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Small-Footprint Capillary UHPLC/MS Technology Significantly Reducing Consumption of PFAS Containing Modifiers for Fast and High-Resolution Separation of Synthetic Oligonucleotides

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Introduction

Synthetic oligonucleotides are important biotherapeutic drugs because of their broad applications in genetic research, drug development, diagnostics, and personalized medicine and has gained popularity as a therapeutic modality in the past few years.

HFIP is a perfluoroalkyl (PFAS) substance

Hexafluoroisopropanol (HFIP) is often used as a mobile phase modifier in cation exchange (ion pairing) LC/MS at high pH to determine the presence of the active pharmaceutical ingredient (API) and various other impurities.[1] HFIP is a perfluoroalkyl substance (PFAS) aka “Forever Chemical”, contributing to PFAS contamination of the lab environment due to the aerosolization of droplets in the electrospray process. Therefore, the reduction of HFIP consumption in routine LC analysis of oligonucleotide drugs is of major interest in the long term.

TEA to adjust pH and augment ion pairing

To adjust pH, Triethylamine (TEA) has been identified as the ideal reagent since it augments the ion-pairing chromatography process. However, in the context of LC/MS operation, ion pairing reagents exist in high concentration, which introduces persistent contamination risk of the overall system over time.

In this work, we present a robust method for oligonucleotide analysis which dramatically reduces the consumption of HFIP and TEA, while maintaining chromatographic and mass spectral performance of typical “standard flow” rates of analytical systems.

Axcend Focus LC ® coupled with the Agilent LC/MSD Pro iQ Plus

Experimental

Instrumentation

Axcend Focus LC with Autosampler

Agilent Pro iQ Plus (G6170A) with ESI source

OpenLab CDS Acquisition and Data Analysis 2.8

LC Method Parameters

LC Parameter	Value
Column	Acquity M-Class HSS T-3 100 mm x 0.15 mm with 1.8 um fully porous particle
Mobile Phase A	100 mM HFIP and 15 mM TEA in water
Mobile Phase B	Methanol
Flow Rate	2 µL/min
Injection Volume	250nL
Gradient Program	Time (min) %B 0.0 20 10 27 11 95 12 95 12.1 20

MS Method Parameters

MS Parameter	Value
Ion Source	ESI
Polarity	Negative
Drying Gas Temp	300 °C
Drying Gas Flow	6 L/min
Nebulizer	15 psi
Capillary Voltage	4000 V

Data Processing

Sample data was processed directly in OpenLab CDS Data Acquisition. Each analyte yielded mass spectra with charge state distributions. Molecular Weight (Measured Mass) was determined using the built-in Deconvolution algorithm

Samples Analyzed

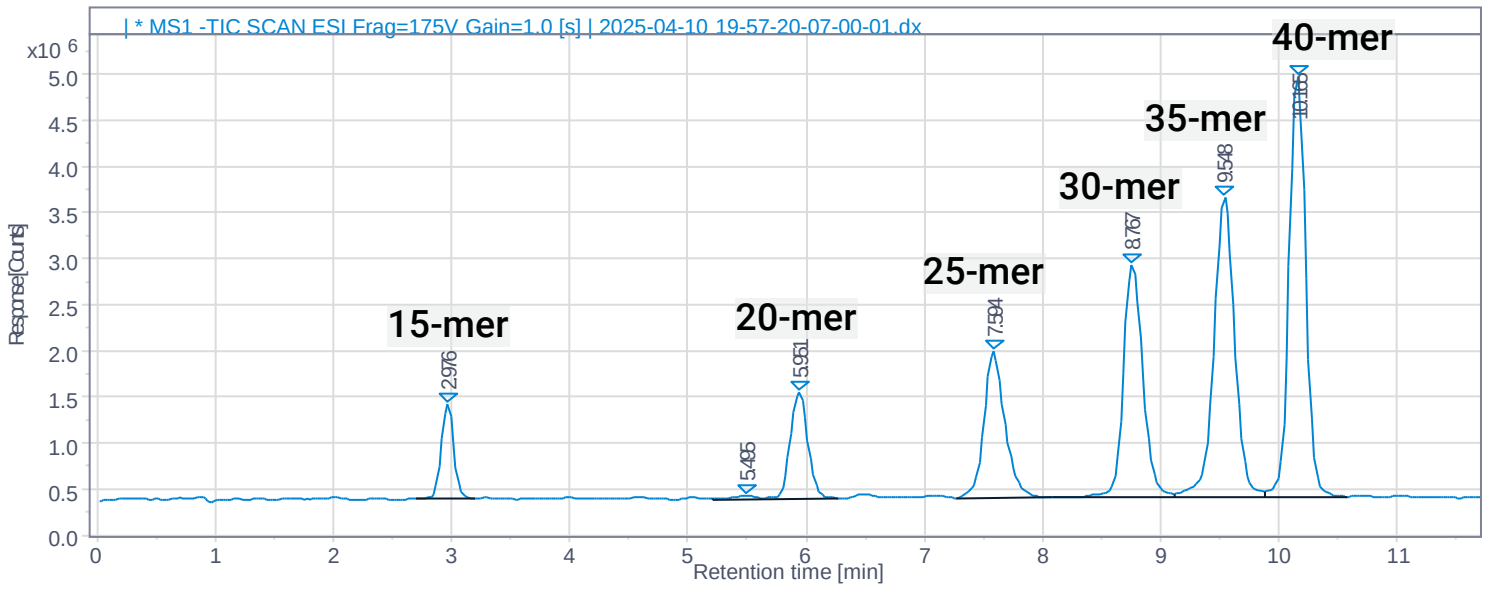
Agilent DNA ladder standard
(15, 20, 25, 30, 35, and 40-mer; Part No. 5190-9029)

Custom 103-mer oligonucleotide

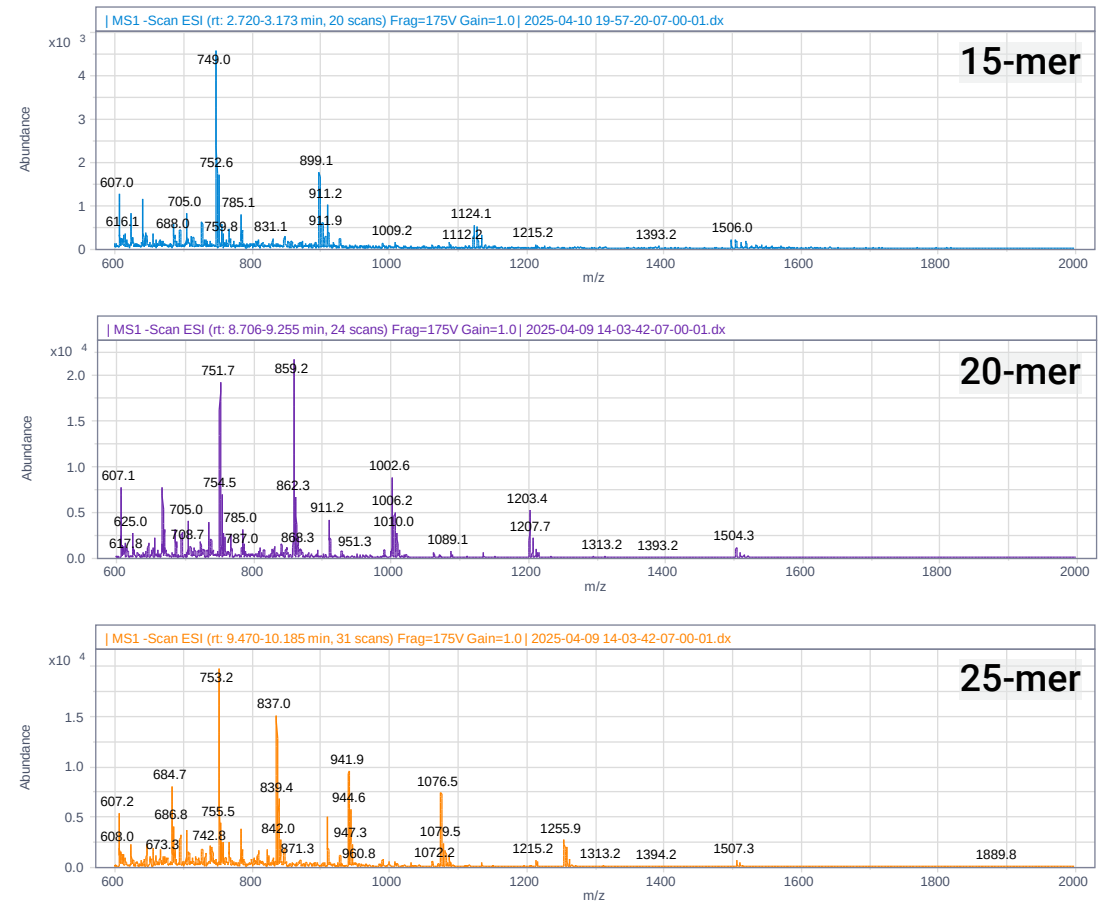
Givosiran Standard

Results and Discussion

DNA ladder standard, 1 mL, with 15, 20, 25, 30, 35, and 40-mer oligos

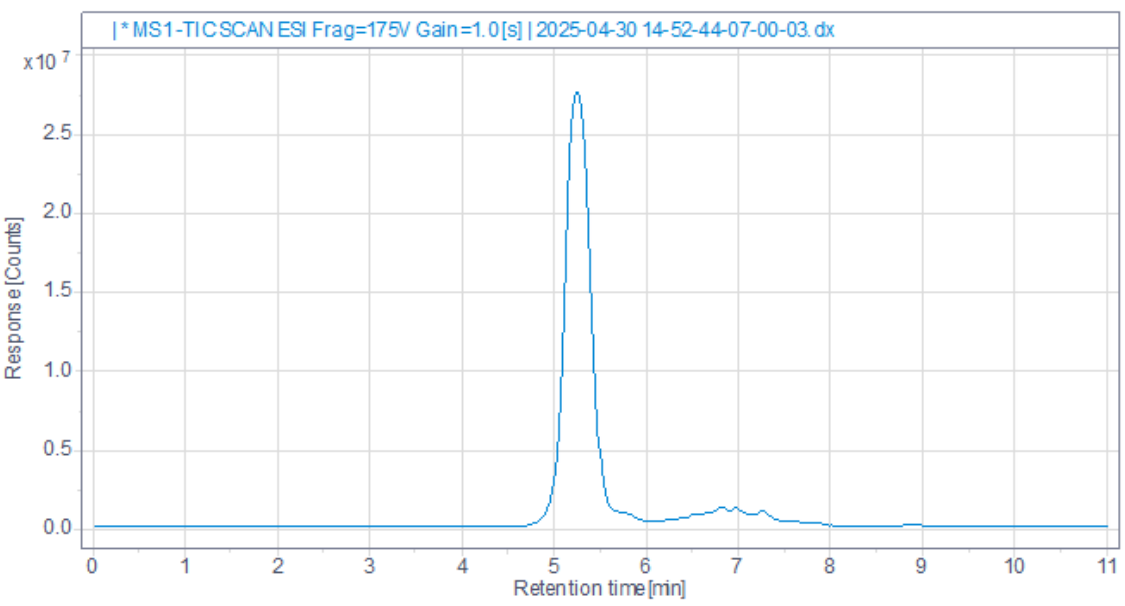


DNA Ladder	Expected Mass	Measured Mass	Delta Mass (Da)
15mer	4501.0	4500.5	0.5
20mer	6022.0	6021.5	0.5
25mer	7543.0	7542.5	0.5
30mer	9063.9	9063.1	0.8
35mer	10584.9	10584.5	0.4
40mer	12105.9	12105.4	0.5

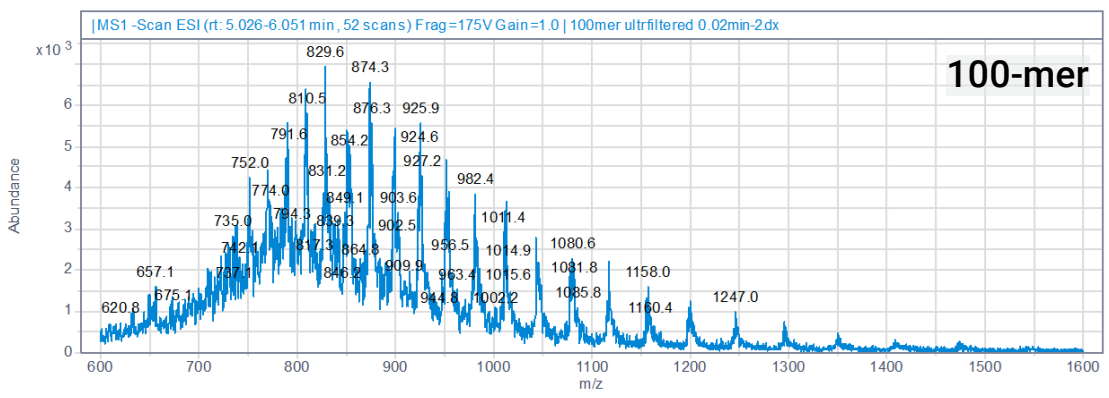


100-mer Oligonucleotide Standard

A custom 103-mer oligonucleotide crude sample of unknown sequence (mass known) was analyzed, demonstrating excellent chromatographic peak shape and generation of mass spectrum.



	Expected Mass	Measured Mass	Delta Mass (Da)
103-mer	32394.3	32394.5	0.2

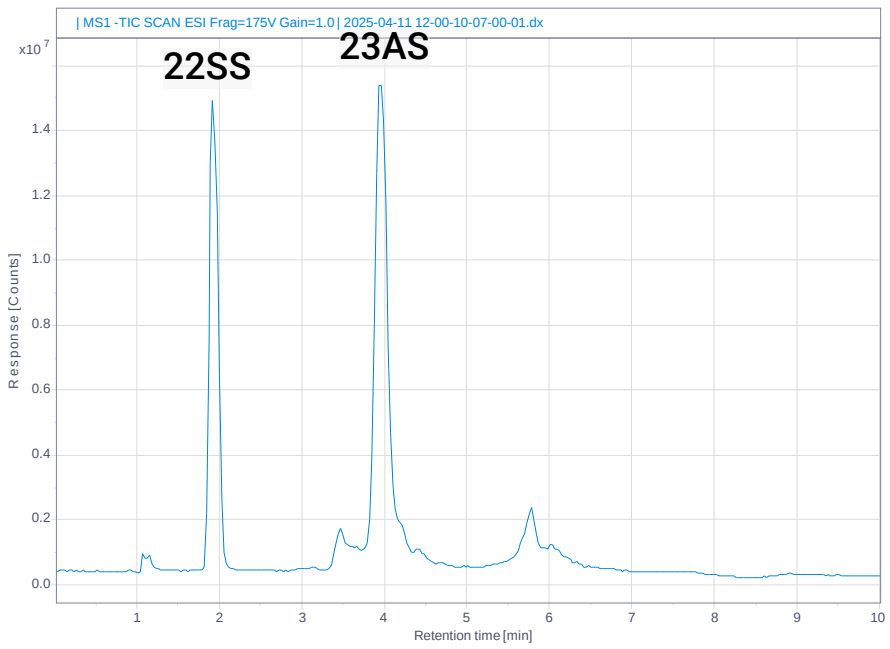


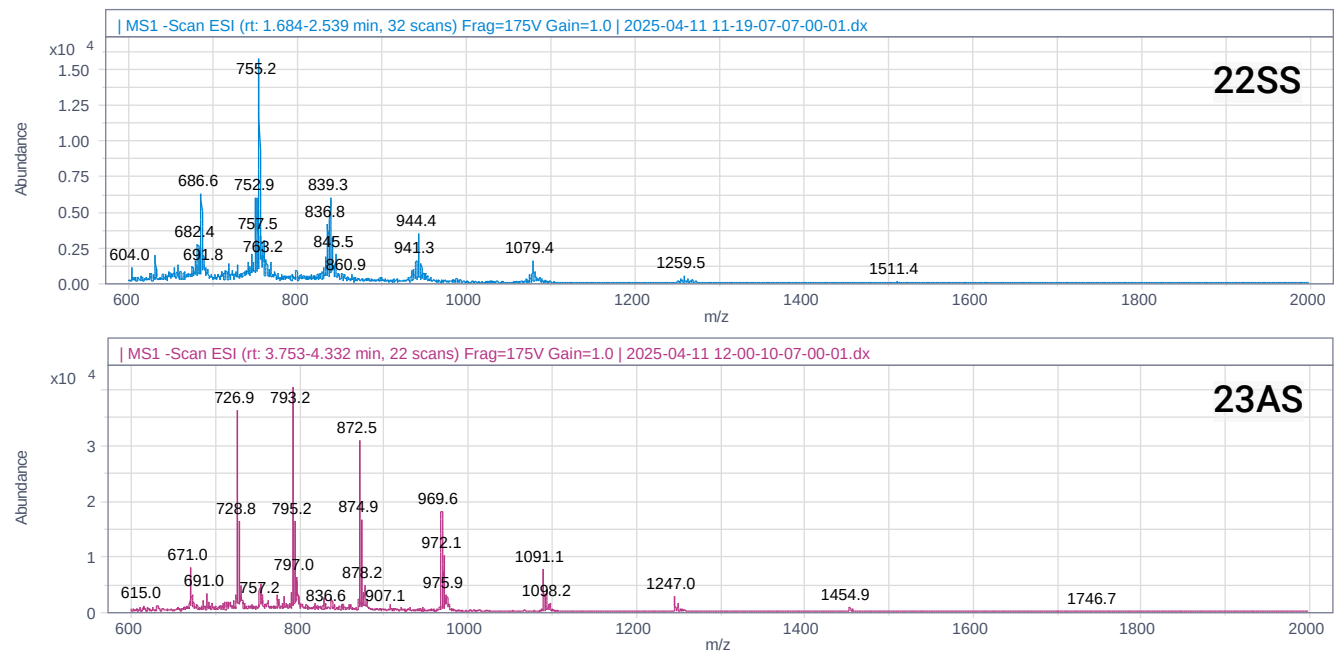
Results and Discussion

Givosiran (Givlaari) a siRNA therapeutic oligonucleotide

Givosiran is an N-acetylgalactosamine (GalNAc)-conjugated siRNA targeting aminolevulinate synthase 1 (ALAS1). This drug contains two “tagged” oligonucleotide strands, sense (SS) and antisense (AS).

Strand	Sequence	Expected Mass	Measured Mass	Delta Mass (Da)
22SS	mC*mA*mGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA/L96/	7563.84	7562.97	0.87
23AS	mU*mG*mGfUmCfUmUfUfCmUfCfAmCfAmGfAmGfUmAmGfA*fA*mU	8736.50	8735.97	0.53





Solvent Consumption Comparisons

A major advantage to microflow based chromatography is the significant reduction in consumption of organic solvents, HFIP, and TEA. Based on the comparison of methods below.

This method results in 220x less (>99.5% reduction) of Methanol, HFIP, and TEA consumption compared to a conventional method.

LC Param.	Microflow Method	Standard Flow Method [2]
Mobile Phase A	100 mM HFIP and 15 mM TEA in water	100 mM HFIP and 15 mM TEA in water
Mobile Phase B	Methanol	Methanol
Flow Rate	2 µL/min	500 µL/min
Injection Volume	250 nL	2 µL
Gradient Program	Time (min) %B 0.0 20 10.0 27 11.0 95 12.0 95 12.1 20	Time (min) %B 0.0 20 10.0 27 11.0 95
Runtime	12-17 minutes	11-15 minutes
Calculation	$12\ \text{min} \times \frac{2\ \mu L\ Solvent}{min} = 24\ \mu L$ $17\ \text{min} \times \frac{2\ \mu L\ Solvent}{min} = 34\ \mu L$	$11\ \text{min} \times \frac{500\ \mu L\ Solvent}{min} = 5500\ \mu L$ $15\ \text{min} \times \frac{500\ \mu L\ Solvent}{min} = 7500\ \mu L$
Solvent Use	**24-34 µL per run**	**5500-7500 µL per run**

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Conclusions

- A standard flow oligonucleotide analysis method containing HFIP and TEA was transferred to microflow chromatography using the Axcend Focus LC.
- This method resulted in a 220x-less consumption (>99.5% reduction) of Methanol, HFIP, and TEA consumption compared to a conventional method.
- This method was able to chromatographically resolve the DNA Ladder Standard (15-40mer oligonucleotides) and custom 103-mer oligonucleotide standard, producing a clean mass spectrum for each analyte.
- This analysis was applied to real sample, Givosiran. Both Sense (SS) and Antisense (AS) components of the drug provided correct MW assignment
- Mass spectral deconvolution resulted in correct MW assignments. ~Δ0.5 Da from theoretical values.

References

[1]Advancements in the characterisation of oligonucleotides by high performance liquid chromatography-mass spectrometry in 2021: A short review (10.1002/ansa.202100066)

[2]Molecular Weight Confirmation of Oligonucleotides Using Agilent LC/MSD XT and OpenLab CDS (5994-7083EN)

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