups, the pKa value ranges from 9 to 9.6. This value decreases significantly for amino groups found in bound amino acids, ranging from 6.8 to 7.9, which was confirmed in the previous study [29].

It is also worth noting that amino acid-based sorbents significantly alter the zeta potential values when the composition of the solution changes. In general, for ACN/water mixtures, when the amount of ACN is less than 80%, they exhibit positive values, while under 100% ACN conditions, their zeta potential takes on negative values. Under conditions of variable pH, these materials show the highest positive zeta potential values in an acidic environment, which results from the protonation of amino groups. An increase in pH from 3 to 5 causes a decrease in zeta potential by about half. Under neutral conditions, zeta potential generally takes on negative values (depending on the type of adsorbent), and an increase in pH to 9 gives negative values regardless of the sorbent structure [29]. This is due to the deprotonation of amino groups on the surface of the adsorbent and the ionization of residual silanols. The exact pH values at which the zeta potential becomes negative depend on the values of the dissociation constants of the amine groups in the sorbent structure. Changes in the zeta potential indicate a change in the surface charge of the sorbents. Thanks to this, pH control makes it possible to change the surface charge, allowing for ionic substances' adsorption and desorption control.

The diverse structure of amino acid-based sorbents means they can interact with potential analytes differently. Previous studies indicated dominant ion–ion electrostatic interactions, hydrogen bonding formation, and hydrophobic/hydrophilic interactions as principal interaction mechanisms [22]. The ability to form various hydrogen bonds, both as a donor and acceptor of this bond, is crucial. Even in the case of sorbents with bound amino acids with a hydrophobic side chain (e.g., Leu), the surface of the sorbent is hydrophilic due to the presence of unmodified amino groups and amide (peptide) bonds. Additionally, the adsorption selectivity may be influenced by π-type electron bonds present in the structure of Tyr. Amino acid-based sorbents have mixed hydrophobic-hydrophilic properties, with an ion exchange mechanism depending on the pH. Weak ion exchange appears to be the most important among the various possible interactions. Significant is the above-mentioned possibility of controlling the surface charge. By changing the pH, it is possible not only to change the net surface charge

and anion exchange capacity in an acidic environment to cation exchange capacity in an alkaline solution (resulting from silanol groups and those of lesser importance), but also to control the number of ion exchange centers through the presence of two types of amino groups with different pKa values.

Given the properties of adsorbents with bounded amino acid molecules and the common knowledge of the interactions between ONs and proteins in cells, it was a natural choice and an interesting challenge to select these materials to extract ASO [5,21,29]. These adsorbents are polar (hydrophilic), similar to ONs, and their potential for weak ion exchange in chromatography (discovered in our team several years earlier) was explored during current research in the extraction of polyanions (synthetic RNA fragments) [21,22,29].

3.2. Optimization of conditions for ONs adsorption/desorption

During the initial phase of experiments, the effect of adsorption solution (i.e., the solvent in which the ON is dissolved) on the effectiveness of adsorption has been studied. The data showed that for all tested conditions (water, pH unadjusted; water, pH 4.0; 5mM AA, pH 4.0; 10mM AA, pH 4.0, and 10mM AA, pH 5.0), more than 99 % of ONs were adsorbed for all tested sorbents, also for aminopropyl one (not modified with amino acid). This observation points out that the adsorption mechanism is probably based predominantly on electrostatic interactions between negatively charged (thio)phosphate backbone of ONs (pKa ~ 2, acidic) and positively charged aminopropyl groups (pKa ~ 9 to 9.6) and amino group in amino acid (pKa ~ 6.8 to 7.9) on the surface of the adsorbent. Since almost complete adsorption was observed for all tested adsorbents, the adsorption efficiency does not depend on the adsorbent surface modification, i.e., on the type of amino acid bonded to the surface of the adsorbent. This is also confirmed by the complete adsorption of ONs on the Amino adsorbent surface. Therefore, the contribution of hydrophobic or π-π interaction to adsorption is negligible. The adsorption does not depend on the nature of the adsorption solution (pure water or salt solution). The adsorption takes place at pH of the adsorption solution, where both amino groups on the adsorbent and (thio)phosphate groups in ONs are charged and allow electrostatic attraction. However, the application of concentrated salts for adsorption can potentially inhibit this electrostatic attraction and cause a decrease in adsorption efficiency. Even though the results showed great potential (adsorption $\geq$ 99%) of pure water usage as an adsorption solution, which would significantly simplify the adsorption procedure, 10 mM AA (pH 4.0) was selected for further studies. The reason was to ensure the exact pH value suitable for the most efficient

adsorption (the pH of pure water may not be stable over time). Ammonium acetate is a buffer in the pH range of 3.8 to 5.8, which is why we used this salt instead of water in the procedure. On the other hand, we assumed that ON's desorption is connected with the loss of electrostatic interaction between ON and the adsorbent. Thus, the application of a solvent with high pH (basically above pKa of amino groups on the surface of the adsorbent) leads to deprotonation of amino groups on the adsorbent surface, the electrostatic attraction is reduced, and ON can be desorbed. To verify these hypotheses, we also conducted a desorption test for the Amino adsorbent, modified only with aminopropyl groups. In our work, the effect of pH on desorption was investigated, and the results are summarized in Figure 1. While using 10 mM AA with pH 8.5 and 9.5, no desorption of ONs was observed. It proves that amino groups on the adsorbent surface are still positively charged, and electrostatic attraction with negatively charged ONs occurs, which is consistent with the calculated pKa values. Increasing AA pH from pH 9.5 to pH 10.5 causes an increase in desorption (from 0% recovery to ~75% recovery), which confirms that between pH 9.5 and 10.5, a loss in positive charge density on the sorbent surface takes place (Figure 1A). An increase to pH 11.0 causes an additional increase in recovery (~80%), resulting from the protonation equilibrium (99% of the deprotonated form is obtained at a pH 2 units higher than pKa) (Figure 1A). Interestingly, these effects were the same for all tested adsorbents regardless of amino acid type (under experiment conditions), even though the pKa values of amino groups at different amino acid residues vary from about 7 to 9, which should result in partial desorption while using pH 9.5. On the other hand, however, desorption under analogous conditions for the Amino adsorbent was 28±2%. This observation confirms the crucial role of aminopropyl groups on the adsorbent surface. They interact strongly with ONs, which is essential during adsorption, but desorption from an adsorbent coated only with aminopropyl groups is incomplete. This proves that the modification of some of the aminopropyl groups is important. Although our research has shown that the type of amino acid does not significantly impact recovery (under experiment conditions), reducing the number of amino groups by blocking them with amino acid molecules is essential, especially during desorption. Still, adsorption/desorption processes are driven by an ion exchange mechanism and do not correlate with the type of amino acid, since no significant differences between recoveries for individual adsorbents were observed (Figure 1A). The results show that for amino acid-based sorbent, around 20-25% of ON remains adsorbed on the surface of the adsorbent after the desorption procedure, while it is around 70% for Amino (Figure 1A). It may correlate with the fact that even at pH 11.0, not all the aminopropyl groups are deprotonated. The reason can be simply explained by the fact that if the desorption solution's pH is about 1 higher than

the pKa of aminopropyl groups, approximately 10% of aminopropyl groups remain protonated. Additionally, steric effects can occur (e.g., not all aminopropyl groups on the surface are deprotonated because they are not accessible for desorption solution through adsorbed ON). Hydrogen bonding between deprotonated aminopropyl group/amino acid residues and ON can also contribute to the adsorption. Even though the recoveries reach values of a maximum of around 80%, the most important thing is the repeatability of the obtained recoveries (standard deviation < 5%) (Figure 1A).

Since we assumed that the adsorption/desorption mechanism is based predominantly on ion exchange, the next steps of the study involved attempts to improve recovery by changing the concentration of desorption solution, which is the most influential parameter in ion-exchange chromatography. Initially, the effect of AA concentration (for pH 11.0, where the recovery was the highest for all tested adsorbents) was studied. The results are depicted in Figure 1B and show that no general trend is applied; however, a slight decrease in ONs recovery between 10mM and 30mM is visible for all tested adsorbents (Figure 1B). This effect reveals that electrostatic attraction between positively charged adsorbent and negatively charged ON at pH 11.0 is not taking place (if so, the increasing concentration would cause inhibition of electrostatic attraction, and the recovery would increase). Thus, sligh changes in recovery with changing of AA concentration are probably connected with changing of partitioning, which was previously described for amide-based sorbent in HILIC [31].

Since the concentration had almost no effect on recovery, in the next step, we tested the impact of the proportion of organic solvent and the type of solvent on desorption efficiency, which is a typical parameter affecting the adsorption in reversed-phase or HILIC-based interaction mechanisms. Figures 1C and D show the effect of organic solvent content in elution solution on ONs desorption/recovery. Generally, comparable results are achieved in the range of 0% to 50% of organic solvent for both ACN and MeOH, except for 50% MeOH using tyrosine adsorbent, where a significant decrease in recovery was observed. The presence of 25% MeOH in elution solution increases the desorption (except for Met), while using 75% MeOH leads to a decrease in recovery (≤0.5 %) (Figure 1C). The presence of ACN in elution solution causes a reduction in recovery for all tested sorbents. Interestingly, even using 75% ACN, 6-8% of recoveries were obtained for Leu and Met adsorbents (Figure 1D). The fact that in the range from 0 to 50% of the organic solvent, the recoveries differ only slightly for most of the adsorbents confirms the negligible contribution of hydrophobic interaction to the sorption processes. Since we assume the main driving force for ONs elution is lack of electrostatic attraction, the effects regarding the organic solvent ratio are probably mostly connected to the

change of solution pH*, because increasing organic content decreases the pH* - for solutions with 75 % of organic, pH* is about 9.7 (for ACN) and 9.8 (for MeOH); pH^* stands for the pH of the hydro-organic solution, measured by pH meter calibrated by standard aqueous solutions. To conclude the adsorption/desorption conditions optimization for dSPE, the results showed minor differences among the tested amino acid-based adsorbents. Contrary results were observed for recovery for Amino and amino acid-based adsorbents, where the differences are large. Together with other observations, it confirms that the primary mechanism responsible for ONs adsorption is ion exchange, which occurs in acidic conditions. On the other hand, the desorption of ONs is caused by the deprotonation of these amino groups at a basic pH. An important parameter, which should be considered, is the crucial role of modifying/blocking part of the aminopropyl groups using amino acids. Even though the ion exchange is the main driving force for ONs adsorption, it can be assumed that some other types of interaction are also involved (e.g., hydrogen bonding), which are consequently responsible for the differences in recoveries for different types of ONs (see the following sections). Based on the described preliminary adsorbent testing, Met adsorbent showed higher recoveries than the other tested adsorbents, which can be connected with the presence of sulfur in the structure of methionine. The standard deviation of recovery for this adsorbent was relatively low (1 - 3%). Additionally, 10mM AA, pH 4.0, was chosen as an adsorption solution, and 10mM AA, pH 11.0, as an elution solution. The conditions for the finally optimized dSPE procedure are shown in Figure S1. The developed procedure is simple and efficient. Moreover, the procedure and environmentally friendly. Through the wole procedure we use only 110 µL MeOH (out of 700 µL), while all other solvents are just a low concentration organic salt, which is not harmful to the environment. What is more, we use environmentally friendly ammonia or acetic acid to adjust the pH. The entire extraction process is carried out using only one plastic Eppendorf tube, so it does not generate much waste.

3.3. Extraction of ASOs with different lengths

Preliminary studies led to the selection of three adsorbents, namely Leu, Tyr, Met (Table 2, based on recovery), for the assessment of the adsorption efficiency of two phosphorothioate-modified ASOs (PS16, PS18) differing in length (16-mer and 18-mer) (Table 1). The recovery of PS16 was higher than for PS18 for all adsorbents (Figure S4). Moreover, in subsequent experiments, also fully PS 20-mer (PS20) was used (see Fig. S5 and section 3.4). The results follows described tendencies, i.e. decrease in recovery with increasing length from 16 to 20-mer, but only for Leu and Tyr adsorbensts. This effect is probably due to the lower number of

nucleotides and thus lower negative charge. The greater charge of longer ASO increases its affinity to amino acid-based adsorbent due to weak anion exchange and hydrogen bonding, reducing desorption. These results are satisfactory for the monitoring therapy using ASO because the main metabolites of ASO are their truncated fragments generated by exonucleases. Extraction is based mainly on weak ion exchange, but small differences in recoveries for Met, Tyr, Leu were noticed, indicating the impact of amino acid bonded to the surface (Figure S4). The aromatic ring in Tyr side chain may interact with nitrogen bases in ASO by π-π interactions; however, they are not advantageous since the lowest recoveries were noticed for this adsorbent (65 ± 2% for 16-mer; 52 ± 1% for 18-mer; 48 ±1% for 20-mer) (Figures S4 and S5). Probably, the strength of these interactions reduces recovery. Utilizing Leu with the hydrophobic side chain increases recovery compared to Tyr, because hydrophobic interactions are not the strongest ones for ONs leading to more efficient release of ASO from Leu surface (83 ± 3% for 16-mer; 68 ± 2% for 18-mer; 62 ±2% for 20-mer) (Figures S4 and S5). The highest recovery values for all ASOs were obtained using the Met adsorbent, and the standard deviation was low (~3%). Moreover, for 20-mer ON, the recovery was higher compared to shorter ones (89 ± 2% for 16-mer; ~ 75 ± 2% for 18-mer; 91 ± 3% for 20-mer)This can be connected with the fact, that sulfur is more electronegative than carbon; thus, in a C-S bond, electron density will be slightly pulled towards the sulfur atom, giving it partial negative charge and the carbon atom partial positive charge. Despite the slight electronegativity difference, sulfur is larger and more polarizable atom than oxygen. The presence of polar solvent molecules (like water in an aqueous alkaline solution) and ions will interact with the thioether molecule. These interactions, primarily through ion-dipole forces and dipole-dipole forces, can induce or enhance polarization in the thioether bond. The lone pairs on the sulfur atom can also interact with cations or the positive ends of polar solvent molecules, which may increase the desorption and thus recovery.

3.4. The influence of ASO modification on dSPE recovery

ASOs 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 increase the ASOs stability and affect their binding properties. Therefore, the next step of the research was to check the impact of ASO modification on the extraction recovery using amino acid-based adsorbents. Leu, Tyr, Met were selected for this investigation together with three ASO of the same sequence, but differing in the type of structural modification, namely PS, ME, MOE (Table 1 and 2). The results are presented in Figure S5. In average, the

lowest recoveries were obtained for the PS (49–91%), contrary to ME (58-88%) and MOE (72-89%). The reproducibility was satisfactory in each case (lower than 3%). Generally, as the hydrophobicity of ASO modification increases, so does its recovery. As discussed in Section 3.1, the surface of adsorbents remains largely hydrophilic due to residual unmodified amine groups and amide (peptide) bonds. Thus, increasing the hydrophobicity of modified ASO causes a reduction of their interaction with the hydrophilic surface, and the recovery increases. These results show that ASO modification affects extraction efficiency, making this result necessary for further application to real samples.

The next step of the study involved the application of the developed dSPE procedure for the extraction of three analogs of nusinersen (the active ingredient of Spinraza, used for the treatment of spinal muscular atrophy): NU, NU PS, NU ME (Table 1). The study involved four adsorbents Leu, Tyr, Ala, Met (Table 2). These 18-mer analogs differ in the chemical modifications used. Additionally, three ASO (13-, 14-, and 18-mer) containing both of the mentioned modifications in one molecule (NU PS-ME) were analysed (Table 1). This ASO with two modificationswas used, because most of therapeutic ones contain more than one type of modification (e.g., nusinersen includes both of them). Figure 2 summarises the recovery of ASOs depending on the adsorbent. The lowest recoveries were obtained for unmodified NU (61-69%), except for Met, where recovery was higher (94%) (Figure 2). In the case of modified ONs, regardless of the adsorbent used, significantly higher recovery values were determined (NU PS 78-84%, NU ME 70-100%). The introduction of modifications to the structure of ASOs leads to an increase in the molecule's hydrophobicity. It limits the ability of ON to form hydrogen bonds, particularly by eliminating hydroxyl groups on ribose or replacing oxygen in the phosphate group. These changes cause increased recovery. It has to be underlined that the reproducibility of the results was high in each case, as SD was lower than 4% (Figure 2). In the next step, NU PS-ME was extracted using Met, since it previously showed the highest recoveries regardless of the type of ASO modification. The introduction of both modifications to the ON sequence increases ASO hydrophobicity significantly; consequently, high recovery values and reproducibility were obtained for 13-, 14- and 18-mer (86-89% with SD 1-2%) (Figure 3). This is of great analytical significance because metabolites of therapeutic ONs are their shorter fragments. These results confirm the suitability of the developed dSPE procedure for the determination of both the active ingredient of the drug and its metabolites in biological samples. Moreover, low values of standard deviations indicate good repeatability of the developed method.

The recoveries of ONs also differed depending on the adsorbent used, indicating a possible influence of the chemical properties of the side chain on the efficiency of the desorption process (as mentioned in Section 3.3). Regardless of the type of ASO modification, the lowest recoveries were observed for Tyr (Figure 2 and S5). This effect can be explained by the possibility of π–π type interactions between the aromatic ring of tyrosine and the nitrogenous bases of ON, which complicates ON desorption. Higher recoveries were observed for Leu and Ala adsorbents, whose side chains are hydrophobic, which limits their ability to form strong interactions with ASO, contrary to Tyr (Figure 2 and S5). The highest recovery, regardless of the type of modification used, was obtained using Met, just like when testing adsorbents.

The tested amino acid-based materials were found to be effective for ASO extraction. Based on the obtained results, Met adsorbent was selected for further studies using serum due to the highest recovery values for this material, regardless of the type of ASO modification and its length. Additionally, low standard deviations indicated good repeatability of the method. This is an important parameter from the point of view of its possible use in the analysis of biological samples.

3.5 Sorption kinetics and sorption capacity

The study of the adsorption over time was performed for Met and NU Me. After the first minute, 100% of NU ME was adsorbed. The observed effect is a consequence of apparently quick electrostatic attraction between negatively charged ON and the positively charged surface of the adsorbent. Quick adsorption is a significant advantage of the developed procedure because it takes less time.

The sorption capacity of Met was determined for a 100 μM solution of NU ME. The process was carried out until ON was detected in the supernatant. The sorption capacity was calculated based on the amount of adsorbed NU ME, equal to 91±5 μg/mg. This value was valuable and essential for appropriately developing the extraction procedure (calculation of mass required for the given concentration of ON).

3.6. Chromatographic method validation

IP RP UHPLC was used for the analysis, enabling the separation and quantitative determination of ONs. The method was validated to confirm its accuracy and repeatability. The validation parameters of the chromatographic method are presented in Table S1. The calibration curve was prepared based on the results obtained for the standard solutions in the 0.16 – 5.00 μM range. The linearity of the method was based on R^2 and was equal 0.999. For NU ME, the

LOQ value was 0.016 µM, and the LOD value was 0.005 µM. The intra- and inter-day variability was determined for two different concentrations, and RSD for the first one was lower than 2.74% for NU ME, while RSD for inter-day was lower than 4.27% (Table S1).

3.7. ASO extraction from enriched serum samples

The developed extraction procedure was applied to extract NU ME from enriched human serum samples. The serum was enriched with NU ME to 5 µM and diluted with water in a ratio of 1:3. In the first attempt, the developed dSPE procedure (using Met) was applied directly to diluted serum; however, proteins were adsorbed together with ON. Both proteins and NU ME were desorbed during the elution step, but recovery for ON did not exceed 20% (Figure S6). Based on this result, we proved that our procedure cannot be used to extract serum samples directly. It can be applied as an additional purification step after the prior removal of serum proteins. ONs have a very high affinity for proteins, and the most commonly used methods for separating these two groups of compounds are LLE or enzymatic degradation. Therefore, in further trials, we removed proteins from serum using LLE extraction with a phenol/chloroform/isoamyl alcohol mixture. This step is usually followed by a subsequent extraction of the LLE with chloroform to remove residual phenol. In our study, we used the developed dSPE method to extract NU ME from serum after the first LLE. We have also made an attempt to use dSPE as an additional purification method after prior LLE/LLE. Figure 4 presents chromatograms from both attempts. The goal of applying LLE/dSPE (Figure 4A) was to reduce the sample preparation steps to just two and purify the LLE extract of phenol and protein residues. The goal was easily achied since LLE allowed the removal of most proteins, and dSPE fully purified the extracts, with selective extraction of NU ME with recovery of 95±1% (Figure 4A). In the second approach, the LLE (using phenol/chloroform/isoamyl alcohol) was subjected to the second LLE using chloroform, and next, dSPE was used for NU ME extraction (Figure 4B). The use of an additional step allowed the extract to be purified from matrix impurities, as evidenced by the lower intensity of the signal (between 2 and 3 min.) visible in the chromatogram. The NU ME was completely adsorbed, in contrast to the substances originating from the matrix, which remained in the solution (Figure 4B). The recovery was high, 91±1%. The above results suggest that the developed method is selective and can be used to extract ASOs from serum samples, especially after prior application of LLE. The method's advantages are high recoveries, a relatively short time, and a minimized amount of adsorbent used for extraction.

4. Comparison with previously published dSPE extraction methods

So far, only a few publications have been published on the use of dSPE for the extraction of therapeutic ONs. The available literature reports on the use of poly(ionic liquids), as well as magnetic nanoparticles coated with crosslinked zwitterionic poly(ionic liquids) as adsorbents in dSPE and magnetic dSPE [32,33]. Moreover, silica-based adsorbents functionalised with amino and carboxylic groups have also been used [34]. These approaches have enabled the efficient isolation of ONs differing in modification type (PS, ME, MOE) and length, similarly to our method [32,33,34]. Nevertheless, our method is applicable also for ON modified in two different structural elements (phosphate group and ribose molecule), typical for therapeutical ONs. Generally, each of these materials was used in weak ion exchange mode with a critical role of pH change. However, application of poly(ionic liquids) required usage of organic solvents for elution, contrary to amino-acid based material as well as adsorbents with amino-carboxylic moieties [32,33,34]. This is a great advantage of our method, because it increases greenness of the extraction procedure. The advantage of magnetic dSPE (magnetic nanoparticles coated with poly(ionic liquids)) significantly shortens the extraction time, while our method unfortunately is more time-consuming [32,33]. On the other hand, it is characterized by simplicity of execution. Regarding the recovery, previously developed dSPE methods provided about ~80% for ONs isolated from serum samples [32,33,34]. The utilization of Met adsorbent for extraction of modified ONs from serum allowed obtaining greater recovery (higher than 90%) with a very good reproducibility.

5. Conclusion

The study examined the dSPE of ASOs using five different amino acid-based adsorbents. For all tested adsorbents, the adsorption process is primarily based on weak ion exchange, i.e., on the electrostatic attraction between positively charged aminopropyl groups and amino groups in amino acid and negatively charged ONs. Consequently, desorption is based on reducing electrostatic attraction by reducing the positive charge at the surface of the adsorbent. Thus, the adsorption/desorption process can be driven only by a pH change (from pH 4.0 to pH 11.0). The salt concentration in the desorption solution does not significantly affect the recovery, while the addition of organic solvents (≥75%) significantly reduces the recovery (below 10%). Regardless of the amino acid in the side chain, applying all adsorbents provided recoveries higher than 70%. Minor differences in recovery are most likely due to additional structural interactions (e.g., hydrogen bonding, π-π interactions) characteristic of a given side chain. The highest recoveries obtained for Met are probably caused by the presence

of sulfur (in the thioether group of the side chain of amino acids). It has a partial negative charge, and it is a larger and more polarizable atom than oxygen. The length of ASO affects the recovery as it is higher for shorter ASO. The increase in ASO hydrophobicity caused by their modifications leads to a higher recovery (e.g., NU 61-69%; NU PS 78-84%; NU ME 70-100%). The above assumption is also confirmed by the results for ASO with two modifications in the sequence, regardless of its length. In addition, the standard deviations indicate that the developed method is repeatable. The developed method was successfully used for ON extraction from enriched serum samples. The use of LLE allowed for the removal of most proteins, and dSPE effectively purified the extracts and selectively isolated NU ME (95±1%). The results indicate the possibility of repeatable extraction and determination of ASO and its metabolites from real samples using amino acid-based materials. It is the first attempt of this kind. The developed method is relatively fast and simple, and ensures the repeatability of results.

The data that support the findings of this study are openly available in open repository *Dispersive solid phase extraction of antisense oligonucleotides with the use of amino acid based adsorbents* at https://doi.org/10.18150/01O0IW.

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Table 1. Oligonucleotides used during the study (their sequences, modification type, molecular masses)

Name	Sequence (5´→ 3´)	Molecular mass [Da]	Type of modification	Used for
DNA	GCCCAAGCTGGCATCCGTCA	6063	Unmodified	Adsorption and desorption optimization
PS18	GCCCAAGCTGGCATCCGT	5734	Phosphorothioate	The influence of ON length
PS16	GCCCAAGCTGGCATCC	5068	Phosphorothioate	
PS	GCCCAAGCTGGCATCCGTCA	6368	Phosphorothioate	The influence of ON modification
ME	GCCCAAGCTGGCATCCGTCA	6622	2′-O-methyl	
MOE	GCCCAAGCTGGCATCCGTCA	7657	2′-O-(2-methoxyethyl)	
NU	GGUCGUAAUACUUUCACU	5655	Unmodified	
NU PS	GGUCGUAAUACUUUCACU	5929	Phosphorothioate	
NU ME	GGUCGUAAUACUUUCACU	5908	2′-O-methyl	The influence of modification and extraction from serum
NU PS-ME 13	UCGUAAUACUUUC	4399	Phosphorothioate and 2′-O-methyl	The influence of modification and length
NU PS-ME 14	UCGUAAUACUUUCA	4759	Phosphorothioate and 2′-O-methyl	The influence of modification and length
NU PS-ME 18	GGUCGUAAUACUUUCACU	6181	Phosphorothioate and 2′-O-methyl	The influence of modification and length

Table 2. The structures and shortcuts of all adsorbents used during the study, together with the carbon and nitrogen load.

Material	Shortcut	Structure	Carbon load [%]	Nitrogen load [%]	Coverage density [μmol/m^2]
Amino	Amino		3.01	0.92	3.13
Aminio-Alanine	Ala		4.04	1.39	1.73
Amino-Leucine	Leu		7.39	1.39	1.92
Amino-Serine	Ser		7.18	1.47	1.31
Amino-Thyrosine	Tyr		9.71	1.46	0.75
Amino-Methionine	Met		5.08	1.55	0.99

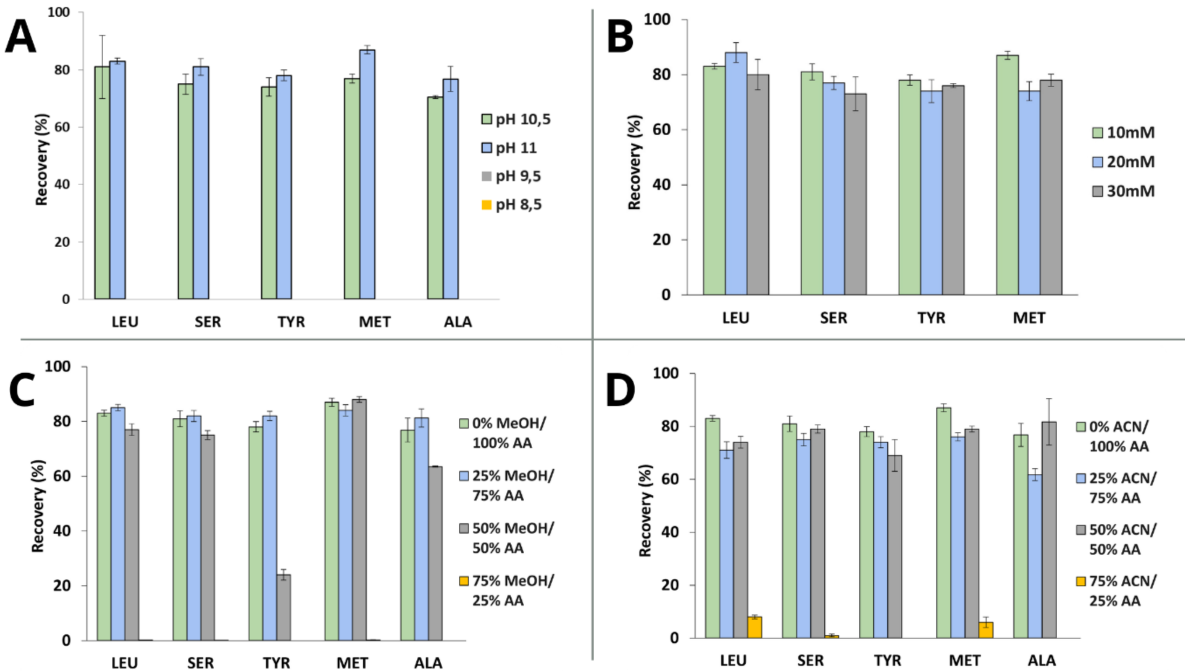

Figure 1. The impact of desorption solvent composition on the recovery: A) the influence of pH of 10 mM ammonium acetate (the recovery for pH 8.5 and 9.5 was equal to zero); B) the influence of ammonium acetate concentration at pH 11; C) the impact of methanol (MeOH) content in the mixture with 10 mM ammonium acetate of pH 11; D) the effect of acetonitrile (ACN) content in the mixture with 10 mM ammonium acetate of pH 11.

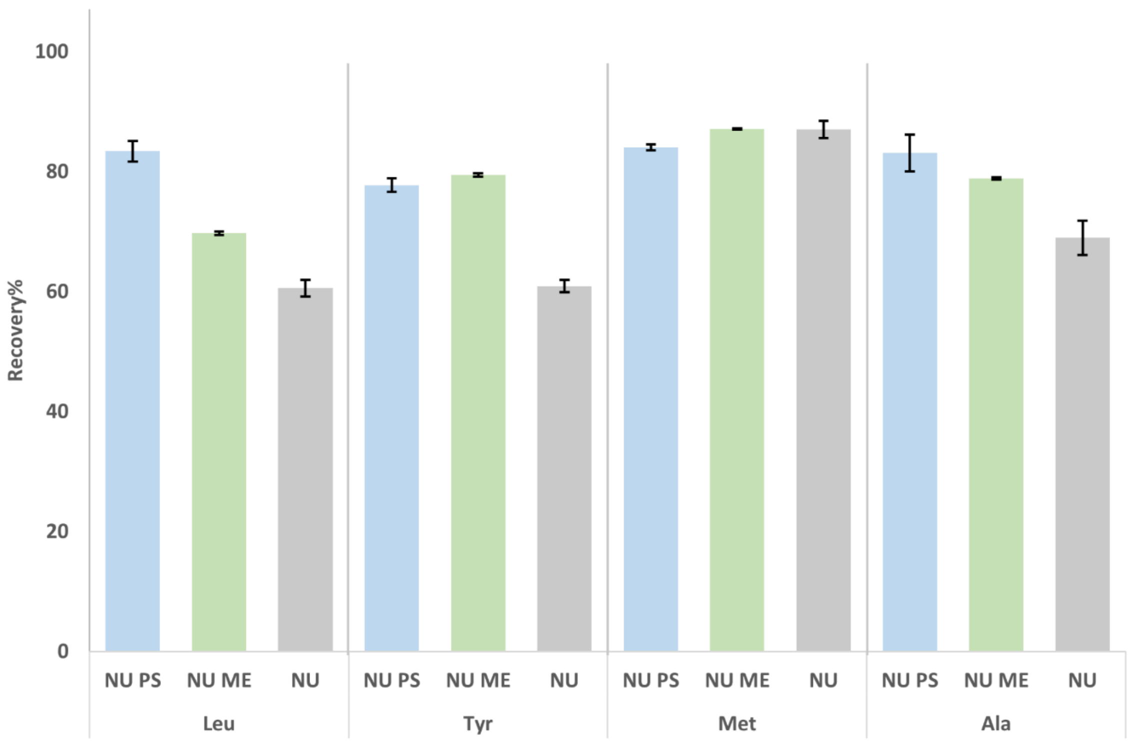

Figure 2. A comparison of the impact of ASO modifications on recovery depending on the adsorbent used.

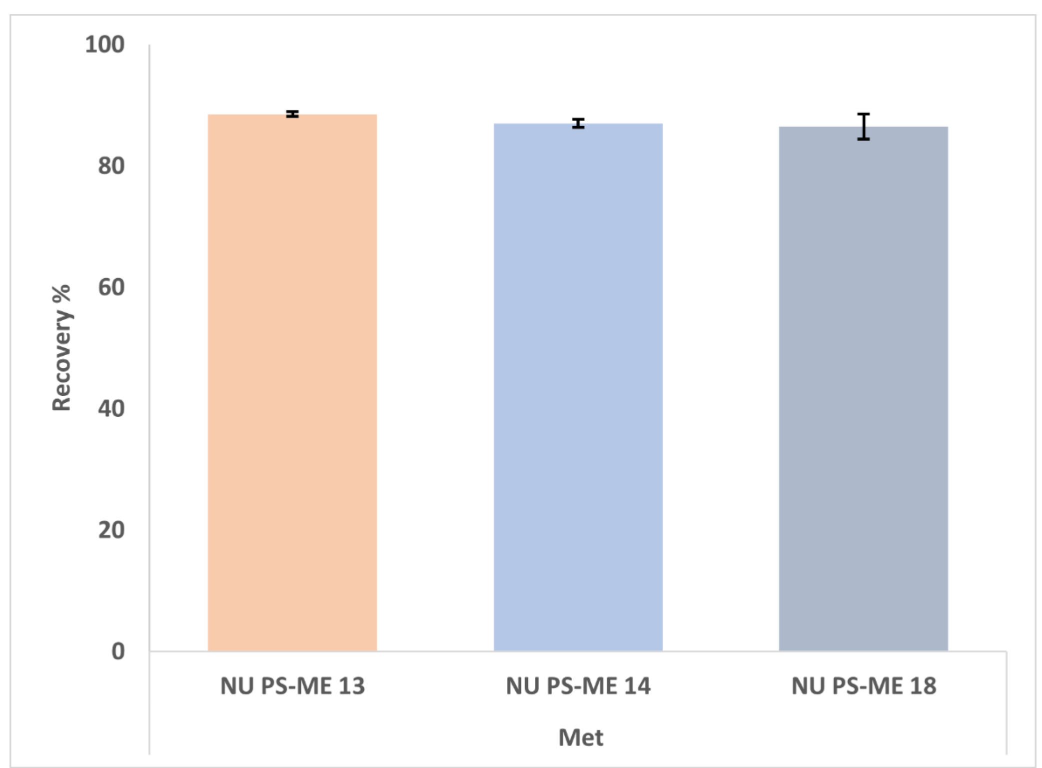

Figure 3. The effect of ASO modification and length on recovery for Met.

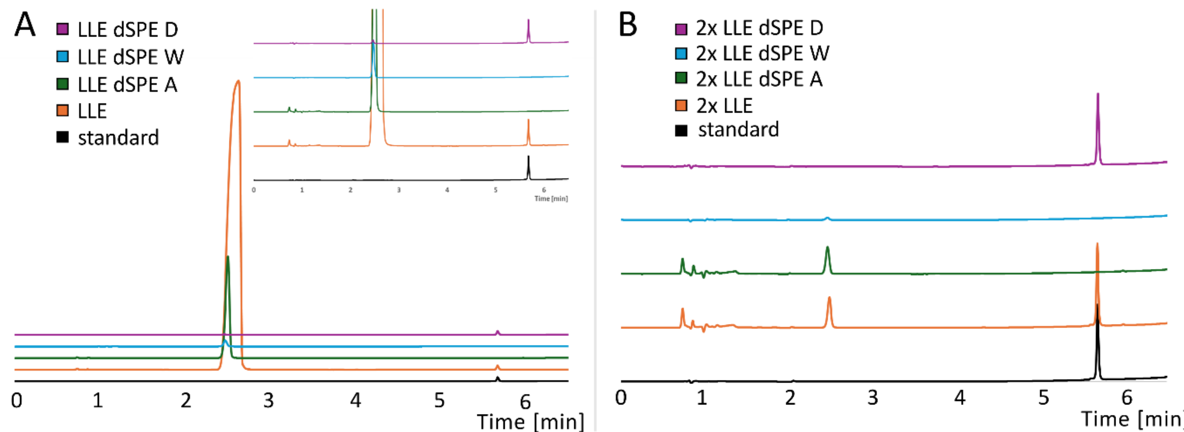

Figure 4. Exemplary chromatograms for serum extracts at various stages of extraction procedures: A) LLE extraction and dSPE purification; B) LLE/LLE extraction and dSPE. Chromatograms shown in the figure are for: NU ME standard, extract after LLE pre-purification and dSPE procedure, where A – supernatant after the adsorption process, W – supernatant after washing, D – supernatant after desorption.