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Oligonucleotide Characterization for Quality Control on the Shimadzu LCMS-2050

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1. Introduction

Nucleic acid-based therapeutics have garnered increased attention in recent years as an innovative class of treatments. 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), offers sensitivity in detection, but lacks 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.

In this poster, we employed a single quadrupole mass spectrometer (**Fig. 1**) for confident mass confirmation and quantitation of impurity levels. Utilization of PDA (photo diode array) detection in series with MS enhances quantitation accuracy due to the strong absorption of nucleic acids at 260nm. The acquired data from these workflows were analyzed considering impurities, including aborted sequences (N-1, N-2) and mobile phase adducts.

Fig. 1. Shimadzu LCMS-2050

2. Methods

Custom designed single-stranded DNA oligomers with lengths ranging from 10-60 nucleotides (**Table 1)** were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) with standard desalting purification. All oligomers were reconstituted to a stock concentration of 100 µM and further diluted in nucleus free water for calibration.

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. The analytical conditions chosen for this analysis are listed in **Table 2** and **Table 3**.

Table 1. Analyzed oligonucleotides.

Length (nt)	Sequence	Avg. mass (Da)
10mer	CACTGAATAC	2996.0
15mer	ACCTGAATACCAATA	4529.0
20mer	TCATCACACTGAATACCAAT	6029.0
25mer	CTATACCGCTGAATACCAATCACTG	7570.0
30mer	ACACTGAATACCAATCACTGAATACTACGC	9112.0
35mer	TCACACTCATGAATACCAATCACTGAATACCAATA	10620.0
40mer	ACACTGAATACCAATTGACACATACTACGCTGAACACTGA	12210.1
45mer	ACAAATCTGAATACCAATCACCGCTGAATACTATGAACAC TGACC	13719.0
50mer	TCATCACACTGAATACCAATCACTGAATACCAATACACTG AATACCAATA	15211.0
60mer	TCAACCTCAATACCAATCACTCACTGAGAATACCAATACA CTGAATACCAATAGAATAAT	18293.1

Table 2. HPLC parameters

Column	Shimadzu Scepter-Claris C18 120 (150mm x 2.1mm I.D x 1.9µm)
Mobile Phases	A) 1% HFIP, 0.1% TEA in water B) 1% HFIP, 0.1% TEA in MeOH
Gradient Program (Quantitation)	B 10% (0-0.4min); B 24% (0.4-4.65min); B 24% (4.65-4.90min); B 99% (4.91 6.40min); B 10% (6.41-9.0min)
Gradient Program (Mass Confirmation)	B 1% (0-0.5min); B 99% (0.5-6.5min); B 99% (6.5-8.0min); B 1% (8.10-10.0 min)
Flow Rate	0.4 ml/min
Column Temp.	50ºC
Injection Volume	0.5µL (mass confirmation), 1 µL (calibration)

Table 3. LC-MS/MS parameters

Ionization	ESI/APCI (DUIS) (-) mode
Mode	SCAN (Mass Confirmation) SIM (Quantitation)
Mass Range	550-2000 *m/z*
Event Time	0.4 sec (SCAN), 0.013 (SIM)
Cycle Time	0.5 sec
Interface Voltage	-2.0 kV
Nebulizing Gas Flow	2 L/min
DL Temp.	250ºC
Desolvation Temp.	500ºC
Qarray Voltage	-20 V
Detector Voltage	1.3 kV

3. Results

A linear relationship with an R^2 value of 0.998 was observed for the 30mer oligonucleotide from 0.1 to 50 µM with absorbance at 260 nm (**Figure 2**, left). The MS-based calibration curve, generated using the peak area from a SIM 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 (**Figure 2**, right). Both the UV and MS calibration curves demonstrated accuracy levels within 30%.

Oligomers ranging from 10-60 nucleotides were injected in triplicate at 50 pmol mass on column 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 a single quadrupole mass spectrometer is a low-resolution detector, average mass was used for mass confirmation. The data shown in **Table 4** indicates that mass accuracy is ≤0.2Da for all analyzed oligonucleotides. The overlay of extracted to ion chromatograms (EIC) of the most abundant charge state of each oligomer is shown in **Figure 3.**

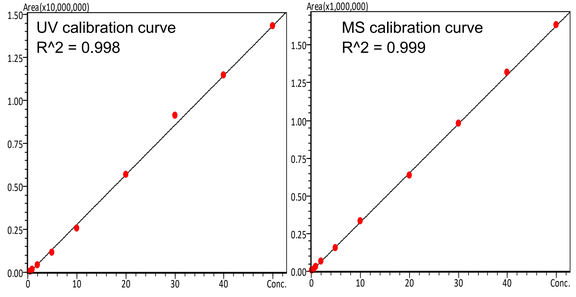

Figure 2. (left). UV calibration curve with linear dynamic range from 0.1- 50 µM as measured by peak area at 260nm. (right) MS calibration curve with linear dynamic range from 0.5- 50 µM as measured by peak area of the -10-charge state (910.2 *m/z*) from the intact oligonucleotide.

Table 4. Mass confirmation for 10-60mer oligonucleotides using respective high-abundance charge state.

Nucleotide Length	Abundant charge state (z)	Theoretical *m/z*	Observed *m/z*			Mean (n=3)	Mass Accuracy	Std. Dev (n=3)	RSD (%)
			run 1	run 2	run 3				
10	-3	997.7	997.6	997.6	997.6	997.6	0.1	0.00	0.00
15	-6	753.8	753.8	753.8	753.8	753.8	0.0	0.00	0.00
20	-9	668.9	668.9	668.9	668.9	668.9	0.0	0.00	0.00
25	-10	756.0	755.9	755.9	756.0	755.9	0.1	0.05	0.01
30	-13	699.9	699.9	699.9	699.9	699.9	0.0	0.00	0.00
35	-14	757.6	757.5	757.6	757.6	757.5	0.0	0.04	0.00
40	-16	762.1	762.1	762.1	762.2	762.1	0.0	0.05	0.01
45	-18	761.2	761.2	761.2	761.3	761.2	0.0	0.08	0.01
50	-19	799.6	799.5	799.8	799.6	799.6	-0.1	0.15	0.02
55	-23	724.5	724.5	724.3	724.5	724.4	0.1	0.12	0.02
60	-23	794.3	793.9	794.3	794.1	794.1	0.2	0.20	0.03

Potential impurities such as aborted sequences (N-2, N-3) and solvent adducts were identified using extracted ion chromatograms (EIC) from 550-2000 m/z. Impurities were extracted to ion chromatograms (EIC) within the 100 ppm range as shown in. **Figure 4.**

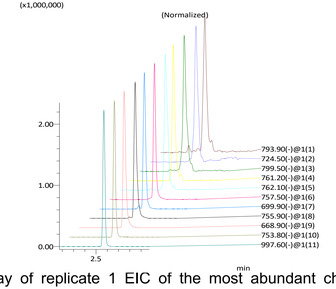

Figure 3. Overlay of replicate 1 EIC of the most abundant charge state for each oligomer from 10-60mer

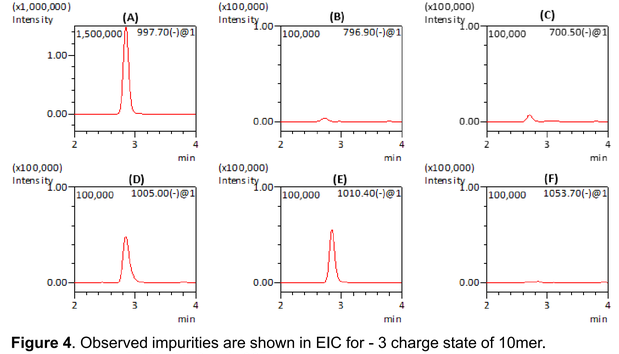

Figure 4. Observed impurities are shown in EIC for - 3 charge state of 10mer.
A; [M-3H]-3, m/z 997.7.
B; -3 charge state of [N-2] 5' aborted sequence, [M-3H]-3 m/z 796.9.
C; -3 charge state of [N-3] 5' aborted sequence [M-3H]-3, m/z 700.5.
D; [M+Na+-3H]-2, m/z 1005.0.
E; [M+K+-3H]-2, m/z 1010.4
F; [M+K+-3H]-2, m/z 1053.7.

4. Conclusion

- Single quadrupole MS detector coupled with UV detector offers simple and reliable MS and UV based quality control analysis for oligonucleotides.
- MS data shows a single quadrupole detector can achieve higher mass accuracy for a wide range of oligonucleotides.
- Excellent quantitation accuracy is achievable in both MS and UV based analysis under optimized source conditions.

Conflicts of interest statement : The authors are affiliated and funded by Shimadzu Corporation.