Evaluation of a novel SPE sorbent for the extraction of oligonucleotides from biological matrices prior to UPLC-MS/MS analysis

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Introduction

Oligonucleotide therapeutics have gained popularity as drug candidates due to high specificity for the treatment of various genetic diseases and disorders. As a result, routine methods are required for analysis and quantitation. However, antisense oligonucleotides (ASO) present various analytical challenges particularly when using LC/MS analysis. Sample preparation is generally required to remove unwanted endogenous matrix components that interact with targets, thereby limiting availability for detection or interfering with the analytical response. This poster investigates the use of a novel polymer-based mixed-mode weak anion exchange solid phase extraction sorbent for improved oligonucleotide detection from various biological matrices. Method performance was evaluated using plasma, urine and liver matrices and assessed for ASO extraction efficiency and overall extract cleanliness.

Experimental

Reagents

Oligonucleotide (OGN) standards were sourced from ATDBio (Oxford, UK). Plasma was purchased from the Welsh Blood Service (Pontyclun, UK), tissues obtained from local butchers and urine supplied by healthy human volunteers. Analytical reagents were from Sigma-Aldrich. All solvents were HPLC grade from Rathburn Chemical Ltd (Scotland, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

Sample Preparation

Plasma and urine:
Up to 100 µL plasma samples were pre-treated with lysis buffer 1:2 or digested with Proteinase K, followed by dilution with 5 parts ammonium acetate buffer, 50 mM pH 5.5. Urine samples were simply pre-treated 1:5 with ammonium acetate buffer, 50 mM pH 5.5.

Tissue Matrix Preparation:
100 mg of liver samples were weighed into 1.5 mL Biotage® Lysera centrifuge tubes. 500 µL of H_2O and 5 x 2.4 mm stainless steel beads were added for homogenization.

Micropulverization (MPE) Procedure:
Biotage® Lysera: 1 x 1-minute cycle at 5.5 m/sec.

Post Pulverization: 100 µL of supernatant was removed and mixed with 200 µL of lysis buffer followed by dilution with 500 µL of ammonium acetate buffer, 50 mM pH 5.5. Full procedure available upon request.

***Figure 1**. Biotage® Lysera Bead Mill Homogenizer.*

Solid Phase Extraction:
Pre-treated matrix was extracted with the Biotage® OLIGO SPE 30 mg 96-well plates (654-0030-PX01), processed using a positive pressure 96 manifold. Final extraction protocol is demonstrated in ***Table 1.***

***Table 1**. Oligo SPE Extraction Procedure.*

Step	Buffer	Volume
Conditioning	MeOH	1 mL
Equilibration	50 mM NH_4OAc pH 5.5 aq	1 mL
Load	Pre-treated matrix	Up to 800 µL
Wash 1	50 mM NH_4OAc pH 5.5 aq	1 mL
Wash 2	50/50 50 mM NH_4OAc pH 5.5 aq/ACN	1 mL
Wash 3	50/50 100 mM NH_4HCO_3 pH 9 aq/ACN	1 mL
Dry	1 minute @ 20 psi	
Elute	50/50 200 mM NH_4HCO_3 pH 9.5 aq/ACN	500 µL

Post extraction: 500 µL extracts were evaporated in 96-well plates at 60 °C using a TurboVap 96-Dual system, followed by reconstitution in 200 µL of 5/95 mobile phase A:B prior to LC/MS analysis.

UPLC Conditions

Instrument: Waters ACQUITY Premier UPLC (Waters Assoc., Manchester, UK).
Column: ACQUITY Premier BEH Amide VanGuard FIT UPLC 1.7 µm; (50 x 2.1 mm) (Waters Assoc., Manchester, UK).
Mobile phase: 30mM NH_4OAc/0.04% NH_4OH in H_2O:ACN pH8.8; 70/30 (A) and 30/70 (B) **Flow rate:** 0.5 mL/min
Column temp: 40 °C **Injection volume:** 10 µL
Gradient: Initial 5/95 A/B. Linear ramp 5 > 35% A 0-1.5 min; 35 > 90 1.5-5 min; resume initial conditions at 6 min.

MS Conditions

Instrument: Waters Xevo TQ Absolute triple quadrupole mass spectrometer equipped with an ES interface for mass analysis (Waters Assoc., Manchester, UK). Negative ions were acquired in the SIR mode. PDA channel monitored at 260 nm.
Desolvation Temp: 600 °C **Ion Source Temp:** 150 °C

Results

Ion pair reagent use was not an option, so HILIC chromatography was investigated. Initial compound optimisation demonstrated differing charge distribution when using generic reversed phase solvent conditions compared to high ACN/salt concentrations used in HILIC. ***Figure 2.*** illustrates charge distribution differences observed for eluforsen.

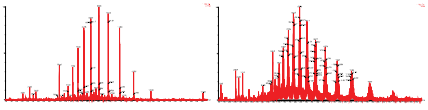

***Figure 2**. Eluforsen charge distribution comparison: HILIC vs RP.*

The analyte panel consisted of a range of ASOs: 14-34 nucleotides; deoxyribose or modified ribose (2'-OMe or 2'-MOE) sugars; phosphodiester (PO) and/or phosphorothioate (PS) backbones; naked, lipid or GalNAc conjugated. Final HILIC separation, both PDA (260 nm) and SIR chromatograms are detailed in ***Figure 3.***

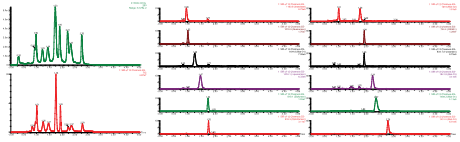

***Figure 3**. HILIC chromatograms: PDA-TIC (LHS) individual SIR (RHS).*

OGNs demonstrate high degree of non-specific binding to glass so all work was performed using plastic components. Evaporative effects were investigated comparing air vs nitrogen (data not shown), various antioxidants and common elution solvent combinations. Nitrogen evaporation in the presence of antioxidant demonstrated reduced evaporative losses. ***Figure 4.*** demonstrates the effect of antioxidant (TCEP) concentration on OGN analyte signal.

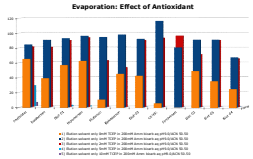

***Figure 4**. Evaporation comparison using TCEP antioxidant.*

Figure 5. illustrates OGN recovery and matrix factor breakdown with respect to plasma and lysis buffer effects.

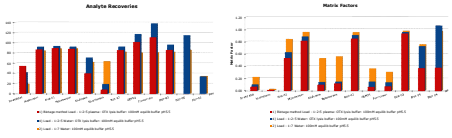

***Figure 5**. OGN recovery (Left); matrix factor breakdown (Right).*

Figure 6. demonstrates effect of wash combination on OGN recovery and matrix factors.

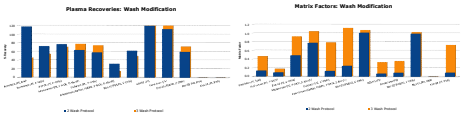

***Figure 6**. Effect of wash combination on OGN recovery (left) and matrix factors (right).*

Figure 7. illustrates OGN recovery in various matrices: plasma, liver and urine. Lipid and GalNAC conjugations can be improved further with subtle method modification of wash and elution steps.

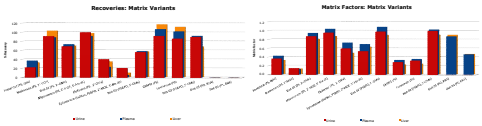

***Figure 7**. OGN recovery and matrix factor comparison from plasma, urine and liver.*

Extract cleanliness was investigated with respect to protein, phospholipid and surfactant removal. ***Figure 8.*** demonstrates gel profiles before and after Oligo SPE extraction. Lysis or proteinase K pretreatment options (left) and tissue matrices are demonstrated. Excellent protein removal was observed for all options due to the optimised pore size distribution of the SPE sorbent.

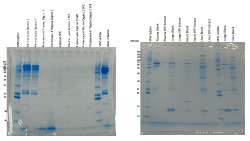

***Figure 8**. Gel electrophoresis profile comparing protein removal under various conditions.*

Figure 9. demonstrates phospholipid profiles for the final SPE extraction protocols compared to a simple protein precipitated extract.

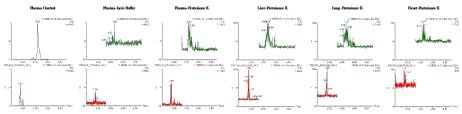

***Figure 9**. Phospholipid profile comparing PPT vs OLIGO SPE extracts.*

Additives to stabilise (reducing agents/anti-oxidants), lyse or digest samples provide added complexity to already complex matrices. ***Figure 10.*** demonstrates the degree of clean up using full scan MS.

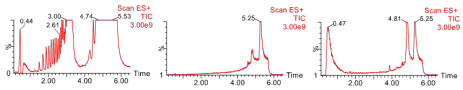

***Figure 10**. Full scan chromatograms comparing additive/surfactant removal: Direct inject; Blank inject; OLIGO SPE extract.*

Excellent buffer removal was observed providing comparable baseline to blank injections. The early eluting peak in the OLIGO SPE extract is a result of the TCEP antioxidant.

Automation

Figure 11. shows a comparison of recoveries between optimised methods for lipid and GalNAC containing OGNs, performed manually and automated.

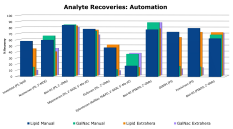

***Figure 11**. Automated vs manual recovery.*

Further development is required however early work indicates suitability of using the Biotage® Extrahera™ Classic as an automated sample preparation system.

***Figure 12**. Biotage® Extrahera™ Classic.*

Conclusion

- This poster demonstrates the applicability of a novel SPE sorbent with targeted pore size distribution to the extraction and analysis of various Oligonucleotides from multiple biomatrices.
- High reproducible recoveries for a range of OGN targets were observed when analysing manually and automated on the Biotage® Extrahera™ Classic.
- Final extraction protocols demonstrated excellent removal of matrix components, specifically proteins, phospholipids and exogenous stabilizers/surfactants.

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