

ANALYSIS OF OLIGONUCLEOTIDE IMPURITIES ON AN UHPLC-TOF MS SYSTEM WITH A MODIFIED SURFACE TECHNOLOGY

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Catalin Doneanu¹, Christopher Knowles², Jonathan Fox², Emma Harry², Ying Qing Yu¹, Joseph Fredette¹ and Weibin Chen¹
¹Waters Corporation, Milford MA USA; ²Waters Corporation, Wilmslow UK

INTRODUCTION

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics [1]. Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC/MS methods for impurity identification and quantification. Oligonucleotides contain a negatively charged phosphate backbone known to interact with metal surfaces typically found in the fluidic path of conventional stainless steel LC systems. These interactions are often responsible for oligonucleotide losses, poor chromatographic peak shapes or poor data reproducibility. To address this challenges, Waters has developed a family of new technologies containing a more inert surface specifically designed to address difficult to analyze analytes – MaxPeak™ High Performance Surfaces (HPS) [2]. The recently launched ACQUITY™ Premier UPLC BSM System has this technology implemented across the entire fluidic path in order to provide a very effective barrier that reduces significantly analyte interactions with all type of metal surfaces. Here we investigated the capabilities of this UPLC system for intact mass confirmation of oligonucleotides and their associated impurities [3]. 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.

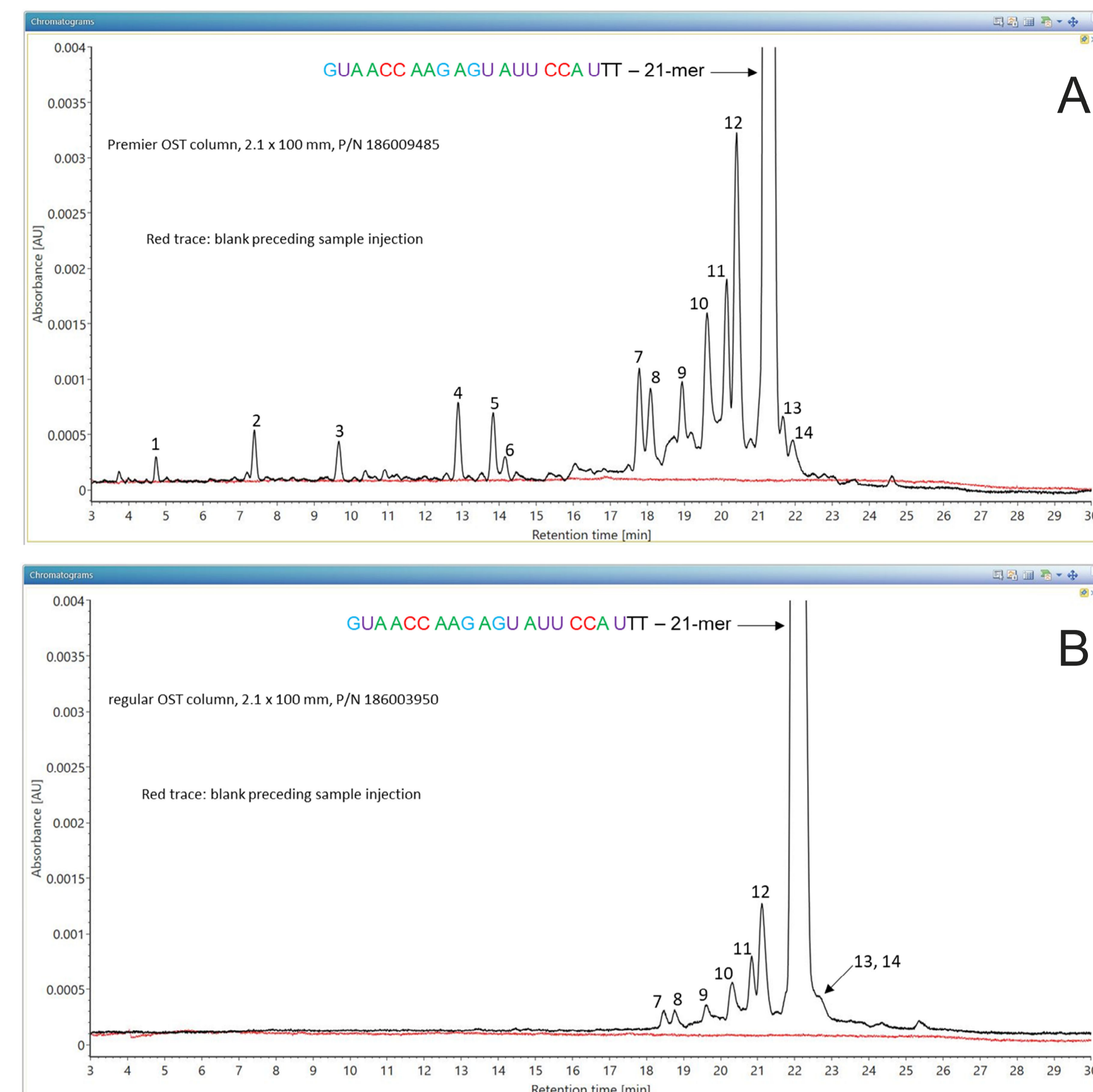


Figure 1. TUV chromatograms showing the separation of oligonucleotide impurities from a 21 nt sample: (A) UV chromatogram recorded on an ACQUITY Premier OST Column; (B) UV chromatogram recorded on an extensively conditioned conventional column. The red traces from each figure correspond to the blanks preceding each injection.

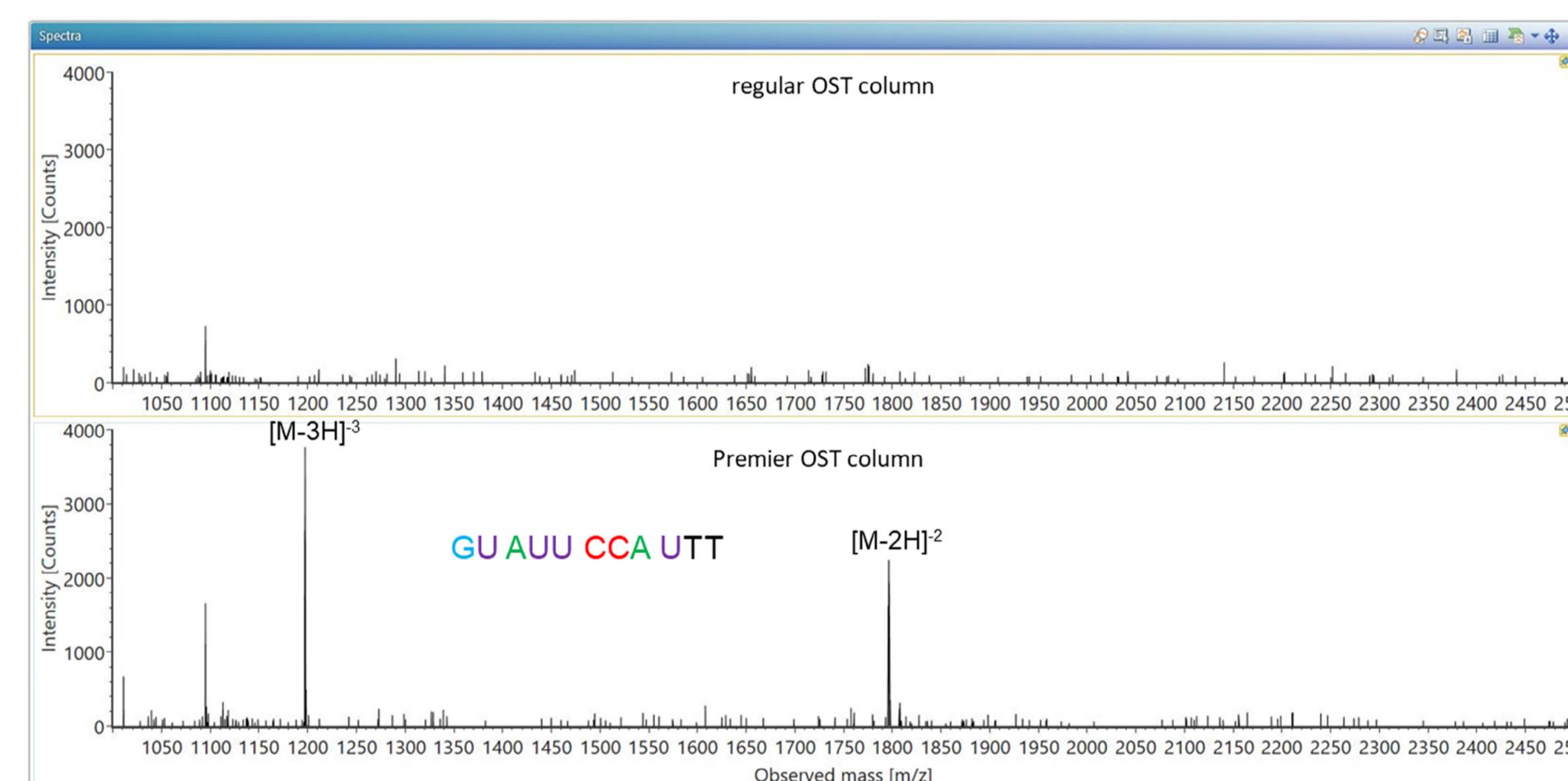


Figure 2. Ion pairing reversed-phase (IP RP) ESI-MS spectra recorded for the least abundant sample component, an 11-mer oligonucleotide impurity (see Table 1 for its sequence), present at 0.18% according to the UV peak area measurement.

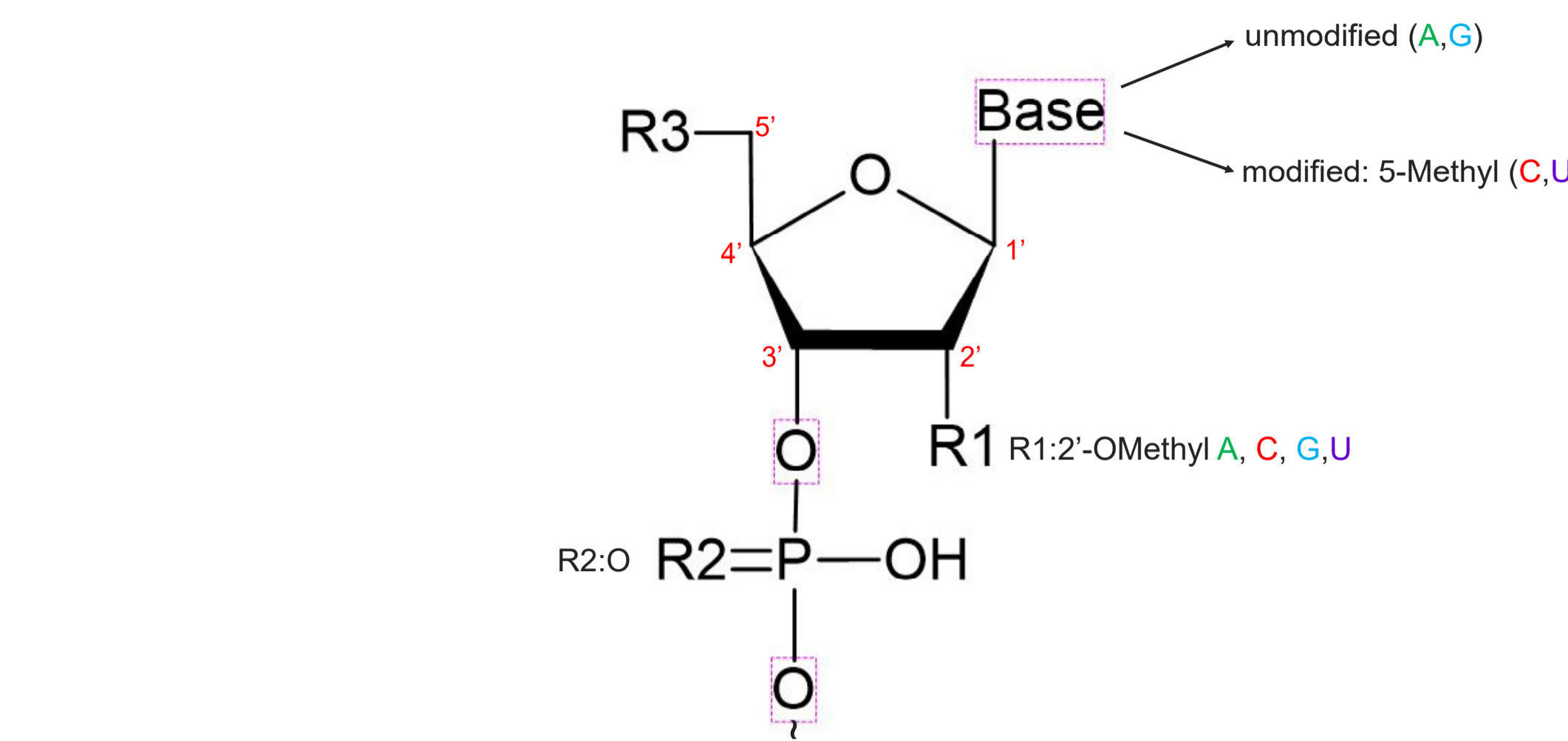


Figure 3. Modifications of the 21-mer oligonucleotide.

Impurity label	Oligonucleotide length	Oligonucleotide modification (Da)	Retention time (min)	Oligonucleotide sequence	Elemental composition	Most abundant monoisotopic mass	Charge state	Accuracy average molecular weight
1	11-mer	-	4.73	GU AUU CCA UTT	C119 H161 N33 O77 P10	1795.8464	2	3595.4641
2	12-mer	-	7.39	AUU AUU CCA UTT	C130 H175 N38 O83 P11	1911.2512	3	3638.6867
3	14-mer	-	9.67	AG AUU CCA UTT	C152 H203 N48 O98 P13	2145.2950	3	4641.1911
4	15-mer	-	12.89	AAG AGU AUU CCA UTT	C168 H217 N53 O102 P14	2659.6510	3	4984.3936
5	16-mer	-	13.83	C AAG AGU AUU CCA UTT	C174 H233 N56 O109 P15	2770.6752	3	5317.6280
6	Modified 17-mer	+19 Da	14.15	CC AAG AGU AUU CCA UTT	C184 H261 N59 O116 P16	2881.7307	3	5650.9473
7	18-mer	-10 Da	17.78	ACC AAG AGU AUU CCA UTT	C196 H283 N64 O122 P17	2996.6555	3	5984.0950
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 N73 O136 P19	2227.0762	3	6687.5045
11	Modified 20-mer	+334 Da	20.15	UA ACC AAG AGU AUU CCA UTT	C220 H283 N71 O136 P19	2230.0997	3	6686.5160
12	Modified 20-mer	+333 Da	20.41	UA ACC AAG AGU AUU CCA UTT	C220 H284 N71 O136 P19	2230.4356	3	6687.5839
MAIN PEAK	21-mer	-	21.25	GUA ACC AAG AGU AUU CCA UTT	C229 H306 N76 O143 P20	2341.4514	3	7030.7786
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 1. Fourteen oligonucleotide impurities identified in a 21-mer extensively modified oligonucleotide with the sequence GUA ACC AAG AGU AUU CCA UTT.

Component	Protein name	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	TUV Peak Area	TUV Area Percentage (%)
1	Peak 1 11-mer oligo	3595.4240	3595.46410	-40.1	-11.1	4.78	1161.31	0.18
2	Peak 2 12-mer oligo	3938.6446	3938.69670	-52.1	-13.2	7.42	3243.55	0.51
3	Peak 3 14-mer oligo	4641.1368	4641.16110	-24.3	-5.2	9.67	2886.75	0.45
4	Peak 4 15-mer oligo	4984.3676	4984.39360	-26.0	-5.2	12.93	5993.75	0.89
5	Peak 5 16-mer oligo	5317.6013	5317.62800	-26.7	-5.0	13.89	5441.62	0.85
6	Peak 6 Mod 17-mer oligo	5650.8583	5650.94710	-88.8	-15.7	14.16	2183.12	0.34
7	Peak 7 18-mer oligo	5994.0855	5994.09500	-9.5	-1.6	17.82	8859.49	1.39
8	Peak 11 Mod 20-mer oligo	6686.5592	6686.57600	-16.8	-2.5	20.14	16066.20	2.52
9	Peak 12 Mod 20-mer oligo	6687.5438	6687.56390	-40.1	-6.0	20.41	29405.55	4.61
10	Peak 8 Mod 21-mer oligo	7011.7480	7011.71230	35.7	5.1	18.13	7615.09	1.19
11	Peak 9 Mod 21-mer oligo	7011.7467	7011.71230	34.4	4.9	18.95	6534.93	1.02
12	MAIN PEAK 21-mer oligo	7030.7784	7030.77860	-0.2	0.0	21.28	529488.11	83.03
13	Peak 13 Mod 21-mer oligo	7031.7655	7031.78650	-21.0	-3.0	21.70	1641.51	0.26
14	Peak 14 Mod 21-mer oligo	7031.7646	7031.78650	-21.9	-3.1	22.00	2175.47	0.34

Figure 4. Screenshot showing the waters_connect software 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 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).

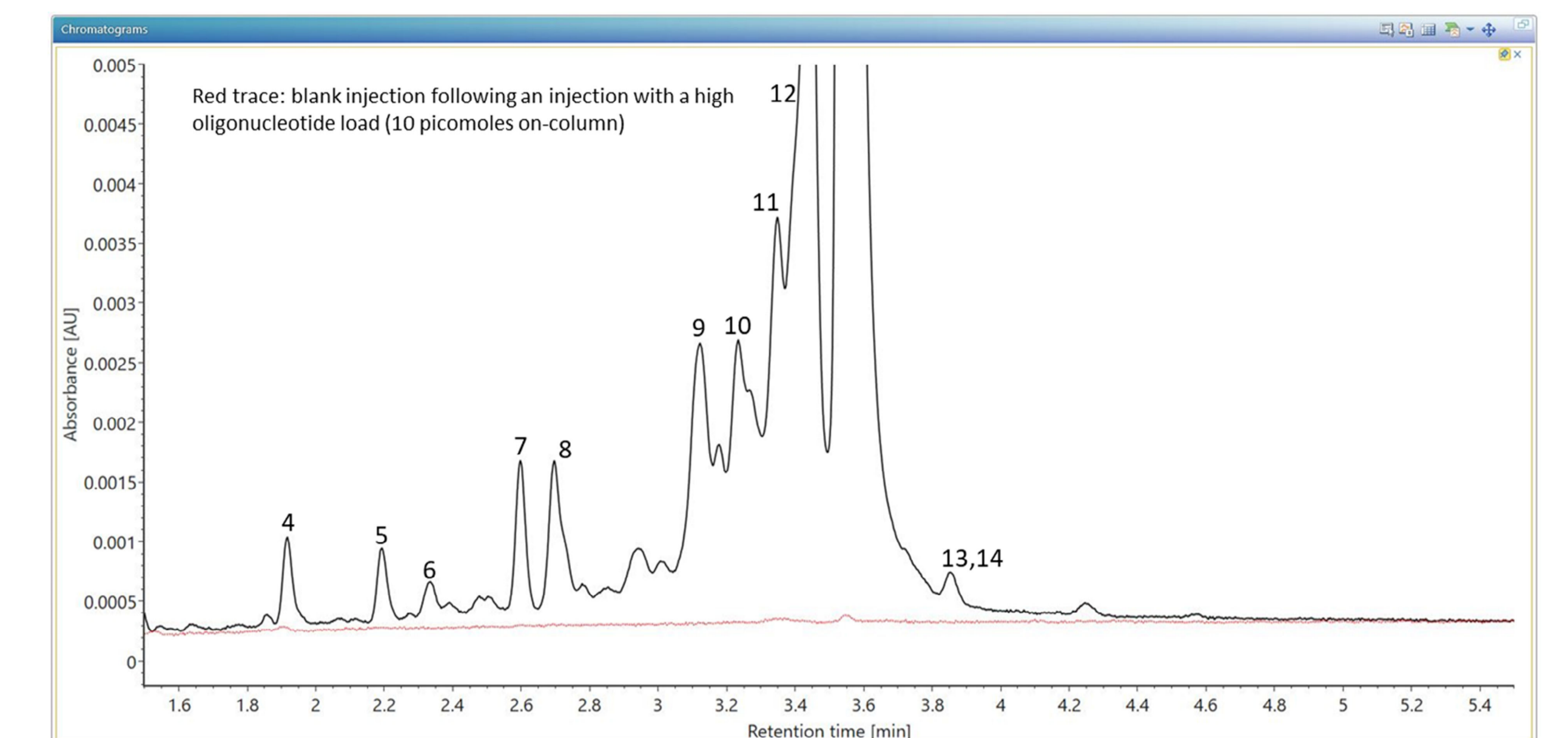


Figure 5. Carryover evaluation, showing the UV chromatogram of a blank injection (red trace) following the injection of the highest sample concentration (1000 nM or 10 picomole oligonucleotide loaded on-column). There is no detectable signal from the 21-mer in the blank, suggesting that the UPLC system and column do not retain any analyte through non-specific adsorption to the various coated metal surfaces found in the fluidic flow-path.

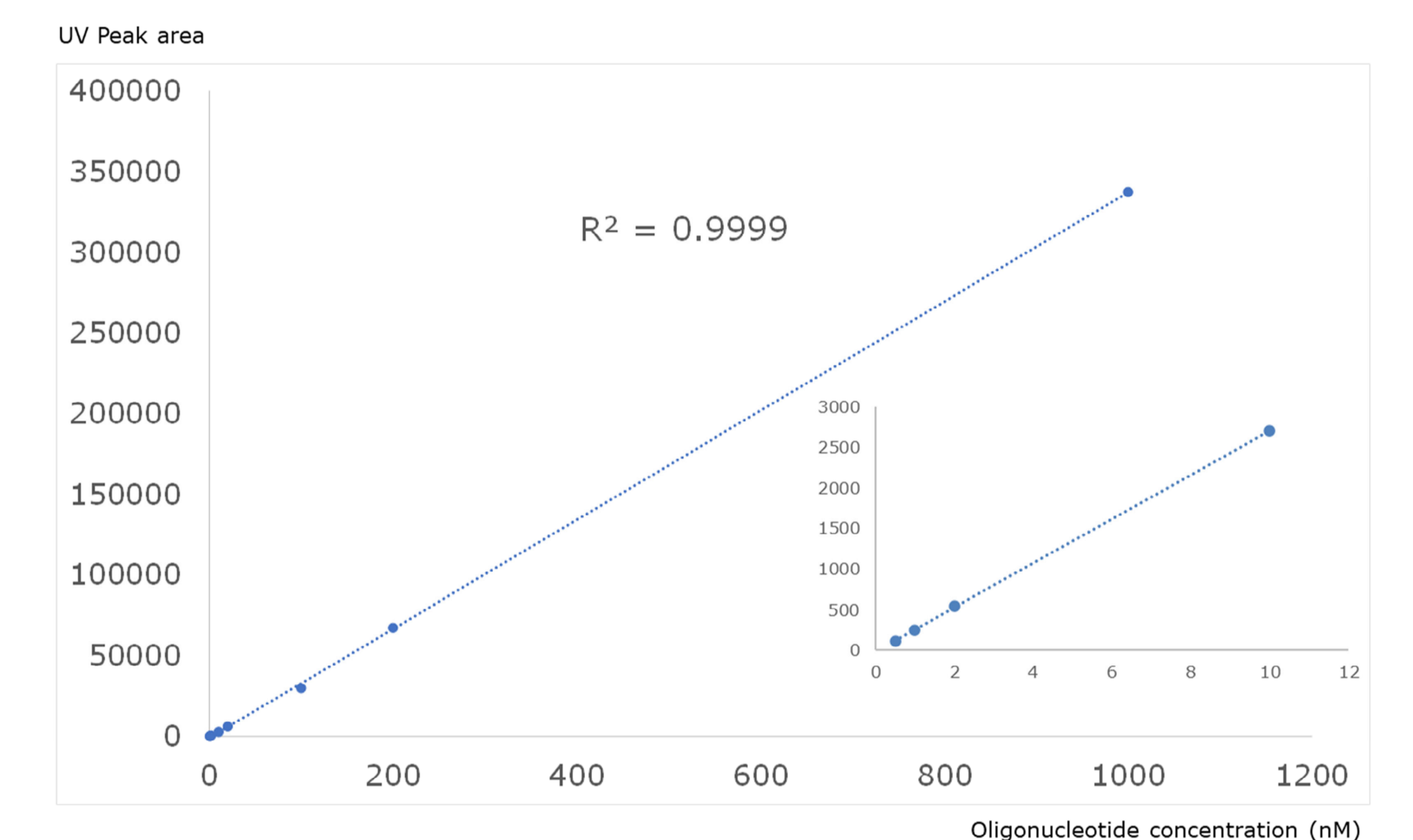


Figure 6. Calibration curve of the 21-mer oligonucleotide showing linearity over three orders of magnitude. Peak areas obtained from the TUV detector were plotted against a wide range of oligonucleotide concentrations, including 0.5, 1, 5, 10, 20, 100, 200 and 1000 nM. Excellent signal linearity was 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. The inset shows the calibration curve in the 0.5 - 10 nM concentration range.

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- DeLano M, Walter TH, Lauber MA, Gilar M, Jung MC, Nguyen J, Boissel C, Patel AV, Bates-Harrison A, Wyndham K Using Hybrid Organic-Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC, *Anal Chem*, 2021, 93, 5773-5781.
- Doneanu C, Knowles C, Fox J, Yu YQ, Fredette J, Chen W Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier, Waters application note 720007301, 2021.

CONCLUSIONS

- A category of products incorporating the MaxPeak High Performance Surfaces (HPS) including the ACQUITY Premier UPLC BSM System and the ACQUITY 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 System with ACQUITY Premier System operated under compliant-ready software
- The oligonucleotide impurity analysis workflow provides intact 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) while the LC-UV information allows for the measurements off all sample components, down to 0.2% relative abundance levels

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BioAccord™ LC-MS System with ACQUITY Premier

METHODS

LC method: A 21-mer oligonucleotide containing a variety of low-level oligonucleotide impurities, was separated on two C18 columns with the same dimensions (2.1 x 100 mm), a regular 2.1 x 100 mm OST column (P/N 186003950) and a recently introduced ACQUITY Premier 2.1 x 100 mm OST Column (P/N 186009485). 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 at a flow rate of 0.3 mL/min at a column temperature of 60° C.

MS method: The data acquired on the BioAccord LC-MS platform in negative ESI mode from $m/z = 400-5000$ using 1 sec MS scans.

Informatics

Data processing: Datasets were processed using the waters_connect™ software.

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