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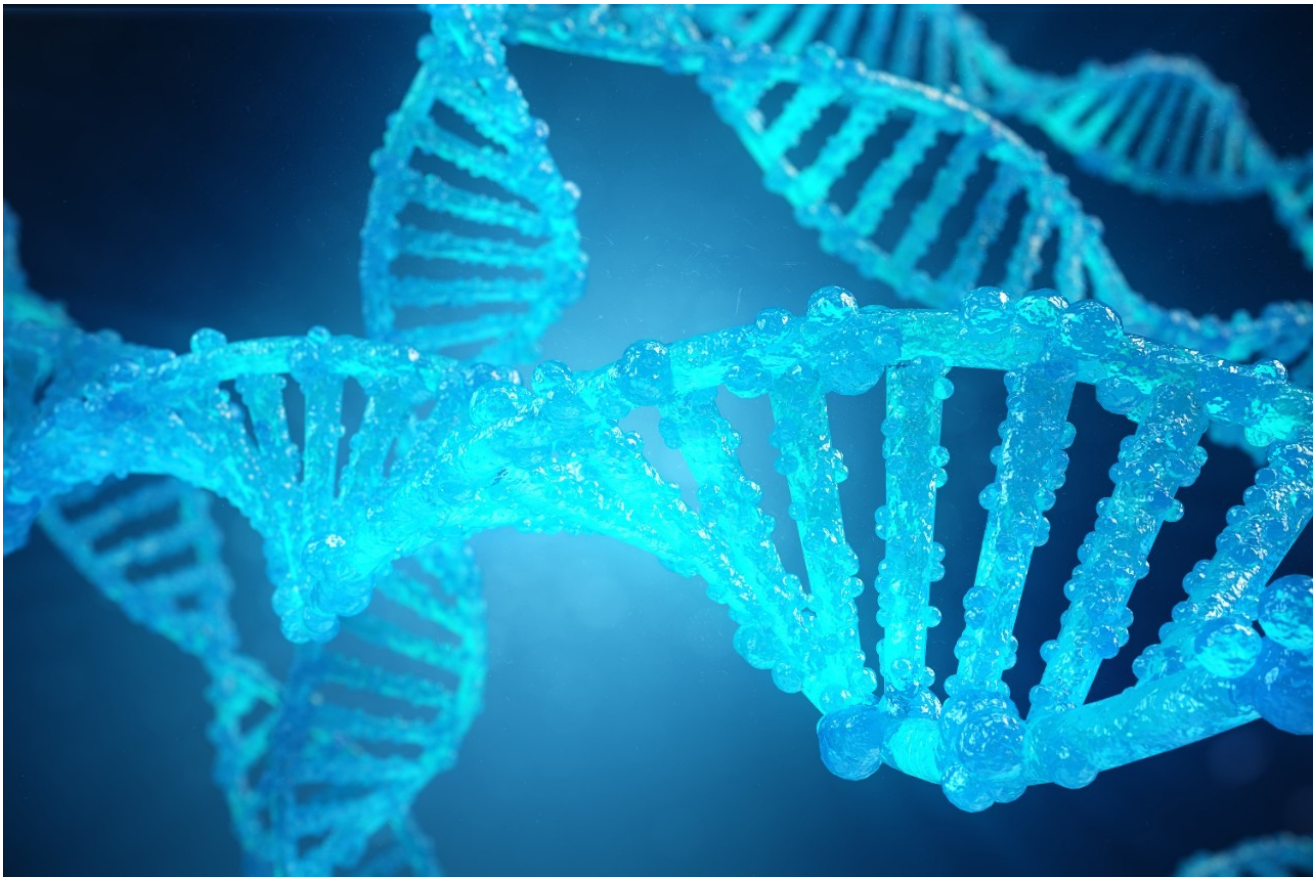
應用手冊

Adding Mass Detection to Synthetic Oligonucleotide Analyses with the ACQUITY QDa Detector

Robert E. Birdsall, Ying Qing Yu

Waters Corporation



Abstract

This application note demonstrates that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting oligonucleotides across a wide molecular weight range and can be readily integrated into existing UV-based workflows.

Benefits

- Addition of mass data to synthetic oligonucleotide assays using MassLynx enabled with MaxEnt deconvolution algorithm
- Complementary mass information using traditional IP-RPLC mobile phases
- Increased productivity through the use of on-line orthogonal detection techniques

Introduction

The Waters ACQUITY QDa Detector has been established as a cost-effective means for obtaining mass spectral data within existing optically-based LC workflows in the biopharmaceutical manufacturing environment when used as an orthogonal detection technique.[1-3] Research into therapeutic oligonucleotides has received steadily increasing attention from the pharmaceutical industry due to potential applications using deoxyribonucleic acid (DNA) sense/antisense oligonucleotides and interfering ribonucleic acid- (iRNA) based therapies.[4-5] IP-RPLC has become a prevalent technique in the analysis of synthetic oligonucleotides in part due to the selectivity offered by such techniques as well as its ability to incorporate MS-friendly reagents and buffers as first demonstrated by Apffel and colleagues.[6-7] Mass information afforded by MS detection offers an efficient means of identifying challenging base modifications for improved productivity in synthetic therapeutic oligonucleotide workflows. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability as an orthogonal detection technique in the analysis of synthetic oligonucleotides.

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting oligonucleotides across a wide molecular weight range and can be readily integrated into existing UV-based workflows. A set of polyT standards ranging from 15 nt to 35 nt in length were used in this study to evaluate the accuracy and compatibility of the ACQUITY QDa Detector in oligonucleotide analyses using traditional IP-RPLC mobile phases comprised of triethylamine (TEA) and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).

Experimental

Triethylamine (99.5% purity) and 1,1,1,3,3,3-Hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. LC-UV grade solvents (Optima series) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were purchased from Waters and prepared at a concentration of 10 pmol/uL. Mass loads on column were kept constant at 50 pmol or 5 µL injections.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC Oligonucleotide BEH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 50 mm
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

*mobile phases prepared gravimetrically

Gradient:

Time	Flow(mL/min)	%A	%B	%C	%D
Initial	0.200	81.0	19.0	0	0
15.00	0.200	73.5	26.5	0	0
16.00	0.200	50.0	50.0	0	0
17.00	0.200	81.0	19.0	0	0
21.00	0.200	81.0	19.0	0	0

QDa Detector settings

Sample rate:	2 points/sec
Mass range:	410–1,250 Da
Mode:	negative
Collection mode:	continuum
Cone voltage:	20 V
Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt1

Results and Discussion

IP-RPLC has become the prevalent separation technique in the analysis of oligonucleotides in part due to the high separation efficiency afforded by such methods when compared to alternative charge-based separations.[5] The use of MS-friendly buffers such as TEA/HFIP – as first demonstrated by Apffel and colleagues – provides an efficient means in the identification of challenging base modifications of oligonucleotides based on orthogonal mass spectrometry data.[6-7] To demonstrate that the ACQUITY QDa is compatible with such IP-RPLC methods, a separation of five polyT standards of increasing length were separated using a TEA/HFIP buffer. As shown in Figure 1, the optical chromatogram (TUV) and response from the mass detector (QDa) – which was in a serial configuration post optical detector – show a high degree of correlation. From this data, it is evident that the ACQUITY QDa Detector is capable of providing mass spectral data using traditional IP-RPLC methods that incorporate ion pairing agents such as TEA buffered in HFIP. As shown in Figure 1, the intensity profile of the ACQUITY QDa Detector is similar but not identical to the UV profile. Interrogation of the raw MS spectrum was performed to gain insight into intensity profile differences.

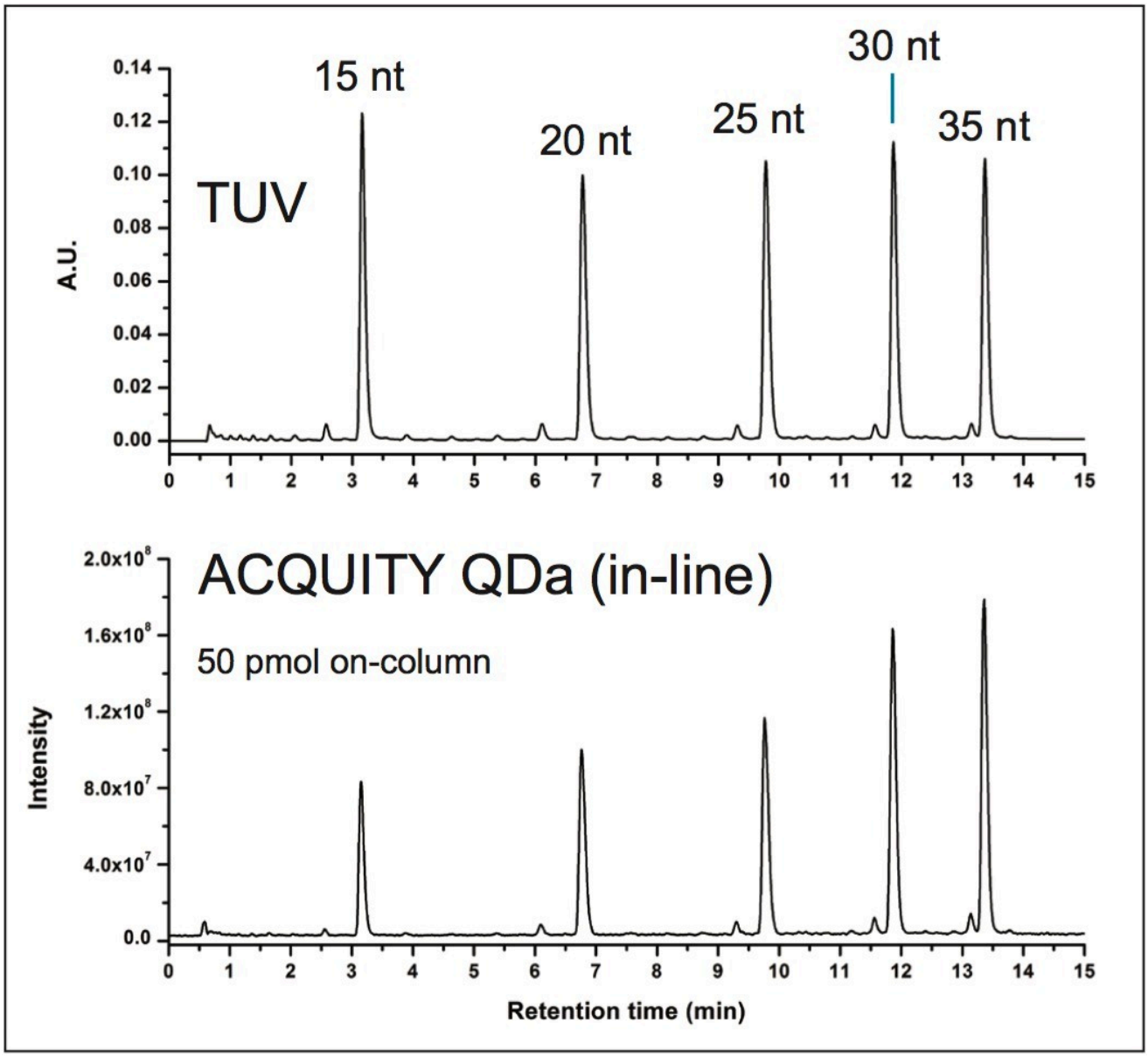


Figure 1. Oligonucleotide analysis with QDa. An IP-RPLC separation of five polyT standards with the ACQUITY QDa in a serial configuration post UV detection.

Table 1 shows that multiple charge states are observed (green highlight) for each of the oligonucleotide standards. Closer inspection of the 35 nt standard indicates at least nine charge states were observed. The shorter sequences, which had greater disparity in intensities, were observed with fewer charge states, indicating potential lower charge states may not be observed as they are outside the detector scan range (>1250 *m/z*). This phenomena does not affect mass data for therapeutic oligonucleotides, as even the shortest standard (nt 15) had five charge states observed.

green = observable charge state

nt	Avg. MW	$[M-4H]^{-4}$	$[M-5H]^{-5}$	$[M-6H]^{-6}$	$[M-7H]^{-7}$	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$	$[M-16H]^{-16}$	$[M-17H]^{-17}$
15	4,500.9	1124.2	899.2	749.1	642.0	561.6	499.1	449.1	408.2	374.1	345.2	320.5	299.1	280.3	263.8
20	6,021.9	1504.5	1203.4	1002.6	859.3	751.7	668.1	601.2	546.4	500.8	462.2	429.1	400.5	375.4	353.2
25	7,542.9	1884.7	1507.6	1256.1	1076.5	941.9	837.1	753.3	684.7	627.6	579.2	537.8	501.9	470.4	442.7
30	9,063.8	2265.0	1811.8	1509.6	1293.8	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2	565.5	532.2
35	10,584.8	2645.2	2116.0	1763.1	1511.1	1322.1	1175.1	1057.5	961.2	881.1	813.2	755.0	704.6	660.5	621.6

Table 1. Oligonucleotide m/z table. A charge state table based on average molecular weight of the polyT standards was used to identify the observed charge states detected with the ACQUITY QDa. Green highlight indicates charge states observed for each standard.

As shown in Table 1, the number of charge states observed across the polyT standards gives rise to the question of instrument accuracy across such a diverse range. An assessment of charge state accuracy of the ACQUITY QDa Detector was thus performed using the observed charge states for the oligonucleotide standards. For this assessment, the difference between the observed and theoretical charge state *m/z* value was determined using the average molecular weight of each oligonucleotide. The results for a technical triplicate of the 30 nt standard are listed in Table 2 as a representative sample. It can be seen from Table 2 that the derived masses for the observed charge states are within the instrument specification of ± 0.2 Da with a high degree of method repeatability demonstrated by the low RSD of 0.02% or lower for each charge state. Similar results were obtained for observed charge states for the remaining standards (data not shown) demonstrating the ACQUITY QDa is capable of providing accurate mass information for oligonucleotide analyses.

30 nt, N=3	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$
Expected	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2
Observed	1132.1	1006.1	905.4	823.1	754.3	696.2	646.5	603.4
S.D.	0.07	0.12	0.04	0.00	0.04	0.04	0.14	0.08
%R.S.D.	0.01	0.01	0.00	0.00	0.01	0.01	0.02	0.01

Table 2. m/z accuracy evaluation. Expected charge states based on average molecular weight of the 30 nt standard were compared to the observed charge states and were within instrument specification of ± 0.2 Da.

The increased number of charge states observed with oligonucleotides as shown in Figure 2A result in mass spectrums that are not straightforward to interpret. Workflows that can deliver accurate mass information in an efficient manner are highly desirable in the analysis of therapeutic oligonucletoides. To this end, deconvolution algorithms such as MaxEnt1 can be incorporated to provide mass data of oligonucleotide spectra for improved productivity. To evaluate this functionality, a one minute window centered across the peak apex of the 30 nt standard was used to combine the MS spectrum data acquired by the QDa. Deconvolution was performed with a peak width of approximately 0.7 Da and a binning resolution of 0.5 Da. As shown in Figure 2B, the spectrum of

the 30 nt standard was deconvoluted to a zero charge state mass of 9,064.5 Da, which was within 0.7 Da of the expected average molecular weight. A minor sodium (Na^+) adduct was also observed with a relative intensity of 6%. Trace salts are routinely encountered in LC-based separations because of their high affinity towards the phosphodiester backbone of oligonucleotides. Similar to the charge state evaluation, an assessment of the mass accuracy of the deconvolution algorithm for the complete set of standards was performed.

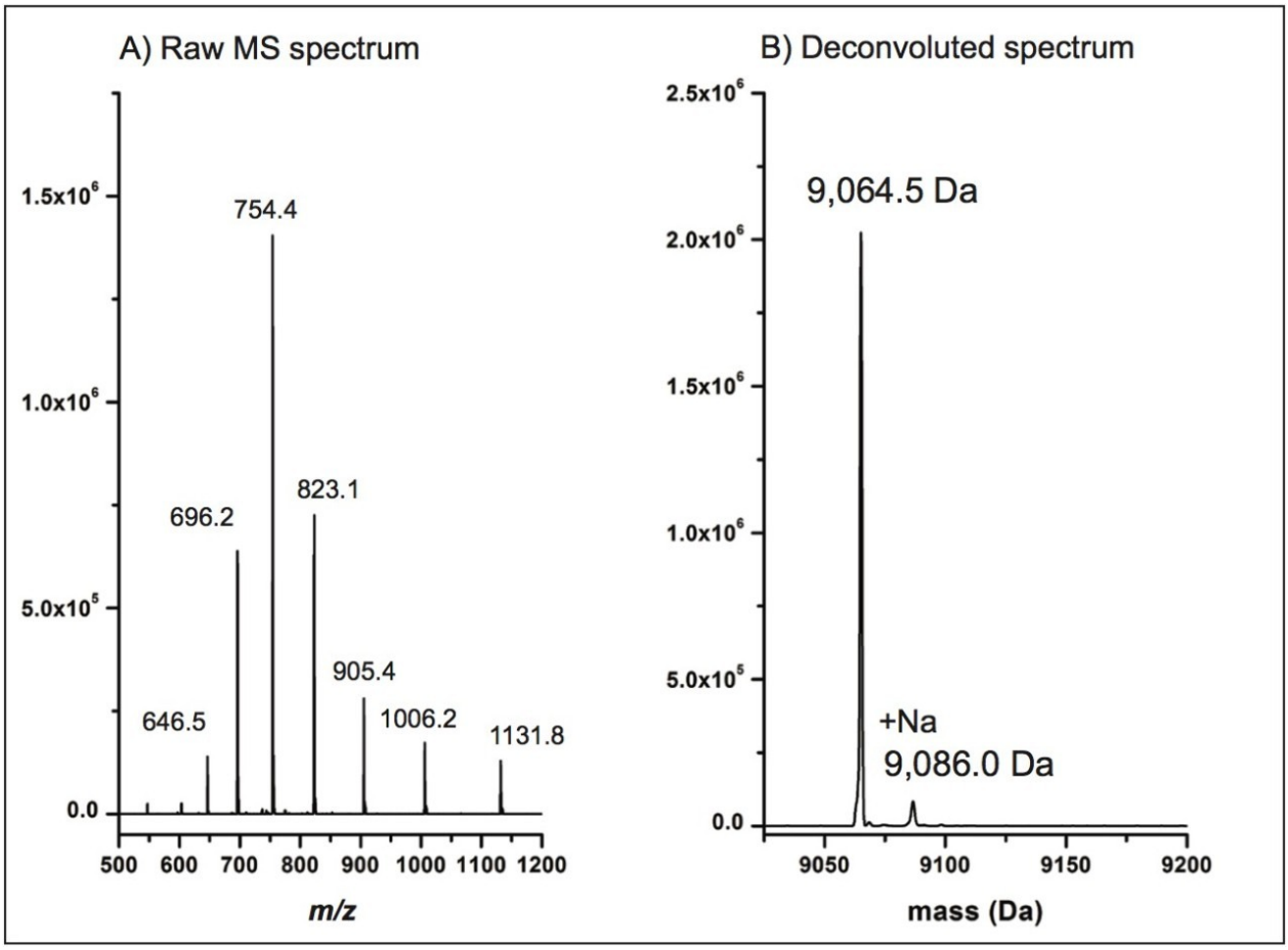


Figure 2. MaxEnt1 Deconvolution of MS spectrum. A) Multiple charge states associated with the raw spectrum of the 30 nt standard when acquiring continuum data using the ACQUITY QDa. B) Deconvolution of the raw MS spectrum of the 30 nt standard resulted in a parent peak mass of 9,064.5 Da (+0.7 Da) and a minor sodium (Na^+) adduct peak with a relative intensity less than 6%.

Using the same methodology as before, the difference between the observed and theoretical deconvoluted mass value was determined using the average molecular weight of each oligonucleotide. An identical number of scans and *m/z* range was used to combine MS spectrum for the polyT standards. Deconvolution parameters were kept constant for the standards with the results listed in Table 3. As shown in Table 3, the deconvolution results were

observed to be highly reproducible across a technical triplicate with no deviation observed in the deconvoluted mass. Mass accuracy was observed from +0.0 Da to +0.7 Da across the polyT standards with increasing deviation associated with increasing sequence length. This is not entirely unexpected, as instrument error increases with higher charge states. In light of this, a mass accuracy of ±1.0 Dalton for oligonucleotides ranging from 15–35 nt demonstrates that the ACQUITY QDa is capable of providing adequate mass information in an efficient manner for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

N=3	nt 15	nt 20	nt 25	nt 30	nt 35
Expected	4500.9	6021.9	7542.9	9063.8	10584.8
Trial 1	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 2	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 3	4500.9	6022.5	7543.5	9064.5	10585.5
Average	4500.9	6022.5	7543.5	9064.5	10585.5
Δ Mass (Da)	0.0	0.6	0.6	0.7	0.7

Table 3. Mass accuracy evaluation of MaxEnt1 deconvoluted mass. Expected mass based on average molecular weight of the polyT standards were compared to the average deconvoluted mass. Mass accuracy ranged from +0.0 Da to +0.7 Da across the set of standards.

Conclusion

Cost-effective techniques that add value and can be implemented into existing workflows with minimal effort are highly desirable in the pharmaceutical industry. The addition of complementary mass information in a single workflow afforded by the ACQUITY QDa Detector provides analysts an efficient means to improve productivity in routine assays. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability with biopharmaceuticals beyond that of mAbs. From this work it has been demonstrated that the ACQUITY QDa is compatible with IP-RPLC mobile phases and is able to detect and report mass information for oligonucleotides over a wide molecular weight range. Collectively, these results establish the ACQUITY QDa as an ideal addition to an analyst's lab for increased productivity and confidence of data analysis for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

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