ANALYSIS OF OLIGONUCLEOTIDE IMPURITIES ON THE BIOACCORD LC-MS SYSTEM WITH ACQUITY PREMIER

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OVERVIEW

Here we demonstrate the use of a novel chromatographic system, equipped with a modified surface technology across its entire fluidic path [1-2], for analysis of modified synthetic oligonucleotides and their impurities. An automated workflow, suitable for both regulated and non-regulated laboratories, was implemented for rapid mass confirmation and purity analysis of modified oligonucleotides.

INTRODUCTION

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics [3]. Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC/MS methods for impurity identification and quantification. The most often used mass spectrometry-based method for oligonucleotide analysis has been reversed-phase chromatography employing a variety of ion-pairing reagents and modifiers in negative ESI-MS mode.
Oligonucleotides contain a negatively charged phosphate backbone known to interact with metal surfaces (like stainless steel, titanium or MP35N – a Ni-Co alloy) typically found in the fluidic path of the UPLC system. These interactions are often responsible for oligonucleotide losses, poor chromatographic peak shapes or poor data reproducibility. The MaxPeak HPS technology implemented along the UPLC fluidic path and the OST column significantly reduced these unwanted interactions, as demonstrated by the results shown in this poster presentation.

A comparison between separations performed on a regular OST column versus a Premier OSTcolumn, showed that the regular column produced poor results in terms of impurity recovery, even after extensive passivation. Only 7 out of 14 oligonucleotide impurities were detected on the regular OST column.

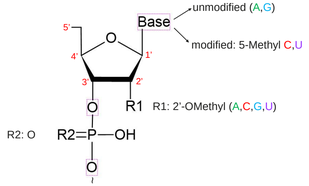
The data presented here demonstrates that a combination of the bio-inert UHPLC system and column is critical for obtaining accurate results in measuring low-level oligonucleotide

BioAccord LC-MS system with ACQUITY Premier

METHODS

Materials

A 21-mer heavily modified oligonucleotide, containing a 2'-OMe modification on 19 of its nucleosides, having the sequence GUA ACC AAG AGU AUU CCA UTT and the elemental composition C229H306N76O143P20 was purchased from ATDBio (Southhampton, UK). Stock solutions were prepared in DI water at a concentration of 1 µM (or 2.34 µg/mL), from which a 10 µL volume was injected, which corresponds to loading 10 picomoles of the 21-mer oligonucleotide on-column.



Modifications of the 21-mer oligonucleotide

LC Conditions

As part of the BioAccord™ LC-MS system, an ACQUITY™ Premier UPLC ™ unit was used for oligonucleotide separations on two columns: 1) a regular OST column (P/N 186003950); 2) a Premier OST column (P/N 186009485). The two columns had the same column dimensions: 2.1 x 100 mm, were packed with the same 1.7 µm BEH (Bridged Ethylene Hybrid) C18 particles and were operated under identical experimental conditions. The column flow rate was 300 µL/min and the column temperature was 60°C. The mobile phase composition was: Solvent A: 7 mM triethylamine (TEA) and 40 mM hexafluoro-2-propanol (HFIP) in Milli-Q water and Solvent B: 3.5 mM TEA, 20 mM HFIP in 50% methanol. Separations were performed using a 25-min gradient from 25-35% B and the total runtime was 40 min.

MS conditions

ESI-MS spectra of oligonucleotides were acquired on the BioAccord LC-MS platform in negative ion mode over the m/z range of 400-5,000 with a full scan rate of 2Hz. The optimized ESI source parameters include: capillary voltage 0.8 kV, cone voltage 40V, source temperature 120°C and desolvation temperature 400°C.

Informatics

Data acquisition and processing was performed using waters_connect software. The LC-MS datasets were processed automatically using the

RESULTS

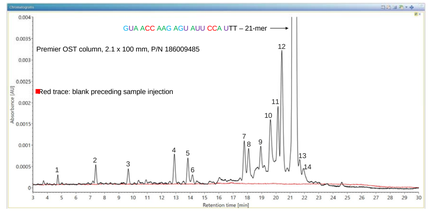


Figure 1A. LC-UV chromatogram recorded on an ACQUITY Premier OST column.

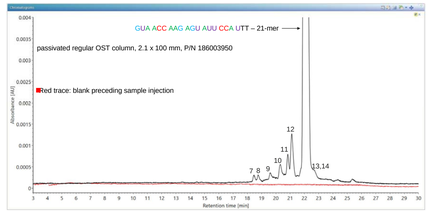


Figure 1B. LC-UV chromatogram recorded on a regular OST column. Analysis of the same sample on a regular column (with stainless steel casing), having the same dimensions and packed with the same stationary phase (C18 1.7 µm particles, 130 Å pores), resolved only half (seven) of the same impurities, completely missing a significant portion of the early eluting impurities. These results can be explained by considering the adsorption effects of oligonucleotides on the inlet and outlet frits of the regular column [2]. When the sample is loaded at the inlet of the column, the major oligonucleotide component can be used to passivate the inlet frit, such as that minor oligonucleotide impurities are not adsorbed to this frit. However, after undergoing the IP-RP separations, because most of the oligonucleotide impurities elute before the major component, there is a great possibility that the outlet frit would retain some of these impurities until it gets fully passivated. It is very likely that the first seven impurities (peaks labeled 1-7) were not detected because they were adsorbed to the outlet frit of

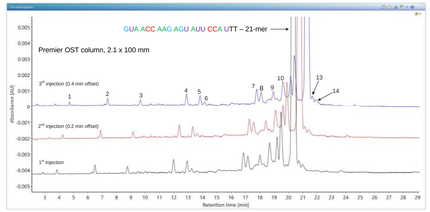
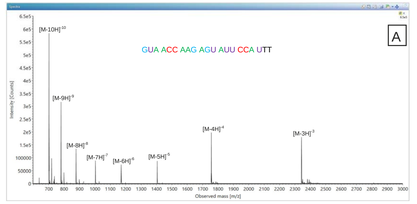


Figure 1C. Three replicates injections performed on the ACQUITY Premier OST column. For better clarity, the red and blue traces are offset by +0.2 min from the previous trace.



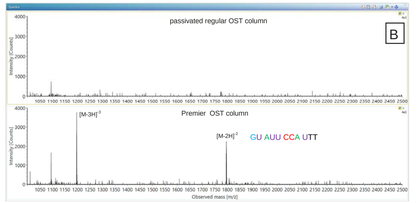


Figure 2. Ion pairing reversed-phase (IP RP) ESI-MS spectra recorded for: (A) the most abundant sample component, the 21-mer heavily modified oligonucleotide; (B) the least abundant sample component, an 11-mer oligonucleotide impurity (see Table I for its sequence), present at 0.18% according to the UV peak area measurement.

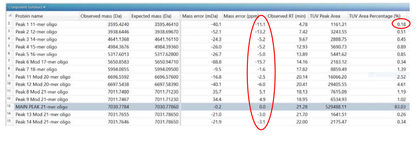
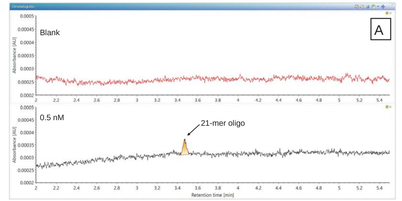
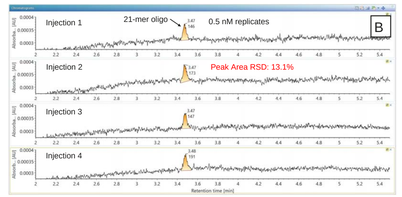


Figure 3. Screenshot showing the waters_connect processing results obtained after BayesSpray charge deconvolution of the ESI-MS spectra recorded for the 21-mer major component and fourteen of its oligonucleotide impurities. The mass accuracy error for measuring the accurate average masses was better than 15 ppm for all sample components. The row corresponding to the main component is highlighted in blue and it indicates a purity of 83.03%, while the abundance of the lowest abundant species (an 11-mer oligo) was 0.18% (highlighted by a red circle).

Impurity label	Oligonucleotide length	Oligonucleotide modification (Da)	Retention time (min)	Oligonucleotide sequence	Elemental composition	Most abundant monoisotopic mass	Charge	Accurate average molecular weight
1	11-mer	-	4.73	GU AUU CCA UTT	C119 H361 N33 O77 P10	3795.8464	2	3595.4643
2	12-mer	-	7.39	AGU AUU CCA UTT	C130 H375 N38 O83 P11	1351.2512	3	3938.6867
3	14-mer	-	9.67	AG AAG AGU AUU CCA UTT	C152 H435 N48 O96 P13	1545.2950	3	4641.9413
4	15-mer	-	12.89	AAG AGU AUU CCA UTT	C160 H437 N50 O102 P14	1659.6510	3	4984.3936
5	16-mer	-	13.83	C AAG AGU AUU CCA UTT	C174 H455 N56 O109 P15	1778.6752	3	5327.6280
6	Modified 17-mer	+19 Da	14.15	CC AAG AGU AUU CCA UTT	C184 H461 N58 O110 P16	1881.7307	3	5650.9471
7	18-mer	+ 10 Da	17.78	ACC AAG AGU AUU CCA UTT	C196 H463 N64 O112 P17	1996.0555	3	5994.0950
8	Modified 21-mer	+19 Da	18.08	GUA ACC AAG AGU AUU CCA UTT	C228 H299 N76 O143 P20	2335.0998	3	7011.7123
9	Modified 21-mer	+19 Da	18.94	GUA ACC AAG AGU AUU CCA UTT	C228 H299 N76 O143 P20	2335.0998	3	7011.7123
10	Modified 20-mer	+343 Da	19.62	UA ACC AAG AGU AUU CCA UTT	C220 H284 N71 O136 P19	2227.0762	3	6687.5045
11	Modified 20-mer	+334 Da	20.15	UA ACC AAG AGU AUU CCA UTT	C220 H291 N71 O136 P19	2230.0997	3	6696.5760
12	Modified 20-mer	+333 Da	20.41	UA ACC AAG AGU AUU CCA UTT	C220 H294 N71 O136 P19	2230.4356	3	6697.5839
MAIN PEAK	**21-mer**	-	**21.25**	**GUA ACC AAG AGU AUU CCA UTT**	**C229 H306 N76 O143 P20**	**2341.4514**	3	**7030.7798**
13	Modified 21-mer	+1 Da	21.65	GUA ACC AAG AGU AUU CCA UTT	C229 H307 N76 O143 P20	2341.7874	3	7031.7865
14	Modified 21-mer	+1 Da	21.93	GUA ACC AAG AGU AUU CCA UTT	C229 H307 N76 O143 P20	2341.7874	3	7031.7865

Table I. Fourteen oligonucleotide impurities identified in a 21-mer extensively modified oligonucleotide with the sequence GUA ACC AAG AGU AUU CCA UTT.





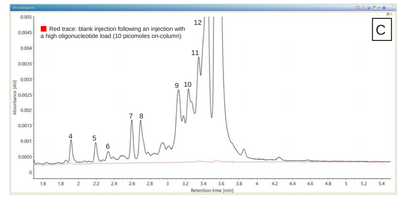


Figure 4. Testing the inertness of the ACQUITY Premier UPLC BSM system and OST Premier column using the 21 nt oligomer: (A) UV chromatogram of the lowest detectable concentration (0.5 nM, or 5 femtomoles of the 21-mer oligonucleotide loaded on-column) compared against the preceding blank injection; (B) replicate UV chromatograms obtained for the lowest detectable concentration (0.5 nM), indicating that the UV peak areas had RSDs below 15%; (C) carryover evaluation, showing the UV chromatogram of a blank injection (red trace) following the injection of the highest sample concentration (1000 nM or 10 picomole oligonucleotide loaded on-column). There is no detectable signal from the 21-mer in the blank, suggesting that the UPLC system and column do not retain any analyte through non-specific adsorption to the various coated metal

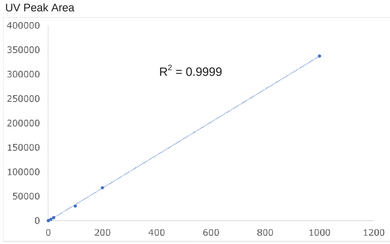


Figure 5. Calibration curve of the 21-mer oligonucleotide showing linearity over three orders of magnitude. Peak areas obtained from the TUV detector were plotted against a wide range of oligonucleotide concentrations, including 0.5, 1, 5, 10, 20, 100, 200 and 1000 nM. Excellent signal linearity was obtained for this assay, indicating that very low oligonucleotide concentrations can be recovered completely from a very inert LC system that does not interact in any way with the analyte.

CONCLUSIONS

- A category of products incorporating the MaxPeak™ High Performance Surfaces (HPS) including the ACQUITY™ Premier UPLC BSM System and the Premier OST Columns provide critical advantages for oligonucleotide impurity analysis
- Improved oligonucleotide analysis in terms of low detection limit and chromatographic reproducibility is demonstrated using the BioAccord Premier LC-MS system operated under compliant-ready software
- The oligonucleotide impurity analysis workflow provides mass confirmation for oligonucleotide impurities as well as their relative abundance. The results from our study indicate that the LC-MS platform provides good mass accuracy (better than 15 ppm) for intact mass confirmation of modified oligonucleotides and their impurities, while the LC-UV information allows for the measurements of all the sample components, down to 0.2%

REFERENCES

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