Development of mixed-mode dispersive solid phase extraction of antisense oligonucleotides using commercial adsorbents

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Abstract

Sample preparation remains a challenge in the bioanalysis of antisense oligonucleotides due to their polyanionic character, structural complexity, and interactions with proteins. In the current study, a novel mixed-mode extraction methodology for oligonucleotides was developed utilizing commercially available sorbents and subsequently applied to serum matrices. Notably, this work constitutes the first evidence that broadly available adsorbents, not specifically designed for oligonucleotide isolation, can nevertheless be effectively employed for their extraction. Five sorbents with various functional groups were systematically evaluated to elucidate their interaction with antisense oligonucleotides. As a result, dispersive solid-phase extraction procedures were developed, with adsorption and desorption controlled by electrostatic, hydrogen-bonding, π-π, and hydrophobic interactions. Elution conditions were optimized using a central composite design, demonstrating that efficient desorption requires simultaneous modulation of salt pH and concentration, and organic solvent content. The results showed that extraction proceeds via a mixed-mode mechanism. Two silica-based sorbents modified with phenylboronic acid and 2-(2-pyridyl)ethyl groups exhibited the most favorable balance of recovery, reproducibility, and sorption capacity, achieving recoveries up to 93%. Kinetic studies revealed rapid adsorption for both sorbents, while differences in desorption influenced method transferability from dispersive solid phase extraction to solid-phase extraction. Finally, the extraction procedure for phenylboronic acid-based material enabled one-step isolation of antisense oligonucleotides from serum without prior protein removal. Although substantial (1:5 v/v) serum dilution was required and recoveries were moderate (59-71%), the approach represents an alternative to currently used methods. Moreover, it expands the bioanalytical tools for mixed-mode oligonucleotide extraction using widely available commercial sorbents.

Key words: antisense oligonucleotides; solid phase extraction; commercial adsorbents; central composite design; recovery

1. Introduction

The extraction of antisense oligonucleotides (ASO) from biological matrices is one of the most critical steps in bioanalytical analysis [1–3]. ASOs are characterised by high polarity, multiple negative charges resulting from the presence of phosphate groups (pKa ~ 1-2), a significant molecular weight (typically 6-10 kDa for 18-25-mer), and numerous chemical modifications [3–6]. They increase stability towards nucleases but also cause strong binding to plasma proteins, complicating sample preparation [3,7]. Methods used so far for ASO extraction from biological samples (serum, urine, etc.) are very diverse, ranging from liquid-liquid extraction (LLE) and enzymatic protein digestion to different types of solid-phase extraction (SPE) [1,3,8–13]. The last one became the most widely used method for isolation of ASO from biological samples, e.g., serum, cerebrospinal fluid [11,14,15].

To date, SPE of ASO has been performed using various modes, including ion pairs, ion exchange, hydrophilic interactions, and hybridization [8,11,14–17]. Each of these approaches has its advantages and disadvantages; however, it should be emphasized that they undoubtedly allow obtaining high recoveries [3]. Furthermore, the use of ion pairs or hydrophilic interactions enables the concentration and direct analysis of extracts using mass spectrometry, but the procedures are time-consuming, and reproducibility is not always high [8,14,18–20]. Anion-exchange extraction, on the other hand, yields extracts with a very high salt content (most often inorganic salts) [13]. Each of these approaches requires an additional extraction step to thoroughly remove proteins from biological samples. Hybridization provides the highest selectivity without the need for sample pretreatment [18,21]. Unfortunately, it requires a greater financial investment, and the recovery of ASO metabolites is problematic [18].

The mixed-mode dispersive and solid-phase extraction methods were recently introduced to ASO analytics and have become an interesting alternative [22]. Both adsorption and desorption in this case are based on different types of interactions, depending on the functional groups used to modify the adsorbent surface, e.g., ionic liquids, dicarboxylic acids, and amino acids [23–26]. Typically, hydrophobic and electrostatic interactions were most influential for ASOs. The application of mixed-mode solid-phase extraction provides high recoveries (80-95%) and reproducibility [12,22,26]. Moreover, low concentrations of organic salts and low content of organic solvents are used during the extraction process, making this method

advantageous and directly applicable to mass spectrometry detection [24–26]. The sorbents used so far for this mode are mainly home-made, specially synthesized materials [24,25]. Despite the success of their application, the limited commercial availability and potential batch-to-batch variability constitute significant limitations. At the same time, the growing number of approved ASO drugs, including nusinersen, inotersen, eteplirsen, and golodirsen, results in an increased demand for standardized, accessible, and reproducible bioanalytical methods [4,5]. Therefore, we believe that one potential direction in current oligonucleotide (ON) bioanalytics could be to evaluate the suitability of currently available commercial materials (given their availability and fully characterized properties) for ASO extraction in mixed-mode [12]. Among the sorbents that have been used with great success to date, Oasis HLB [7,11,14,15] and Clarity OTX [11,12,22] are undoubtedly worth mentioning.

In this study, the potential of five commercially available adsorbents containing various functional groups (e.g., amide, sulfoxide, alkyl chains, aryl rings, carboxyl groups) for the extraction of ONs has been investigated. These materials have not yet been systematically evaluated for ASO extraction, and, therefore, the study is novel. The use of commercial materials offers advantages over homemade materials, such as rapid and widespread availability, high reproducibility across batches, and elimination of variability and costs associated with laboratory synthesis. This study aims to evaluate for the first time the suitability of these adsorbents for mixed-mode dSPE extraction of ASO. The application of widely available sorbents may facilitate the broader adoption of ASO analytical methodologies in both research and clinical laboratories, especially where access to specialized materials is limited.

2. Materials, reagents, and methods

2.1. Materials and reagents used during the study

Methanol (MeOH, Chromasolv®, gradient grade, ≥ 99.9%) was supplied from Honeywell (Charlotte, NC, USA). Ammonium acetate (AA, ≥ 99%), acetic acid (≥ 99%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, ≥ 99.9%), and hexylamine (HA, 99%) were purchased from Merck (Warsaw, Poland). The 25% ammonium hydroxide solution (NH_4OH) and formic acid (FA, ≥ 99%) were purchased from Avantor (Gliwice, Poland). Deionised water was obtained from a Milli-Q system (Millipore, Burlington, MA, USA).

The following adsorbents were selected for this study: Discovery® DSC-WCX, 2-(2-Pyridyl)ethyl-functionalized silica gel, PK30 Discovery® DPA-6S SPE Tube, PK30 Supelclean® Sulfoxide (Merck, Warsaw, Poland), Bond Elut PBA (Altium, Warsaw, Poland). Their basic parameters were presented in Table 1, while their structure is shown in Figure S1.

All tested ONs (Table S1) were supplied by Sigma Aldrich (Dorset, UK) in lyophilized form. ON solutions at 100 µM were prepared with the appropriate volume of deionized water. Subsequent dilutions were performed with a conditioning solution, as described below. 18-mer NuDNA is an unmodified ON, while the remaining four compounds are 18-, 16-, 14-, 12-mer ONs (with similar, 3'-shortened sequence) and 2′-O-methyl and phosphorothioate modifications (NuMePs) (Table S1).

2.3. Instrumentation and chromatographic conditions

A Dionex UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) system (Dionex Corporation, CA, USA) was used to perform quantitative and qualitative analysis of ASO during the dSPE method development. Moreover, the Thermo Scientific™ Vanquish™ Horizon ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific Inc., Germany) was used for quantitative analysis and the application of the dSPE procedure to serum samples. The chromatographs were equipped with a diode array detector, and 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 Chromeleon 7.2.10 software. A Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Milford, MA, USA) was used in this study. The experiment was conducted using ion-pair ultra-high-performance liquid chromatography (IP RP UHPLC). Two different chromatographic methods were applied. The first one was used during the development of dSPE procedure with the following conditions: flow rate 0.35 mL/min; column temperature 65°C; mobile phase composition: 5mM HA, 150 mM HFIP, MeOH; gradient program: 10-90 % (v/v) of MeOH in 8 min. The second chromatographic method was used during the application of a new, mixed-mode dSPE method to serum samples. The following conditions were used: flow rate 0.30 mL/min; column temperature 50°C; mobile phase composition: 5 mM HA, 150 mM HFIP, MeOH; gradient program: 15–55% (v/v) MeOH in 10 min.

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

2.3. Development of dSPE procedure for ONs

During experiments, 2 mg of adsorbent was used (each extraction in duplicate, due to the screening nature of the experiment). NuDNA was used to optimize adsorption and

desorption conditions. For sorbent conditioning, 100 µL of MeOH, 100 µL of H_2O, and 100 µL of the solution used during sample load were used.

The following solvents were tested during optimization of adsorption (sample load) conditions: H_2O, H_2O acidified to pH 4 using FA, H_2O alkalized to pH 10 using NH_4OH, 10mM AA (pH 4 and 10). The 100 µL of 5 µM NuDNA was loaded on the adsorbent surface. The washing step was performed using 100 µL of a 10/90 % v/v MeOH/adsorption solution. The composition of the elution solution was optimized in a complex manner, first by screening studies and then by using a central composite design (CCD). The influence of three independent variables on ASO recovery was examined, namely: pH of AA solution (7-11), organic solvent content in the solution (0-100% v/v MeOH), and AA concentration (0-125 mM for DPA and 10-210 mM for the remaining adsorbents) (Table S2 and S3). NuDNA recovery was the dependent variable. The CCD parameters were determined using the Numiqo e. U. online tool. calculator. The resulting experimental designs included 15 experiments differing in eluent composition, presented in Tables S2 and S3. Next, two-dimensional response surface plots were generated using Statistica software (StatSoft, Inc., Tulsa, OK, USA).

After each dSPE step, the sample was shaken using a vortex for 5 minutes and then centrifuged for 10 minutes (14,000 rpm).

2.4. Final extraction procedure

The finally optimized mixed-mode dSPE procedure for the PBA adsorbent (2 mg) was as follows: 1. conditioning: 100 µl of 10 mM AA (pH 4); 2. sample load: 5 µM ON diluted with 10 mM AA (pH 4) to a final volume of 100 µl; 3. washing: 100 µl 90/10 % v/v/ 10 mM AA (pH 4)/MeOH; 4. elution: 100 µl 50/50 % v/v 110 mM AA (pH 11)/MeOH. After each step, the sample was shaken with a vortex for 5 minutes and then centrifuged for 10 minutes (14,000 rpm).

2.5. Adsorption and desorption kinetics and sorption capacity

The effect of time on adsorption and desorption was investigated. The study was conducted for PBA and PE adsorbents, as well as NuDNA. Five adsorption and desorption times (1, 2, 5, 10, and 15 min) were tested. Individual adsorbents were conditioned according to a developed procedure (section 2.4).

The sorption capacity was determined using 1 mg of the adsorbent and three different volumes (20, 40, 70 µl) of 100 µM NuDNA. Samples were shaken using a vortex for 15 minutes and then centrifuged for 10 minutes (14,000 rpm). The supernatant was analyzed by UHPLC to

determine the amount of unadsorbed NuDNA in solution. The sorption capacity was calculated by the following equation:

$$Q_e = \frac{(C_0 - C_e) * V}{\mathrm{m}}$$

where Q_e is sorption capacity [μg/mg], C_0 is the initial concentration [μg mL^{-1}], C_e is the equilibrium concentration [μg mL^{-1}], V is the initial volume of sample solution [mL], and m is the mass of the adsorbent [mg].

2.6. Transferring dSPE methods to SPE

An experiment was conducted to evaluate the applicability of PBA and PE adsorbents for the SPE extraction of 5 μM NuDNA. The PBA adsorbent was purchased in SPE cartridges (Table 1). PE was individually packed into an empty 1 ml cartridge, with 30 mg of adsorbent per cartridge. SPE procedures for the individual adsorbents were developed based on the conditions described for dSPE and presented in Figure S2. The gravimetric flow was used during sample loading and elution.

2.7. Validation of the chromatographic method

IP RP UHPLC was used for the quantitative determination of a mixture of four ASO (NuMePs12, NuMePs14, NuMePs16, NuMePs18) (Table S1). Calibration curves were constructed based on the results obtained for standard solutions of the four-component mixture in concentrations from 0.15 (0.31 μM for NuMePs12) to 10 μM (0.15, 0.31, 0.625, 1.25, 2.5, 5.0, 7.5, 10.0 μM). The linearity of the method was assessed by determining the determination coefficient (R^2). Inter-day repeatability was determined by performing 7 injections of a mixture of ASOs at 3 different concentrations (0.625, 5.0, and 10.0 μM) on the first, third, and seventh days. Intra-day precision was evaluated using 7 injections of an ASO mixture at 3 concentrations (0.625, 5.0, 10.0 μM) within 1 day. Precision was expressed 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 was at least 3:1, while LOQ was defined as the concentration at which the signal-to-noise ratio was at least 9:1.

The matrix effect (ME) was evaluated using the post-extraction spiking approach. A blank serum sample was processed through the complete dSPE procedure, but without adding ASO standards. After extraction, the ASO mixture was added to the blank extract at concentrations identical to those used for the standards. ME was then assessed by comparing

the peak areas of the standard solutions with those of the spiked blank extracts. The following formula was applied:

$$ME = \frac{P_{Ape}}{P_{As}} * 100\%$$

where: ME – matrix effect; P_{Ape} – peak area of ASO in post-extraction spiked matrix; P_{As} – peak area of ASO standard.

2.8. Application of mixed-mode dSPE procedure to the extraction of ASOs mixture from serum

Serum samples were collected in accordance with protocols approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (approval no. KB/236/2025). Serum was first fortified with a mixture of NuMePs12, NuMePs14, NuMePs16, and NuMePs18 to 5 µM each. Four attempts were tested: undiluted serum and serum diluted using 10 mM AA (pH 4) at the following ratios: 1:1, 1:3, 1:5. The samples were vigorously mixed, and ASOs were extracted using the developed dSPE procedure (described in 2.4 section).

3. Results and discussion

3.1. Selection and characteristics of commercial adsorbents

Previous achievements in ASO extraction indicate that the selection of sorbent surface functionalization is crucial for the strength and nature of interactions [24,26]. A practical aspect of developing extraction methods using commercial materials is their availability (without the need to synthesize dedicated adsorbents) and the repeatability of the procedures. Therefore, five commercially available adsorbents with different chemical structures were chosen (Figure S1). Despite their use in the extraction of other classes of compounds [27–35]. These materials have not been used in ASO extraction to date. The selection was based on their potential to interact with ASO via various interactions (hydrogen bonds, electrostatics, hydrophobicity), enabling the possible application of mixed-mode extraction. The present study is the first of its kind.

DSC contains carboxyl groups with pKa ~ 3, and nitrogen with pKa of 6.5. These groups can undergo reversible ionization depending on the solvent pH [27,28]. In addition to electrostatic interactions, hydrogen bonds can occur between the functional groups of the adsorbent and nitrogen bases and riboses in the ASO structure. PE with 2-(2-pyridyl)ethyl groups can accept a proton and form an anion exchanger (Figure S1) [29,36]. This material is designed to efficiently extract acyl-coenzyme A and its esters, but in our opinion, it could be

useful for ASO extraction [29,36]. Since the pKa of nitrogen in the 2-pyridyl ring is equal to 6, the electrostatic interactions depend on the pH. Moreover, an aromatic pyridine ring can interact by π… π interactions. DPA is a polyamide resin containing amide groups capable of forming hydrogen bonds both as a proton donor and acceptor (Figure S1) [30–32]. It became useful for the isolation of oligosaccharides or polyphenols [31,32], while the polar nature may confer benefits during ASO extraction. SULF possesses the greatest number of different groups, such as polarized sulfoxide, secondary amine, amide group, and short alkyl chains (Figure S1) [37]. Sulfoxide groups make this material particularly useful for the extraction of polychlorinated biphenyls from environmental matrices, due to interactions between the electrophilic sulfur atom and the p-electron cloud of the analytes[37,38]. SULF adsorbents can interact by dipole-dipole interactions (sulfoxide), hydrogen bonds (amide), electrostatic (amine), hydrophobic (alkyl), similarly to ASO. PBA is a silica-based sorbent functionalised with phenylboronic acid groups widely used for the extraction of oligosaccharides, glycans, nucleosides, polyphenols, etc. [33–35] (Figure S1). The extraction mechanism may involve the reversible formation of boronate complexes with diol groups present in the analytes' structures (depending on solvent pH) [33–35]. Additionally, hydrogen bonding and π-π interactions (phenyl ring) may occur during extraction with PBA. In our opinion, they have potential for application in ASO isolation.

The diversity of possible interactions indicates the potential of selected sorbents to operate in a mixed-mode, encompassing electrostatic, π-π, hydrogen bonding, and hydrophobic interactions. This is a promising selection of adsorbents that may, for the first time, identify new commercially available materials for use in mixed-mode extraction of ASO.

3.2. Development of dSPE procedure

The development of the dSPE procedure aimed to determine the conditions enabling complete adsorption and efficient, repeatable desorption of ASO using commercial adsorbents. The diverse surface functionalisation enables multiple types of interactions between the adsorbent surface and the ASO, necessitating careful, individual selection of solvent composition. During the optimization of the procedure, 2 mg of sorbent was used with a 5 µM solution of NuDNA.

3.2.1. Sorbent conditioning

The conditioning step involved using MeOH, H_2O, and the solvent used during sample load (100 µL each).

3.2.2. ON sample load

The following solvents were tested to select the optimal adsorption conditions: H_2O, aqueous solution of FA (pH 4); aqueous solution of NH_4OH (pH 11); 10 mM AA (pH 4 and pH 11). Results were presented in Table 2. Complete adsorption of NuDNA from water was noticed only for PBA; however, a high amount of this ON was also retained at the surface of DPA. This material is based on polyamide units with amide groups interacting by hydrogen bonds with polar compounds, e.g., saccharides (Figure S1) [31]. In the case of ONs, adsorption is likely to involve interactions with the hydroxyl groups of ribose and nucleobases. PBA material retains NuDNA via interactions between hydroxyl groups in a phenylboronic molecule (Figure S1) and riboses and bases. Moreover, hydrophobic interactions with alkyl spacers in DPA structure may interact with NuDNA by hydrophobic interactions, while the phenyl ring in PBA provides π-interactions for nucleobases in ON (Figure S1).

Complete adsorption was observed for four of the five adsorbents with an aqueous solution of FA and 10 mM AA, both at pH 4 (Table 2). For DPA and PBA, the interactions at low pH are analogous to those in water. For SULF and PE, NuDNA adsorption occurs only at low pH (Table 2). The structures of both adsorbents contain nitrogen atoms that can accept a proton (in the pyridine ring of PE and in a secondary amine group of SULF), which makes them weak ion exchangers at low pH (Figure S1). We assume that the pKa of secondary amine in SULF is lower than 10, due to the effect of neighbouring amide and sulfoxide groups adjacent [39,40]. Still, it will be protonated at pH 4, and consequently, electrostatic attraction will be the basic force of adsorption (Table 2). The pKa of the nitrogen in the pyridine ring equals 6, and NuDNA sorption for PE is also based on electrostatic interactions [41]. Furthermore, π-π interactions will take part in the NuDNA adsorption at PE, while hydrogen bonding and dipolar interactions occur for NuDNA and SULF (sulfoxide and amide groups) (Figure S1). It is important to note that both an aqueous FA solution and a low-pH salt solution can be used to achieve complete NuDNA adsorption.

Low (below 30% for DPA) or non-adsorption (for PBA, PE, SULF, DSC) of NuDNA was noticed when aqueous NH_4OH solution or 10mM AA at pH 10 was applied (Table 2). Under these conditions, deprotonation of the PE and SULF occurs, which disrupts electrostatic attraction. The PBA surface becomes negatively charged due to the transformation of phenylborate groups into the boronate ones [41]. It causes electrostatic repulsion between phosphate groups in NuDNA and boronates.

The only exception in our study is the DSC sorbent, as adsorption did not exceed 15% regardless of the solvent used (Table 2). This effect probably results from the ion-exchange mechanism and the negatively charged carboxyl groups. For this reason DSC sorbent was excluded from further study.

3.2.3. Sorbent washing

The 100 µL of 90/10 % v/v 10 mM AA (pH 4)/MeOH was used during the washing step. No NuDNA desorption was observed.

3.2.4. ON elution

The composition of the eluent used during desorption was optimised using CCD. During screening tests, the two salts, namely AA and sodium perchlorate, were tested. Results showed that both enabled NuDNA desorption; consequently, we decided to use an organic salt. The impact of salt concentration and pH on NuDNA recovery was also preliminarily tested. Both parameters appeared to be influential, and recovery increased with increases in salt pH and concentration. The basic pH was tested, due to the results obtained during adsorption studies (adsorption at low pH of solvent). Regarding the choice of concentration range, the higher concentrations for DPA were related to the results we obtained during preliminary studies. It was found that an application of 125 mM DPA did not yield high recoveries. Moreover, the addition of an organic solvent to the elution solution also increased recovery for all of the studied adsorbents. Finally, pH, salt concentration, and MeOH content were selected as independent variables for the CCD design of experiments, with recovery as the dependent variable. Table S2 (for DPA) and Table S3 (for SULF, PE, PBA) present CCD results, namely sets of 15 experiments differing with independent values. Each extraction was performed twice. The basic statistics for CCD models were described in section S1 and Table S5 in the Supplementary Materials. Based on the experimental data, two-dimensional response surface plots were generated to illustrate the dependence of recovery on two independent variables (Figure S3). The experiments were carried out in a wide pH range, up to pH 11. This may raise concerns about the stability of ONs. ASOs are fully modified with a 2′-O-methyl group and a phosphorothioate backbone, which substantially enhances resistance to chemical degradation. While extreme alkaline conditions can induce backbone cleavage, such effects are generally associated with prolonged exposure rather than the short contact times used during elution. Furthermore, it should be underlined that no degradation was observed experimentally. In

particular, chromatograms did not show the presence of shorter fragments or additional peaks indicative of degradation.

For the DPA (Figure S3A), high NuDNA recovery can be achieved with an AA concentration greater than 110 mM and a high pH, while the MeOH content should be kept in the range of 60-70% v/v. On the other hand, high recovery is also possible at a pH of 6.5 and 140 mM AA (Figure S3A). MeOH disrupts hydrogen bonding and hydrophobic interactions, which are probably essential for desorbing NuDNA from DPA. The amide group may undergo protonation (carbonyl oxygen), but only under very low pH conditions (~ 2) [42]. Therefore, we suppose that electrostatic interactions between NuDNA and the DPA surface did not occur. Nevertheless, high salt concentrations combined with high pH are necessary for elution. Both parameters probably reduce nucleobase-amide hydrogen bonding. Bases act as hydrogen bond acceptors, and their interactions with amide N-H bonds are weaker, facilitating desorption from the DPA surface.

For the PBA adsorbent, high NuDNA recoveries require the use of 100-150 mM AA (pH 10.5-11) and 30-60% v/v MeOH (Figure S3B). The phenylborate groups transform into the boronate under basic conditions (pH greater than 10), resulting in a negative charge at PBA surface [43]. Under these conditions, presumably electrostatic repulsion between NuDNA and PBA increases recovery (Figure S3B). Furthermore, high AA concentration also reduces NuDNA interactions with the sorbent surface, because more counterions are present in the solution (ammonia cations). Conversely, the impact of MeOH on recovery is likely due to hydrophobic and π-π interactions between the ON and the phenyl ring in the PBA structure (Figure S1). Increasing the organic solvent content destabilises these interactions, affecting the polarity and dielectric properties of the eluent.

High recoveries of ON from SULF surface were noticed for the mixture of 60-80% v/v MeOH and 20-40% v/v AA (Figure S3C). However, high salt concentration (>100 mM) and pH (>10) should be used (Figure S3C). As mentioned in 3.2.2 section, NuDNA adsorption may be partially driven by electrostatic attraction between the positively charged secondary amine (Figure S1) and phosphate groups in the ON [44]. High pH of the solvent used for elution presumably deprotonates the secondary amine group and consequently reduces electrostatic interactions [39,40,44]. Sulfoxide and amide groups in SULF (Figure S1) probably rely on hydrogen bonding and dipolar interactions with NuDNA. The high salt concentration disrupts hydrogen bonding. Since an electrophilic sulfur atom may bind nucleobases by π-electron cloud, MeOH can be used for desorption.

The efficient desorption of NuDNA from PE surface requires high pH (above 9) and concentration (above 180 mM) of AA, as well as 40-60% v/v of MeOH (Figure S3D). Since the pKa of the nitrogen in the pyridine ring equals ~6, its deprotonation occurs at high pH [41]. Therefore, probably electrostatic attraction is disrupted, facilitating NuDNA elution at a high pH of solvent. Moreover, at high pH, when pyridine becomes neutral, the salt presumably reduces nonionic polar interactions, e.g., hydrogen bonding occurring between nitrogen and NuDNA. MeOH reduces hydrogen bonds and π-π interactions, so it must be added to the elution solvent.

The proposed interaction mechanisms are inferred from systematic trends in pH, ionic strength, and organic solvent composition and should be regarded as plausible dominant contributions rather than direct experimental proof of individual interaction types.

Based on the results of optimizing desorption conditions for ON from the surfaces of selected commercial adsorbents, it can be concluded that mixed-mode extraction is employed. This is evidenced by the variety of interactions involved in adsorption, especially in desorption.

Optimal eluent compositions were selected for each tested adsorbent based on Figure S3. The selected conditions should provide the greatest possible recoveries. The following eluent compositions were ultimately selected:

DPA: 30/70 % v/v 140 mM AA (pH 11)/MeOH;

PBA: 50/50% v/v 110 mM AA (pH 11)/MeOH;

SULF: 20/80% v/v 200 mM AA (pH 11)/MeOH;

PE: 40/60% v/v 200 mM AA (pH 11)/MeOH.

The selected conditions were then experimentally verified, and recoveries are presented in Figure 1. These values were higher than the maximum recoveries obtained during the CCD experiments. The lowest recovery was noticed for DPA, while the greatest was for PBA and PE (95% and 86%, respectively) (Figure 1). Consequently, these materials were selected for further studies.

The extraction method was optimized using an unmodified oligonucleotide due to cost considerations. However, the method should apply to ASOs, so in the next step, a mixture of four ASOs of varying lengths (12-, 14-, 16-, and 18-mers) was extracted. Their sequences are modified in two structural elements: the phosphorothioate group and the ribose (2′-O-methyl) (Table S1). This allows us to assess the suitability of the developed mixed-mode procedures for extracting modified therapeutic ONs as they are usually modified in several structural elements. The results are shown in Figure 2. For PBA, recovery rates ranged from 82% to 93%, while for PE they were lower, ranging from 75% to 86%. Furthermore, greater reproducibility was

achieved for PBA (although it is not low for PE). We observe one characteristic trend for both materials: recovery increases with increasing ASO length (Figure 2). This is likely due to the increased hydrophobicity of ONs with longer sequences, resulting from a greater number of hydrophobic modifying groups (sulfur atoms and methyl groups). Greater hydrophobicity likely enables more efficient desorption of ASOs from the surfaces of polar sorbents compared to shorter ASOs.

The results presented above demonstrate that commercially available sorbents can be successfully used in the mixed-mode extraction of therapeutic ONs. This applies in particular to PBA and PE. This has been demonstrated for the first time and is very important because it expands the possibilities for ASO extraction using mixed-mode adsorbents that are widely available. The currently available commercial material Clarity OTX is specifically designed for ON extraction, which is both an advantage and a disadvantage. The limitation is the cost; the advantage is its efficiency. Our research has shown that other materials, not originally intended for ON extraction, can also be successfully used for this purpose.

3.3. Study of adsorption, desorption kinetics, and sorption capacity

The adsorption of NuDNA over time was studied for PE and PBA. After 2 minutes, 100% of NuDNA had been adsorbed onto the surfaces of both adsorbents (Figure S4). This rapid adsorption may be due to electrostatic attraction for PE and hydrogen bonding for PBA. Desorption of NuDNA from PE was completed within 15 minutes, while it took just 2 minutes for PBA. The differences in the time required to reach desorption equilibrium for the tested materials most likely result from the different nature of the interactions during elution (described in the 3.2.4 section). Rapid adsorption and desorption are advantageous because they shorten the extraction procedure. The sorption capacity of both materials was determined. It was 23.11 μg/mg for PBA and 29.32 μg/mg for PE. This parameter indicates that a large amount of ON can be adsorbed onto the surface, which is advantageous when applying the developed procedure to real samples.

3.4. Transferring methods from dSPE to SPE

The developed dSPE procedures were transferred to SPE to expand the applicability of the developed extraction procedures. During the procedure, we decided to use gravity flow for the sample load and elution steps to increase contact time. We collected the eluates from the cartridge during loading and washing. The ON was not washed off the surface during any of these steps. The finally adopted SPE procedures are presented in Figure S2. The recovery of

NuDNA in SPE was 98±4% with PBA, demonstrating that this sorbent can be easily used in both SPE and dSPE without compromising repeatability or recovery. This suggests that the dominant interactions are quickly destabilised and do not require a long contact time with the eluent. Moreover, we believe that high recoveries are due to the rapid desorption kinetics of NuDNA from the adsorbent (1 minute). More than 40% of the difference in recovery between dSPE and SPE was noticed for PE (40±3.8% for SPE). This effect probably results from the short contact time between NuDNA and the sorbent surface (in dSPE, the time is longer, due to, e.g. 5 min of mixing and 10 min of centrifugation). Our desorption kinetics results showed that it takes 15 minutes for PE; consequently, recovery in SPE is lower. Moreover, the static hold-up volume test was performed, stopping eluent flow from the PE surface for 10 and 20 minutes. However, this did not significantly change the recoveries, which were 43.2±4.1% and 45.5±3.3%, respectively.

At this stage of the research, we selected PBA for further studies because it offers broader applicability (dSPE and SPE). Nevertheless, it should be emphasized that PE can also be successfully used for ASO extraction via mixed-mode dSPE, as described in our publication.

3.5. Quantification method

IP RP UHPLC was used to determine ASO. This technique ensured the efficient separation and quantitative analysis of the tested analytes. Validation was performed using a mixture of 12-18-mer NuMePs. The procedure was validated to assess its accuracy, precision, and repeatability. Calibration curves for the individual analytes were prepared using standard solutions. The calibration curve ranged from 0.15 to 10 μM (except for NuMePs12, which started at 0.31 μM). The linearity of the method was assessed using R^2 values ranging from 0.9993 to 0.9999 (Table S4). The limits of quantification (LOQ) and detection (LOD) were 0.15 μM and 0.05 μM. Method precision was assessed based on intra- and inter-day variability. For all analytes, the RSD did not exceed 2.35% (intraday) and 2.53% (inter-day), respectively (Table S4). The developed chromatographic method was quick, precise, repeatable, and suitable for effective separation of ASO mixtures.

3.6. Extraction of ASOs mixture using developed mixed-mode dSPE procedure

The developed dSPE extraction procedure was applied to human serum samples. The serum was fortified with a mixture of ASOs of various lengths modified at two structural sites (Table S1). We tested the applicability of the procedure to undiluted serum and serum diluted with 10 mM AA (pH 4) in ratios of 1:1, 1:3, and 1:5 v/v. In the first case, complete adsorption of the

tested compounds was not achieved; it did not exceed 10%. This was likely a consequence of protein adsorption, which was high in this case (although we did not exceed the sorption capacity of PBA). A similar effect was observed for plasma diluted at a 1:1 ratio. At a 1:3 v/v dilution, adsorption of the tested ASOs was 90%, and recovery ranged from 41% to 50%. The best results were obtained when serum was diluted with a 10 mM AA solution at low pH in a 1:5 ratio. Adsorption of the four ASOs was complete, and the recoveries were as follows: NuMePs18 - 71±3, NuMePs16 - 65±3, NuMePs14 - 59±1, NuMePs12 - 65±2. These were not high, but the reproducibility was satisfactory, and the procedure could be applied directly to serum without the need for prior protein removal (using proteinase K or LLE), making it a one-step procedure. Figure 3 shows the chromatograms obtained for: a mixture of ASO standards, a solution after sample load, after sorbent washing, and after ASO elution. During the sample load and washing steps, peaks originating from matrix compounds are visible (Figure 3). There is a small amount of these in the eluate, which indicates that the method we developed can be used for the extraction and purification of ASO in biological matrices. However, this is possible with significant serum dilution, which may constitute a limitation of the method. On the other hand, the ME was low, between 94±2% for NuMePs12 and 96±3% for NuMePs18. The recoveries for post-spiking serum were following: NuMePs18 - 68±2, NuMePs16 - 62±2, NuMePs14 - 55±1, NuMePs12 - 63±1.

It may be concluded that the developed dSPE method using PBA may be applied to the extraction of ASO from serum, but recoveries will be low (lower than 70%) compared to other solid phase extraction methods used to date.

3.7. Comparison of our method to SPE methods used so far for ASO extraction

A comprehensive comparison of the most commonly used commercial adsorbents and solid-phase extraction methods, along with a newly developed procedure, is summarised in Table 3. It takes into account recovery, matrices, solvents, advantages, and limitations. High recoveries and repeatability are typically observed with Oasis HLB material in ion-pair mode. This is one of the most commonly used SPE methods for ASO extraction today. On the other hand, the method requires protein removal using LLE or Proteinase K. Another disadvantage is the need for amine utilization, which is not a popular trend in ASO analytics nowadays. The same concern about the application of weak ion-exchange extraction using Waters WAX material (amine utilization in the procedure). Moreover, the procedure requires preliminary protein removal, which is simple, and recoveries may be as high as 100% even from biological matrices. The gold standard became the application of Clarity OTX for mixed-mode extraction

of ONs. The method is robust, and in most cases may be used as a one-step procedure, similar to our method. Unfortunately, the application of our method requires significant dilution of the serum before extraction (contrary to Oasis HLB or Clarity OTX), making it a disadvantage of the procedure. Furthermore, the recovery is much lower when using PBA, compared to other methods used so far. Nevertheless, we believe that our method can be competitive with those used to date, as it is very simple and reproducible in both SPE and dSPE. Our studies have shown that ASO extraction can be extended to other commercially available sorbents, in particular PE and PBA, without compromising extraction quality.

4. Conclusions

Our study demonstrates, for the first time, that commercially available adsorbents not originally designed for ON extraction can be successfully applied to the mixed-mode extraction of ASOs. Comprehensive evaluation of five adsorbents with diverse surface chemistries revealed that the efficiency of ASO adsorption and desorption depends on interactions between ONs and the sorbent surface, including electrostatic, hydrogen bonding, π-π, and hydrophobic interactions. Therefore, the selection of commercial sorbents and solvent composition must be optimized to balance these interactions and enable both complete adsorption and efficient elution. ASO desorption from PE, PBA, SULF, and DSC requires high pH to disrupt electrostatic and hydrogen bonding interactions, while high salt concentrations provide charge screening, and organic solvents weaken hydrophobic and π-π interactions. The need to optimize all these parameters indicates that the extraction proceeds via a mixed-mode mechanism rather than a single dominant interaction. Among the tested materials, PBA and PE sorbents exhibited the most favorable extraction features, providing high recoveries, reproducibility, high sorption capacities, and rapid adsorption kinetics. However, differences in desorption kinetics significantly affected method transferability from dSPE to SPE. While PBA showed high recovery, PE exhibited reduced recovery in SPE due to slow desorption kinetics. Despite this, both sorbents can be used for ASO extraction in a mixed-mode. To the best of our knowledge, this is the first demonstration that PBA and PE sorbents can be used for mixed-mode extraction of ASO.

The developed mixed-mode dSPE procedure using PBA was successfully applied to the extraction of structurally modified therapeutic ASOs from human serum without prior protein removal. The disadvantage of the developed method is the need for significant serum dilution before extraction and low recovery from the biological sample. On the other hand, the method provided acceptable reproducibility and matrix tolerance while maintaining simplicity. We hope

our findings facilitate the rational design of future extraction protocols for therapeutic ONs and encourage the wider investigation of commercial adsorbents for mixed-mode extraction. The obtained results significantly broaden the scope of sorbent chemistries applicable to ASO sample preparation.

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Author contribution statement:

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

Karolina Ostrowska: Investigation, data curation, writing – original draft, visualisation

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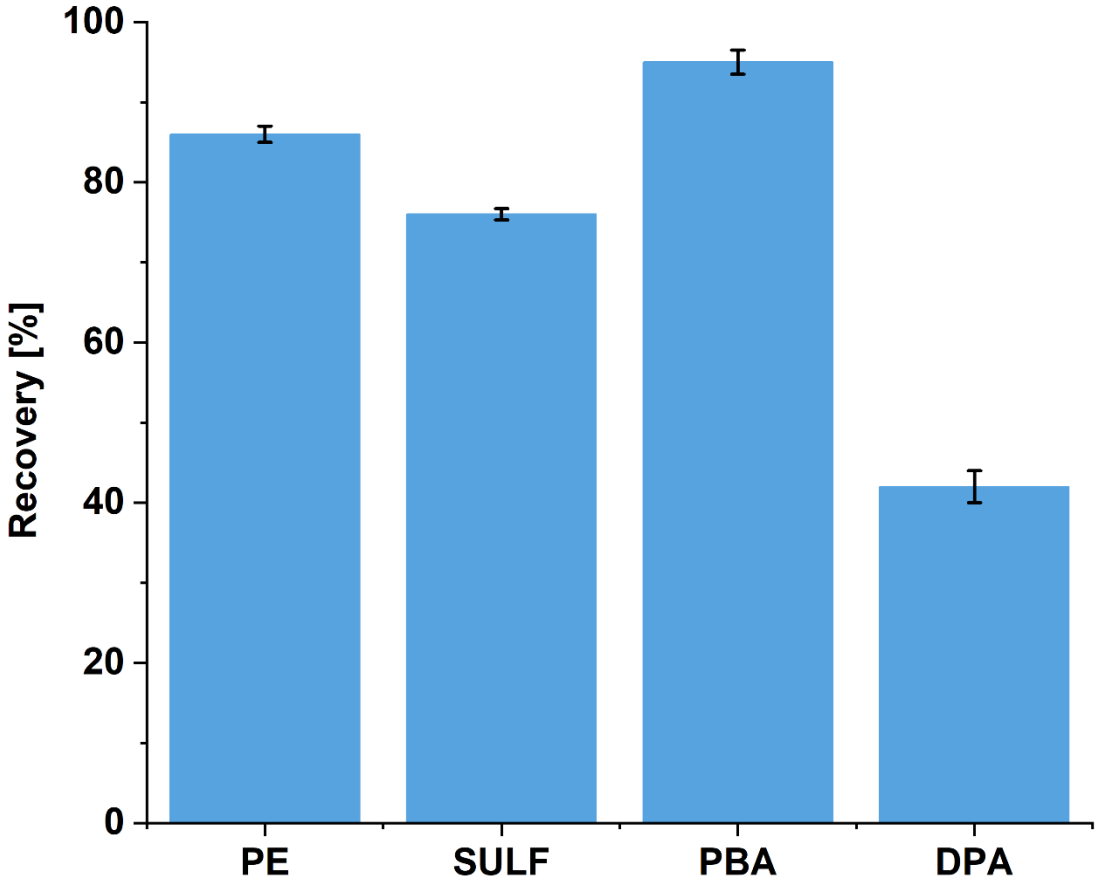

Figure 1. Recoveries of NuDNA for all tested commercial adsorbents using optimized dSPE procedures.

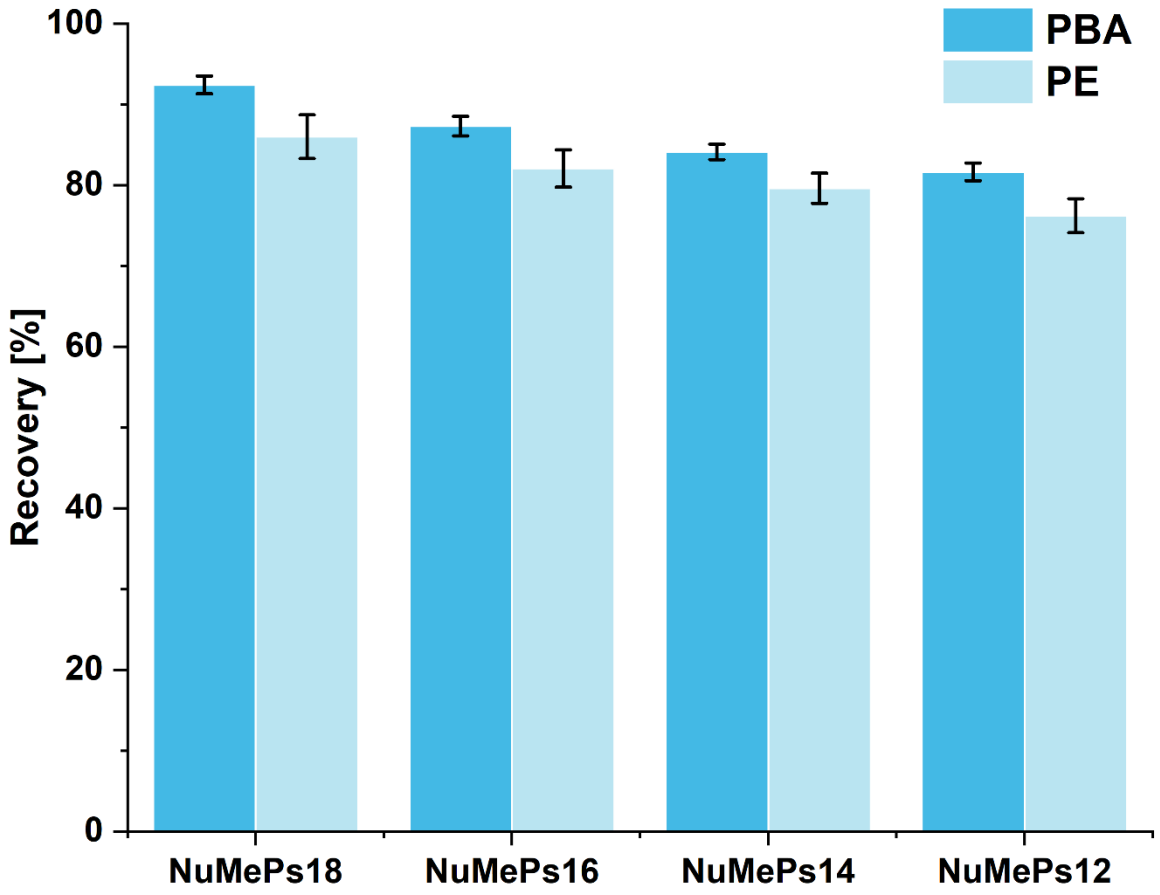

Figure 2. Recoveries of a four-ASO mixture from the surface of PBA and PE adsorbents using optimized dSPE procedures (described in 3.2.4). Experimental conditions: Waters Acquity UPLC BEH C18 column, mobile phase composition: 5mM HA, 150 mM HFIP, MeOH; gradient elution program: 15–55 % (v/v) of MeOH in 10 min, autosampler temperature 10°C, column temperature 50°C, flow rate 0.30 mL/min.

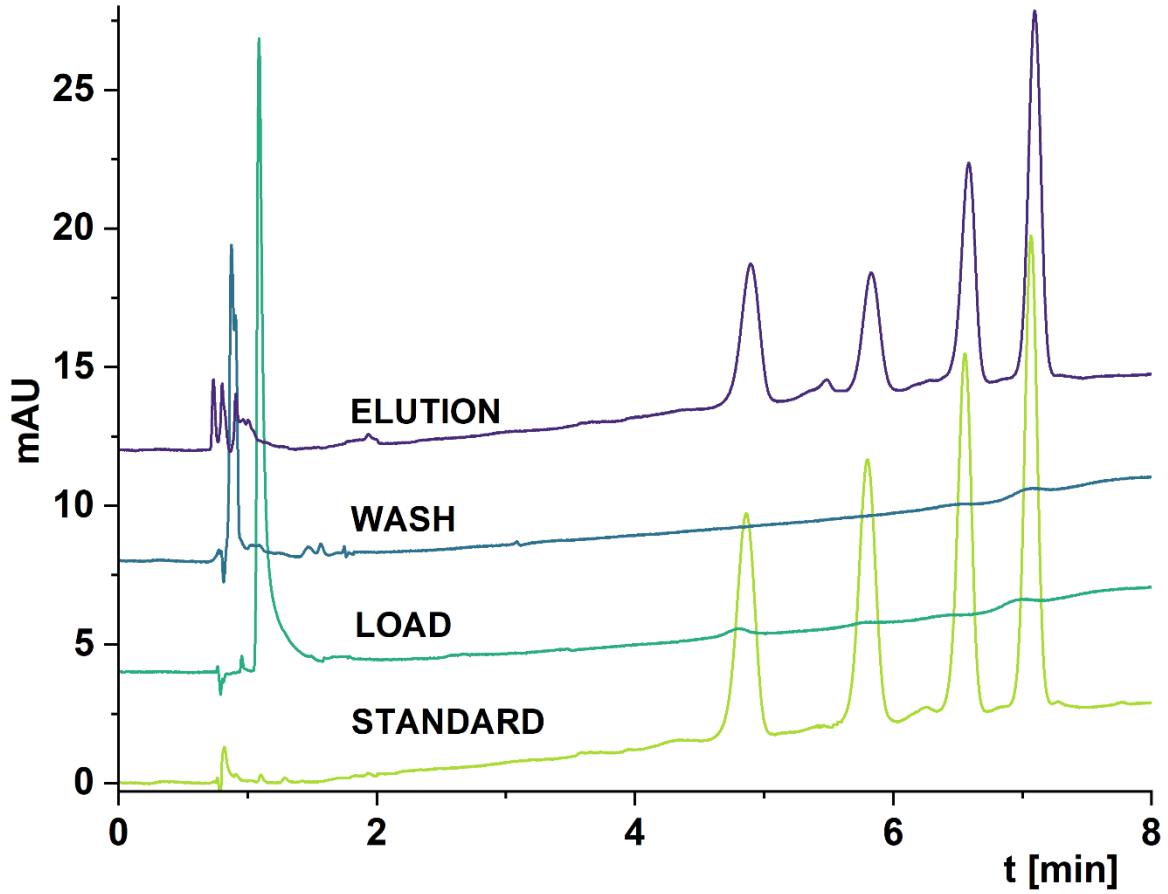

Figure 3. Exemplary chromatograms for serum extract at various stages of the dSPE procedure (sample load, washing, elution) using PBA adsorbent for the ASOs mixture. Elution order: NuMePs12, NuMePs14, NuMePs16, NuMePs18.

Table 1. The characteristics of sorbents selected for the study.

Sorbent	Shortcut	Form	Support	Type of groups	Pore diameter [Å]	Surface area [m²/g]	Particle size [µm]
Discovery® DPA-6S	DPA	SPE (250 mg, 6 mL)	polymer	polyamide resin-based material with amide groups	–	–	50-160
Discovery® DSC-WCX	DSC	SPE (50 mg, 1 mL)	silica gel	ethylene diamine triacetate groups	70	480	–
Supelclean® Sulfoxide	SULF	SPE (3 g, 6 mL)	silica gel	sulfoxide groups; amide groups; secondary amine	60	475	~45
Bond Elut PBA	PBA	SPE (100 mg, 1mL)	silica gel	phenylboronic acid groups	60	500	40
2-(2-Pyridyl)ethyl-functionalized silica gel	PE	Bulk	silica gel	2-(2-pyridyl)ethyl groups	60	500	97-74

Table 2. Results of NuDNA adsorption using various solvents and sorbents.

Solution	Adsorption [%]				
	PE	DSC	DPA	SULF	PBA
H_2O	0	0	95±2	20±1	100
H_2O pH 4 (using FA)	100	14±1	100	100	100
H_2O pH 11 (using NH_4OH)	0	0	20±1	0	0
10mM AA pH 4	100	12±2	100	100	100
10mM AA pH 11	0	0	32±1	0	0

Table 3. Comparative evaluation of extraction methods

Adsorbent	Mode	Solvents	Matrix	ASO recovery [%]	Advantages	Limitations	Reference
Oasis HLB	ion pair	5-50 mM amine (TEA, HA), 100-200 mM hexafluoroisopropanol, MeOH	serum, plasma, cerebrospinal fluid	83-98	high recoveries regardless of modification type; possibility of extract concentration; widely established method	application of ion pair reagents (amines); time-consuming procedure (up to 2 hours); the risk of low repeatability; necessity of protein removal (liquid-liquid extraction or proteinase K) before extraction	[11,14,15,19,20]
Waters WAX	weak anion exchange	50-100 mM AA (pH 5.5), 50 mM TEA (pH 11.5), MeOH	plasma	76-100	high recoveries; no ion-pair reagents required; MS-compatible; rapid procedure	application of ion pair reagents; the necessity of protein removal (liquid-liquid extraction or proteinase K) before extraction; recovery may vary for different modifications	[13]
Clarity OTX	mixed-mode	50 mM AA (pH 5.5); 100 mM ammonium bicarbonate, tris(2-carboxyethyl)phosphine hydrochloride, acetonitrile, tetrahydrofuran	serum, tissue	74-96	high recoveries; no ion-pair reagents required; rapid procedure; typically one step procedure	high, inorganic salt concentration for elution; in some cases the necessity of protein removal; desalting is required before mass spectrometry	[11,12,22]

						utilization; a complex solvent mixture for elution	
PBA	mixed-mode	10-100 mM AA (pH 4.0 and 11), MeOH	serum	59-71	high recoveries and repeatability; no ion pair reagents; rapid procedure; one-step procedure for extraction from serum	high salt concentration for elution; desalting is required before mass spectrometry application	–