SHIMADZU

MP 534

Practical LC–MS Workflows for siRNA and sgRNA Characterization Using a Single-Quadrupole Mass Spectrometer

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1. Introduction

The development of oligonucleotide therapeutics has created a growing demand for robust analytical methods for siRNA and sgRNA characterization. With the higher molecular weight and the need to characterize siRNA in both its native duplex form and its dissociated single-strand components, distinct chromatographic and mass spectrometric settings are needed. This work describes practical single quadrupole LC–MS workflows (**Fig 1**) for the analysis of siRNA and sgRNA using a single-quadrupole mass spectrometer, with emphasis on method flexibility, data clarity, and suitability for routine analytical environments.

Fig. 1 Instrumentation and data processing setup

2. Methods

Table 1 Oligonucleotide sample sequence and information

Sample	Sequence	Molecular Weight
sgRNA (100-mer)	G*-sA*-sU*-sGr-pAr-pUr-pCr-pUr-pCr-pUr-pCr-pAr-pAr-pCr-pUr-pUr-pUr-pAr-pAr-pCr-pGr-pUr-pUr-pUr-pGr-pAr-pGr-pCr-pUr-pAr-pGr-pAr-pAr-pAr-pUr-pAr-pGr-pCr-pAr-pAr-pGr-pUr-pUr-pAr-pAr-pAr-pAr-pUr-pAr-pAr-pGr-pGr-pCr-pUr-pAr-pGr-pUr-pCr-pCr-pGr-pUr-pUr-pAr-pUr-pCr-pAr-pAr-pCr-pUr-pUr-pGr-pAr-pAr-pAr-pAr-pAr-pGr-pUr-pGr-pGr-pCr-pAr-pCr-pCr-pGr-pAr-pGr-pUr-pCr-pGr-pGr-pUr-pGr-pCr-sU*-sU*-sU*-pUr	32275.7
siRNA (21-mer)	Ar-pUr-pGr-pGr-pAr-pAr-pmUr-pAr-pCr-pUr-pCr-pUr-pUr-pGr-pGr-pUr-pmUr-pAr-pCr-pTd-pTd Gr-pmUr-pAr-pAr-pmCr-pmCr-pAr-pAr-pGr-pAr-pGr-pmUr-pAr-pmUr-pmUr-pmCr-pmCr-pAr-pmUr-pTd-pTd	Sense: 6764.3 Antisense: 6660.1 Duplex: 13424.4

Nucleobase		Ribose		Linker	
G	Guanine	*	methoxy	s	phosphorothioate
A	Adenine	r	hydroxy	p	phosphate
U	Uracil	d	deoxy		
C	Cytosine			Base Modification	
T	Thymine			m	methyl

Samples were purchased from Integrated DNA Technologies (**Table 1**). All analyses were performed using Nexera XS^{TM} inert LC coupled with LCMS-2050 with extended mass range. Impurity profiling and synthesis confirmation were performed on Insight Biologics (ver.2.2). Chromatographic settings and MS acquisition parameters are shown in **Table 2**.

Table 2 Chromatographic and MS acquisition parameters

Inert LC condition	
Column	Shimadzu Shim-pack Scepter Claris C18-300 (150 mm x 2.1 mm I.D., 1.9 µm)
Mobile Phase Conditions	
TEAA (Triethylammonium Acetate)	(A) 30 mM HFIP/10mM TEAA/1.2mM TEA in water (B) 10 mM TEAA in 50% ethanol: 50% water
TBuAA (Tributylammonium Acetate)	(A) 5mM TBuAA/1µM EDTA in 10% acetonitrile: 90% water (B) 5mM TBuAA/1µM EDTA in 80% acetonitrile: 20% water
HAA (Hexylammonium Acetate)	(A) 100 mM HAA in water (B) 50% acetonitrile: 50% 100mM HAA in water
HFIP/TEA	(A) 50 mM HFIP 2.1 mM TEA in water (B) 50% methanol: 50% 50 mM HFIP 2.1 mM TEA in water
Flowrate	0.2 mL/min
Mode	Gradient mode
Sample	diluted in water
PDA detection	260 nm (SPD-M40, UHPLC inert cell)
MS Condition	
Ionization	ESI/APCI (DUIS), negative mode
Interface Voltage	-2.0 kV
Mode	Scan (profile mode) 500-3000 *m/z*
Nebulizing gas flow	2.0 L/min
Drying gas flow	5.0 L/min
Heating gas flow	7.0 L/min
Desolvation Temp.	450 °C
DL Temp.	200 °C

3. Results and Discussion

3.1. sgRNA analysis

As an example of synthesis confirmation, an sgRNA sample was analyzed using a nominal-mass single-quadrupole LC–MS system with extended mass range up to 3000 *m/z*. Two ion-pairing mobile phase conditions were tested, TEAA/TEA/HFIP and TBuAA mobile phases. The TBuAA mobile phase produced the expected shift toward lower charge states (higher *m/z*, **Fig 2**). However, the magnitude of this charge-state shift was modest. The Quick Picking algorithm on Insight Biologics calculated the molecular mass of the sgRNA as 32,275.06 Da, corresponding to a mass error of −0.66 Da (**Fig 2**). In repeated measurements, mass error was typically under 1 Da under both mobile phase condition.

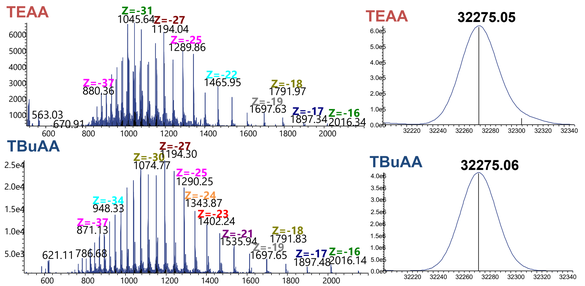

Fig. 2 Mass spectrum and deconvoluted result of sgRNA under two different mobile phase composition.

3.2. siRNA analysis

siRNA forms as a double-stranded molecule. For quality control, two analytical approaches are commonly used: a denaturing method that dissociates the duplex to analyze the sense and antisense strands separately, and a non-denaturing method that analyzes the duplex intact. By keeping the column oven temperature low (e.g. < 28°C), duplex was the predominate peak (**Fig. 3**). By utilizing the extended mass range on the LCMS-2050, the non-denatured duplex can be observed (**Fig. 3**).

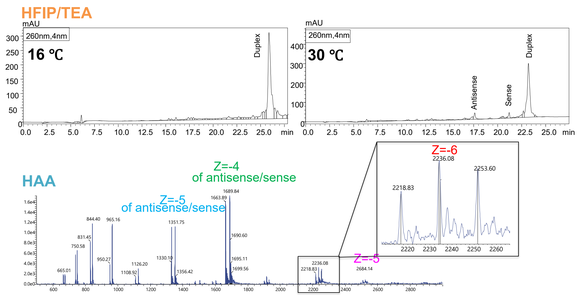

Fig. 3 UV chromatogram of siRNA duplex analysis under different oven temperature (top). Mass spectrum of siRNA duplex (bottom).

Different ion-pairing agents were tested as mobile phase additives (**Table 2**) for optimal chromatographic separation and increased accuracy of impurity identification utilizing single-quadrupole unit resolution LC-MS. Separation optimization was conveniently achieved using LabSolutions MD, which can automatically build optimization methods, rank robust separation conditions, and generate a design space. Among the ion-pairing agents tested, TBuAA and HAA has produce lower charge state for siRNA. Insight Biologics software was able to deconvolute and quickly report impurity profiling of the single stranded siRNA (**Fig 4**).

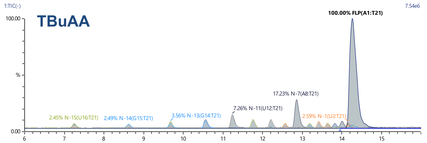

Fig. 4 TIC of the siRNA sense-strand under TBuAA mobile phase, which the impurities were automatically calculated and displayed.

Adduct formation (e.g. HFIP, HA) while using ion-pairing agents are common. An optimized ion source parameters on the LCMS-2050 minimizes the adduct formation leading to increased total ion counts and accurate deconvoluted results (**Fig. 5**).

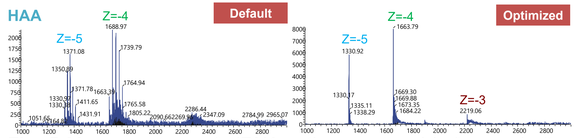

Fig. 5 Mass spectrum of siRNA antisense-strand before and after ion source optimization.

4. Conclusion

- ✓ Single-quad LC-MS with extended mass range delivered sufficient mass accuracy for MW confirmation of both sgRNA and siRNA.
- ✓ Optimized oven temperature, mobile phase, and ion source parameters allowed siRNA duplex to remain intact through the MS.
- ✓ Combining LabSolutions MD and Insight Biologics, a flexible and simple workflow can be used for oligonucleotide analysis.

The authors are affiliated and funded by Shimadzu Corporation.