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Application News

LCMS-2050 Liquid Chromatograph Mass Spectrometer

Oligonucleotide Characterization for Quality Control on the Shimadzu Single Quad Mass Spectrometer LCMS-2050

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User Benefits

- The Shimadzu LCMS-2050 single quadrupole mass spectrometer and SPD-M30A photodiode array detector offer a straightforward workflow for quantitating oligonucleotides, streamlining the analysis process.
- Confident mass confirmation can be obtained over a wide mass range of nucleotide oligomers.
- The Shimadzu LCMS-2050 enables the detection and identification of lower levels of impurities commonly found in synthetic oligomers.

■ Introduction

Oligonucleotide therapeutics have garnered increased attention in recent years as an innovative class of treatments in immunology, virology, and RNA-based therapies. It is crucial to differentiate between full-length and truncated nucleotides, as well as modified and unmodified versions for effective quality control. Traditional hybridization techniques, such as ELISA (enzyme-linked immunosorbent assay) and qPCR (polymerase chain reaction), offer sensitivity in detection, but lack specificity for effective impurity analysis. Liquid chromatography-mass spectrometry (LC-MS) is an advanced technique with inherent specificity that can be utilized to analyze impurities, degradants, and other biological/chemical modifications that cannot be analyzed by hybridization techniques.

Mass spectrometry is commonly perceived as complex, often requiring specialized expertise for routine quality control applications. The Shimadzu single quadrupole LCMS-2050 is a user-friendly instrument capable of acquiring reliable and sensitive data with simplified operation. The automatic setting of the sampling rate from peak width and number of data points enables users of any experience level to generate quality data by streamlining method development. Developing a robust workflow for accurately determining intact mass and quantitation of synthetic oligomers is essential for maintaining high-quality products.

The LCMS-2050, with a mass range of *m/z* 2-2000, is an ideal instrument to address this challenge by facilitating data acquisition over multiple charge states. Utilization of PDA (photodiode array) detection in series with MS can enhance quantitation accuracy due to the strong absorption of nucleic acids at 260nm.

This application note outlines a workflow for confident mass confirmation across the entire range of nucleotide length oligomers (10-60-mer), along with quantitation of yield using both UV and mass spectrometry data. The acquired data from these workflows were analyzed considering impurities, including aborted sequences (N-1, N-2) and mobile phase adducts.

Shimadzu LCMS-2050 Single Quadrupole LC/MS

■ Experimental

Sample Preparation

Custom designed single-stranded DNA oligomers, with lengths ranging from 10-90-mer nucleotides, as shown in **Table 1**, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) with standard desalting purification. All oligomers were reconstituted to a stock concentration of 100 µM and stored at -20 °C when not in use.

Analytical Conditions

All oligomers were eluted with ion pairing, reversed phase conditions using HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) reagent in the mobile phase. Instrument parameters are shown in **Table 2**. A shallow gradient was used to separate the main peak of interest from other interfering peaks. Estimates of impurities were detected by MS as some impurities, such as N-1 aborted sequences, are difficult to separate by chromatography. A steep gradient was used for mass confirmation for the oligonucleotides to allow for quick elution over a large mass range. The MS source conditions (gas flow, temperature, and voltages) were optimized to obtain higher signal intensity for oligomers and to keep the common HFIP adduct abundance low.

Calibration

The 30-mer oligonucleotide calibration curve was prepared by diluting the stock in nucleus free water to 0.1, 0.2, 0.5,1, 2, 5, 10, 20, 30, 40, 50 µM concentrations to create a 10-point calibration curve. Quantitation by MS was performed by selected ion monitoring (SIM) corresponding to the -10-charge state and quantitation by UV was performed by absorbance at 260 nm.

Mass Confirmation

Mass confirmation was analyzed for all oligonucleotides. Triplicate injections of 50 pmol mass on column was performed to verify reproducibility of mass confirmation.

Impurity Analysis

Impurity analysis was performed on the 10-mer oligonucleotide at 50 pmol mass on column.

Instrumentation

Samples were analyzed in series using a photodiode array detector (SPD-M30A) and single quadrupole mass spectrometer (LCMS-2050) coupled to a Nexera UHPLC capable of pressures up to 15,000psi. A Shimadzu Scepter Claris column with bioinert surface treatment on the body and frit was used for analysis.

Table 1: Analyzed oligonucleotides.

Length	Sequence (DNA, 5' -3')	Average mass (Da)
10-mer	CACTGAATAC	2996.0
15-mer	ACCTGAATACCAATA	4529.0
20-mer	TCATCACACTGAATACCAAT	6029.0
25-mer	CTATACCGCTGAATACCAATCACTG	7570.0
30-mer	ACACTGAATACCAATCACTGAATACTACGC	9112.0
35-mer	TCACACTCATGAATACCAATCACTGAATACCAATA	10620.0
40-mer	ACACTGAATACCAATTGACACATACTACGCTGAACACTGA	12210.1
45-mer	ACAAATCTGAATACCAATCACCGCTGAATACTATGAACACTGACC	13719.0
50-mer	TCATCACACTGAATACCAATCACTGAATACCAATACACTGAATACCAATA	15211.0
60-mer	TCAACCTCAATACCAATCACTCACTGAGAATACCAATACACTGAATACCAATAGAATAAT	18293.1

Table 2: Oligonucleotide analysis instrument parameters.

Liquid Chromatography (LC) Conditions			
Column	Shimadzu Scepter-Claris C18 120 (150mm x 2.1mm I.D x 1.9µm)		
Mobile Phase A	1% (95mM) HFIP, 0.1% (4.3 mM) TEA in water		
Mobile Phase B	1% (95 mM) HFIP, 0.1% (4.3 mM) TEA in 50% MeOH		
Injection Volume	0.5 µL (mass confirmation and impurity), 1 µL (calibration)		
Column Temperature	50 °C		
Autosampler Temperature	5 °C		
Gradient (Quantitation)	Time (min)	Flow (mL/min)	B%
	0.40	0.4	10
	4.65	0.4	24
	4.90	0.4	24
	4.91	0.4	99
	4.92	0.45	99
	6.40	0.45	99
	6.41	0.45	10
	6.42	0.4	10
	8.40	0.4	10
Gradient (Mass Confirmation)	Time (min)	Flow (mL/min)	B%
	0.0	0.4	1
	0.5	0.4	1
	6.5	0.4	99
	8.0	0.4	99
	8.01	0.4	1
	10.0	0.4	1
MS Conditions			
Mode	Scan (mass confirmation) SIM (quantitation)		
Mass Range	550-2000 *m/z*		
Ionization	ESI/APCI (DUIS) (-) mode		
Event Time	0.4 sec (full scan), 0.013 sec (SIM)		
Cycle	0.5 sec		
Detector Voltage	1.3 kV		
Nebulizing Gas Flow	2.0 L/min		
Desolvation Temperature	450 °C		
Desolvation Line Temperature	200 °C		
Qarray Voltage	-20V (Auto)		
PDA Conditions			
Wavelength	260 nm		
Slit Width	1 nm		

■ Results and Discussion

Quantitation by UV and MS

The LCMS-2050 has an extended mass range (*m/z* 2-2000), proving to be beneficial in detecting a range of oligomers with multiple charge states. All the oligomers, ranging from 10 to 60-mer, were injected separately with extended gradient elution. The mass detector with SIM events and UV detector were used for quantitation and mass confirmation.

A linear relationship with an R^2 value of 0.998 was observed for the 30-mer oligonucleotide from 0.1 to 50 µM with absorbance at 260 nm (as depicted in **Figure 1A**). The MS-based calibration curve, generated using the peak area from a SIM scan event for the average mass of the -10-charge state from the intact oligonucleotide, also exhibits a strong fit with an R^2 value of 0.999 (shown in **Figure 1B**). Both the UV and MS calibration curves demonstrated accuracy levels within 30%.

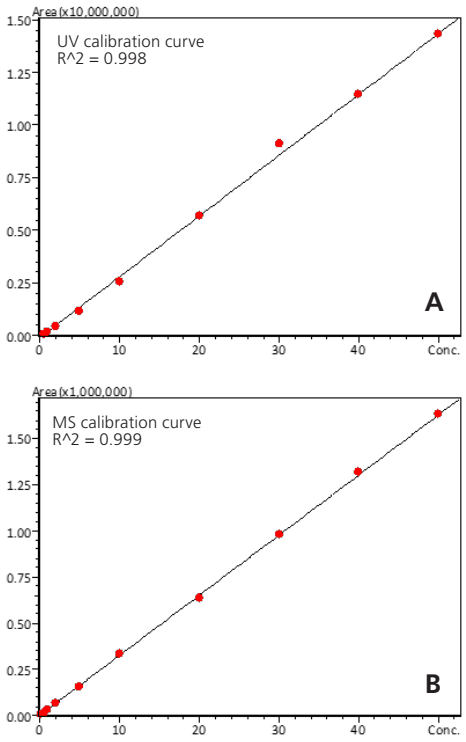

Figure 1: A) UV calibration curve with a linear dynamic range from 0.1-50 µM as measured by peak area at 260 nm. (B) MS calibration curve with a linear dynamic range from 0.5-50 µM as measured by peak area of the -10-charge state (910.2 *m/z*) from the intact oligonucleotide.

Mass Confirmation

Oligomers ranging from 10-60-mer were injected in triplicate at 50 pmol for mass confirmation. Multiple charge states were observed between *m/z* 550-2000 and the most abundant charge state was chosen for each oligomer as shown in **Table 3**. As a single quadruple mass spectrometer is a low-resolution detector, average mass was used for mass confirmation. The data shown in **Table 3** indicates that mass accuracy is ≤0.2 Da for all analyzed oligonucleotides.

The standard deviation (SD) and Relative Standard Deviation (RSD) of observed *m/z* are less than 0.080 and 0.01, respectively, for oligomers ranging from 10-45-mer in length. For longer oligomers, specifically 50-60-mer, the SD and RSD are 0.15 and 0.03, respectively. The overlay of the extraction ion chromatogram (EIC) of the most abundant charge state for each oligomer is shown in **Figure 2**.

Table 3: Mass confirmation for 10-60-mer oligonucleotides using respective high-abundance charge state.

Nucleotide Length	Most abundant charge state (*m/z*)	Theoretical *m/z*	Observed *m/z*			Mean (n=3)	Mass Accuracy	Std. Dev (n=3)	RSD (%)
			run 1	run 2	run 3				
10-mer	-3	997.7	997.6	997.6	997.6	997.6	0.1	<0.01	<0.01
15-mer	-6	753.8	753.8	753.8	753.8	753.8	0.0	<0.01	<0.01
20-mer	-9	668.9	668.9	668.9	668.9	668.9	0.0	<0.01	<0.01
25-mer	-10	756.0	755.9	755.9	756.0	755.9	0.1	0.05	0.01
30-mer	-13	699.9	699.9	699.9	699.9	699.9	0.0	<0.01	<0.01
35-mer	-14	757.6	757.5	757.6	757.6	757.5	0.0	0.04	<0.01
40-mer	-16	762.1	762.1	762.1	762.2	762.1	0.0	0.05	0.01
45-mer	-18	761.2	761.2	761.2	761.3	761.2	0.0	0.08	0.01
50-mer	-19	799.6	799.5	799.8	799.6	799.6	-0.1	0.15	0.02
55-mer	-23	724.5	724.5	724.3	724.5	724.4	0.1	0.12	0.02
60-mer	-23	794.3	793.9	794.3	794.1	794.1	0.2	0.20	0.03

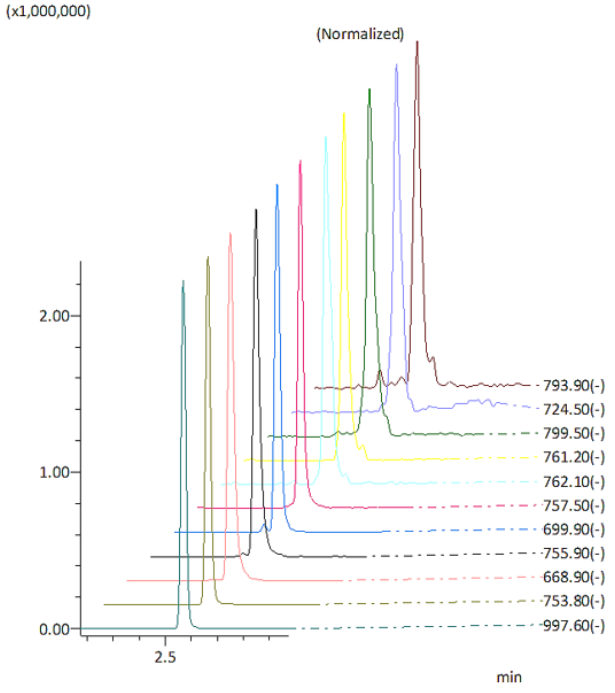

Figure 2: Overlay of replicate 1 EIC of the most abundant charge state for each oligomer from 10-60-mer, represented in **Table 3**.

Impurity Analysis

Potential impurities, such as aborted sequences (N-2, N-3) and solvent adducts, were identified using MS SCAN data from 550-2000 *m/z*. Impurities were identified using extracted ion chromatograms (EIC) with a 100-ppm width. **Figure 3** shows EICs of those impurities and solvent adducts identified for -3 charge state of the 10-mer. Only low levels of N-2 and N-3 were observed. (**Figure 3B** and **3C**, respectively). However, higher abundance of sodium salt adducts $[M+Na^{+}-3H]^{-2}$, (**Figure 3D**) and potassium salt adducts $[M+K^{+}-3H]^{-2}$, (**Figure 3E**) were identified. Trace levels of HFIP adducts from the mobile phase additive were observed (**Figure 3F**) as result of ion source optimization prior to analysis.

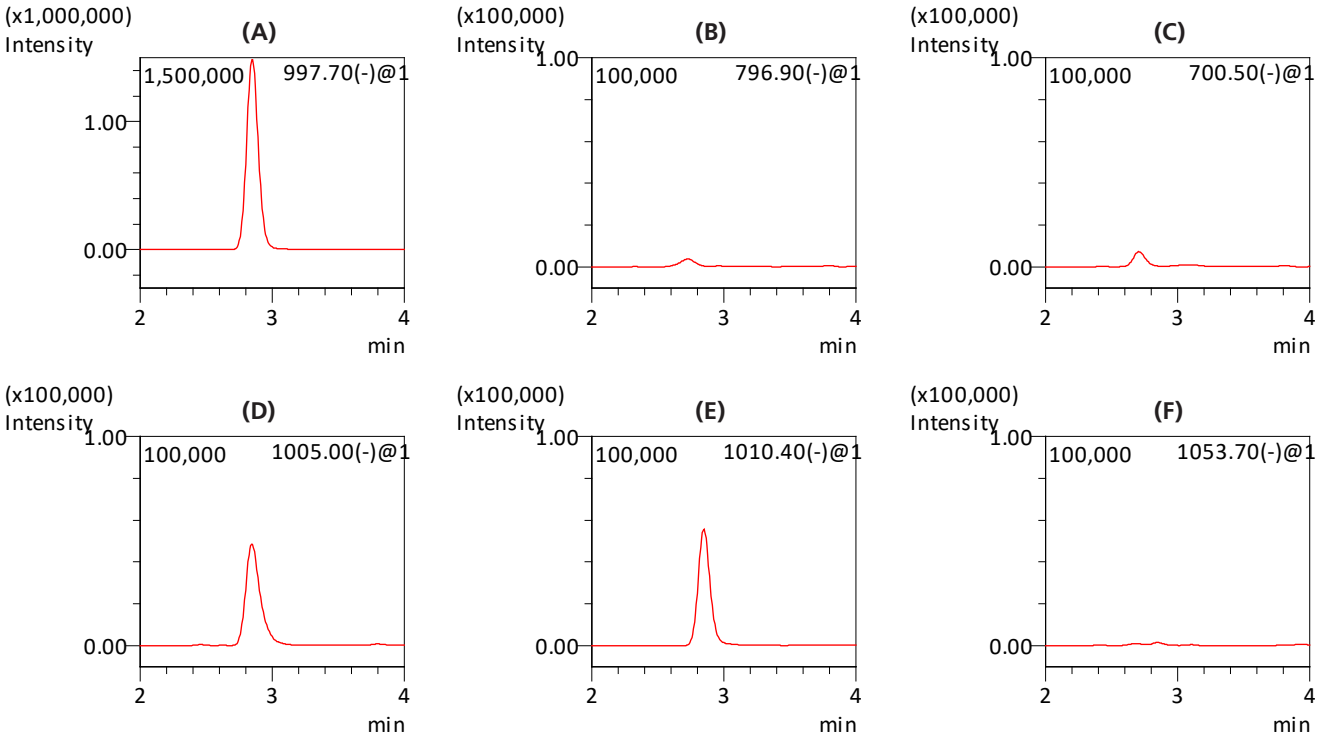

Figure 3: Observed impurities shown in EIC for - 3 charge state of 10-mer.
(A); $[M-3H]^{-3}$, m/z 997.7
(B); -3 charge state of [N-2] 5′ aborted sequence, $[M-3H]^{-3}$ m/z 796.9
(C); -3 charge state of [N-3] 5′ aborted sequence $[M-3H]^{-3}$, m/z 700.5
(D); $[M+Na+-3H]^{-2}$, m/z 1005.0
(E); $[M+K+-3H]^{-2}$, m/z 1010.4
(F); $[M+K+-3H]^{-2}$, m/z 1053.7

■ Conclusion

The Shimadzu single quadrupole LCMS-2050 coupled with a Nexera LC unit with an SPD-M30A PDA detector offers simple and reliable MS and UV-based quality control analysis for oligonucleotides.

MS data shows that a LCMS-2050 can achieve higher mass accuracy for a wide range of oligonucleotides. Additionally, excellent quantitation accuracy is achievable in both MS and UV based analysis under optimized source conditions.

ULTRA FAST MASS SPECTROMETRY


LCMS-8040


LCMS-8045


LCMS-8050


LCMS-8060NX


LCMS-2020


LCMS-2050


Q-TOF LCMS-9030/9050

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