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Application Note

LC-MS Analysis of siRNA, Single Guide RNA and Impurities Using the BioAccord™ System with ACQUITY™ Premier and New Automated INTACT Mass Application

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Abstract

This application note demonstrates an automated, compliance-ready liquid chromatography-mass spectrometry (LC-MS) worflow for intact mass confirmation and purity analysis of synthetic oligonucleotides including small interfering RNA (siRNA) and large single guide (sg) RNA oligonucleotides and their impurities. The data presented here shows the challenging aspects of analyzing very long oligonucleotides, from the chromatographic separation to purity assessment. The new INTACT Mass Application, within the compliance-ready waters_connect™ informatics platform, allows scientists to analyze both short and long oligonucleotides with automated data processing features that streamline nucleic acid analysis.

Benefits

- The BioAccord System with ACQUITY Premier paired with the Premier CSH™ Column is ideally suited for detailed characterization of shorter 20-mer siRNA and longer 100-mer sgRNA oligonucleotides

- An automated, compliance-ready workflow embedded in the waters_connect INTACT Mass application is shown to provide better than 20 ppm mass accuracy for intact mass confirmation of 100-mer single guide RNA (sgRNA) oligonucleotides and their impurities analyzed with an ion-pairing reversed-phase (IP-RP) LC-MS assay
- The workflow described here provides purity information for all the sample components, down to 1% abundance levels

Introduction

During the past decade there has been increased interest in oligonucleotide therapeutics as an alternative to small molecule and protein therapeutics.[1,2] Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods. Despite significant progress in solid phase oligomerization chemistry,[3] synthetic oligonucleotides still contain multiple classes of low-level (0.1–2%) impurities.[4,5] IP-RP is one of the traditional LC-MS methods used for characterizing oligonucleotide products.

Using this approach, an automated workflow for analysis of synthetic oligonucleotides employing the BioAccord LC-MS System operating under the compliance-ready waters_connect platform was recently described.[6,8]

The BioAccord System with ACQUITY Premier featured in Figure 1 is a compact, robust, easy-to-use platform for routine biopharmaceutical analyses. This fully integrated system is comprised of the ACQUITY Premier UPLC™ System, a Tunable UltraViolet (TUV) detector and the electrospray ionization time-of-flight (ESI-Tof)-based ACQUITY RDa Mass Detector.

Here we investigated the capabilities of this LC-MS platform for impurity analysis of both shorter siRNA and longer single guide RNA oligonucleotides (sgRNAs) using a workflow featuring the new INTACT Mass Application.

Figure 1. BioAccord LC-MS System with ACQUITY Premier.

Experimental

Reagents and Sample Preparation

N,N-diisopropylethylamine (DIPEA, 99.5% purity, catalogue number 387649-100ML) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99% purity, catalogue number 105228-100G) were purchased from Sigma Aldrich (St Louis, MO). Acetonitrile (LC-MS grade, catalogue number 34881–1L) was obtained from Honeywell (Charlotte, NC). HPLC grade deionized (DI) water was purified using a MilliQ system (Millipore, Bedford, MA). Mobile phases were prepared fresh and used on the same day. Ten nanomoles of a 100-mer sgRNA oligonucleotide encoding for the HPRT1 enzyme (hypoxanthine phosphoribosyltransferase1) were purchased from Integrated DNA Technologies

(Coralville, IA). The oligonucleotide sequence 5′-G*A*U* GAU CUC UCA ACU UUA ACG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC U*U*U* U-3′ has the elemental composition of C959 H1183 N376 O691 P99 S6 and an average molecular weight of 32,292.5513 Da. The RNA oligonucleotide contains a 2′-OMe modification on its first three 5′ nucleotides (G*A*U*) as well as on its last three 3′ nucleotides (U*U*U*) and the asterisk indicates that all these six nucleotides are phosphorothioated.

Stock solutions of the sgRNA oligonucleotide were prepared in DI water at a concentration of 5 µM, from which a 10 µL volume was injected onto a RP column, which corresponds to loading 50 picomoles of the 100-mer oligonucleotide on-column.

All datasets were acquired and processed using the fully integrated waters_connect INTACT Mass App. The application performs automated 2-D peak detection of UV and total ion chromatogram (TIC) data, combines spectra across the LC peaks and deconvolves the mass spectra using a new automated implementation of the MaxEnt1 or BayesSpray deconvolution algorithms. The deconvolved mass spectra are then automatically searched for product related substances and product related impurities. Finally, the application calculates a purity result using the TUV chromatogram peak areas, TIC peak areas or mass spectrum counts.

LC conditions

LC-MS system:	BioAccord System with ACQUITY Premier
Column:	ACQUITY Premier CSH Column 1.7 µm, 130 Å, 2.1 x 100 mm, (p/n: 186009461)
Column temp.:	50 °C
Flow rate:	300 µL/min
Mobile phases:	Solvent A: 40 mM HFIP (hexafluoroisopropanol), 8 mM DIPEA (N,N-diisopropylethylamine) in DI water, pH 8.8 Solvent B: 4 mM HFIP (hexafluoroisopropanol), 4

mM DIPEA in 75% acetonitrile

Gradient table:

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.3	88	12	Initial
25.00	0.3	83	17	–
30.00	0.3	83	17	6
30.50	0.3	15	85	6
32.50	0.3	15	85	6
33.00	0.3	88	12	6
40.00	0.3	88	12	6

Sample temp.: 6 °C

Sample vials: LC-MS certified, Maximum Recovery Vials (p/n: 186005663CV)

Injection volume: 10 µL

Wash solvents:

Purge solvent: 50% MeOH

Sample manager wash solvent: 50% MeOH

Seal wash: 20% Acetonitrile in DI water

MS Conditions

Acquisition mode:	Full scan
Ionization mode:	ESI(-)
Capillary voltage:	0.8 kV
Cone voltage:	45 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas (N_2) pressure:	6.5 bar
TOF mass range :	400–5000
Acquisition rate:	2 Hz
Lock-mass:	waters_connect lockmass solution (p/n: 186009298)
Data acquisition software:	waters_connect
Data processing software:	waters_connect

Results and Discussion

IP-RP separations typically provide adequate chromatographic resolution for resolving most of the major impurities associated with short synthetic oligonucleotides (20–25 mers) as shown in a recent application note.[8] For this class of shorter oligonucleotides, the BioAccord can resolve the isotopic distributions of all oligonucleotide charge states. A sample utilized in a previous application note,[8] the 21-mer extensively modified

oligonucleotide and its impurities were analyzed on the BioAccord System with ACQUITY Premier. The data was acquired and automatically processed using waters_connect informatics with the INTACT Mass application.

The 21-mer synthetic oligonucleotide, contains a 2′-OMe modification on 19 of its nucleosides, a 5-Methyl modification on four cytidines, a 5-Methyl modification on four uridines, having left only two unmodified nucleotides (thymidines) at the 3′-end. The oligonucleotide sequence is GUA ACC AAG AGU AUU CCA UTT and its elemental composition is calculated as C229 H306 N76 O143 P20.

Chromatographic and mass spectral data for the primary species, the full-length oligonucleotides are presented in Figures 2 and 3. As previously shown,[8] chromatographic data (Figure 2) displays the resolution of individual impurities, enabling UV based quantification. Automatic peak identification was used for spectral summation to produce deconvolved spectra for the main peak and its failed sequence impurities. The deconvolved and the raw spectrum of the main peak is shown in panels A and B from Figure 3. The mock spectrum (panel 3C) represents the raw data used by the algorithm to produce the deconvolution result, and acts as a simple quality check of the deconvolution process.

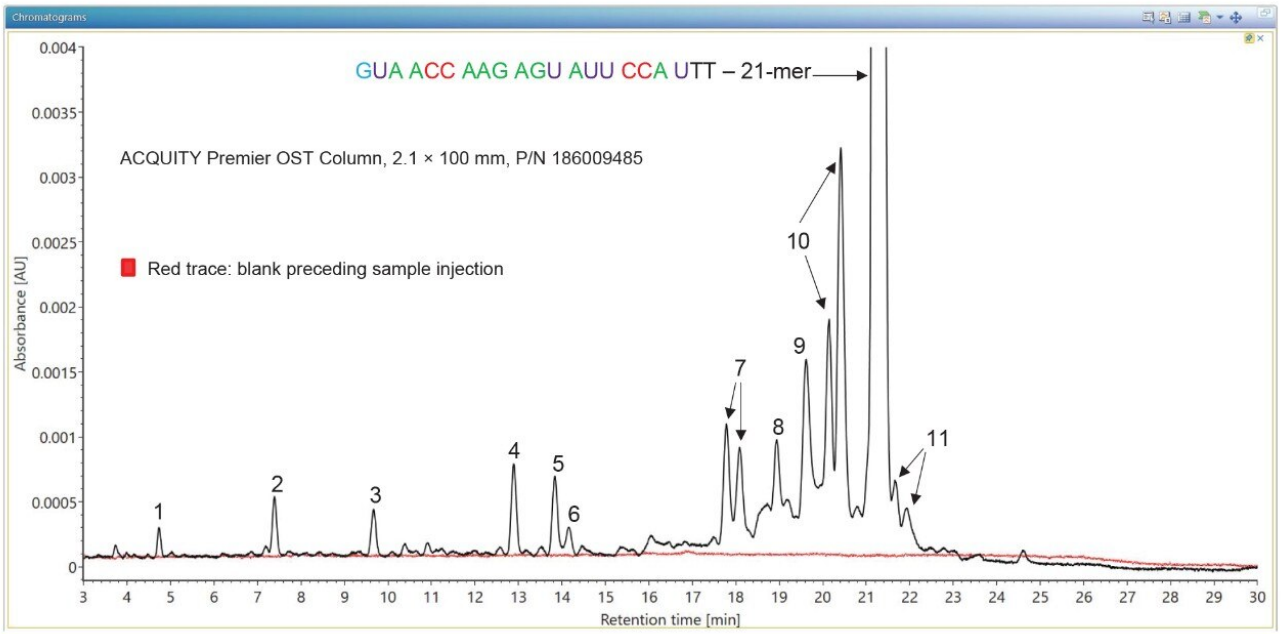


Figure 2. TUV chromatogram recorded at 260 nm, showing the separation of eleven oligonucleotide impurities from a 21-mer extensively modified chromatogram.

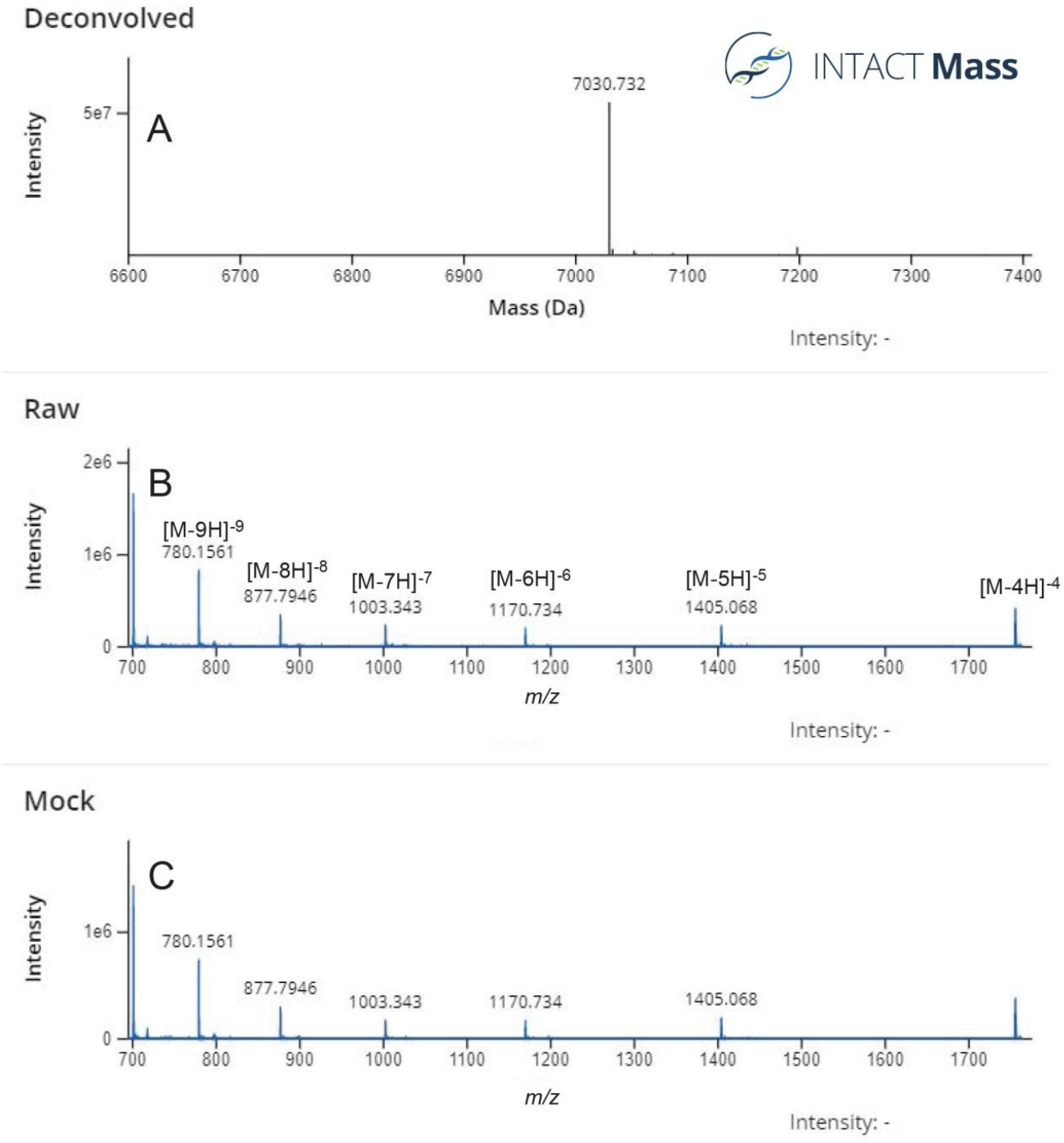


Figure 3. INTACT Mass spectra recorded for the 21-mer siRNA oligonucleotide: (A) MaxEnt1 charge deconvolved spectrum; (B) raw ESI-MS spectrum; (C) mock spectrum which serves as a quality check for the deconvolution

process.

The summarized results of this analysis are displayed in Figure 4. The BayesSpray charge deconvolution algorithm using monoisotopic masses is recommended for processing of isotopically resolved oligonucleotides and using this algorithm with automated settings seven impurities related to failed sequences associated with 5′-end nucleotide losses (11,12,14,15,16,17, and 18-mer impurities) were identified (peaks labeled 1–7 in Figure 2). In addition, four other impurities associated with the 21-mer oligonucleotide bearing different type of modifications were identified as well (peaks labeled 8–11). Each of the three types of impurities present in this sample (identified by the pair of peaks labeled 7, 10, and 11 in Figure 2) have been shown to include two positional isomers.[8] This is a remarkable observation, indicating the ability of the ACQUITY Premier OST Column to chromatographically resolve scrambled oligonucleotide sequences (the dublets corresponding to peaks 7 and 10) and deamination isomers of the full-length product (peaks 11). All the deconvolved oligonucleotide masses were measured with mass accuracies better than 15 ppm. The INTACT Mass Application detected these low-level oligonucleotide impurities, down to 0.2%, according to their UV response.

INTACT Mass

Component	Peak no:	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	Identity result	Observed TIC RT (mins)	Observed UV RT (mins)	LC area	LC amount (%)
D1423 n-OMeA[5] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU	1	3,593.702	3,593.707	-1.5	Pass	4.74	4.69	1,222	0.2
D1423 n-OMeA[4] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU	2	3,936.762	3,936.775	-3.4	Pass	7.37	7.34	2,737	0.5
D1423 n-OMeA[3] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU	3	4,638.916	4,638.907	1.9	Pass	9.66	9.61	2,625	0.5
D1423 n-OMeA[2] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU	4	4,981.988	4,981.975	2.7	Pass	12.88	12.83	5,276	0.9
D1423 n-OMeA[2] & n-OMe5MeC & n-OMeG & n-OMe5MeU	5	5,315.046	5,315.048	-0.3	Pass	13.80	13.77	4,704	0.8
D1423 n-OMeA & n-OMeG & n-OMe5MeU	6	5,991.181	5,991.188	-1.2	Pass	17.76	17.72	7,942	1.4
D1423 n-OMeA	7	6,684.310	6,684.308	0.3	Pass	19.59	19.55	13,473	2.4
D1423 n-OMe5MeU	9	6,693.332	6,693.320	1.9	Pass	20.13	20.08	13,283	2.3
D1423 n-OMe5MeC	10	6,694.315	6,694.304	1.8	Pass	20.39	20.34	26,001	4.5
D1423 unknown[-NH5]	8	7,008.354	7,008.334	2.9	Pass	20.13	20.08	13,283	2.3
D1423	MAIN PEAK	7,027.390	7,027.376	2	Pass	21.22	21.19	469,430	82.0
D1423 Deamination	11	7,028.315	7,028.360	-6.5	Pass	20.39	20.34	26,001	4.5

Figure 4. Screenshot with the processing results generated by the INTACT Mass software for the analysis of a 21-mer oligonucleotide and its impurities. The dataset was deconvolved using the BayesSpray charge deconvolution algorithm and eleven oligonucleotide impurities were identified with mass accuracies of under 15 ppm. The first impurity displayed in this table, an 11-mer oligonucleotide, has the lowest detected abundance at 0.2% according to the UV measurement.

Analysis of a 100-mer sgRNA

In the case of medium to larger size oligonucleotides (50–100-mers), these analytes are more difficult to analyze by LC-MS, because the IP-RP chromatographic separations are not able to achieve full resolution of all major impurities, and because the oligonucleotide ESI-MS spectra have overlapping isotopic distributions. One such example is the case of guide RNAs (gRNAs) which function within the Cas9 gene editing process.

Guide RNAs are RNA molecules used to “guide” the Cas9 endonuclease in CRISPR applications to a precise genomic location for gene editing for gene editing. In nature, there are two individual RNA molecules required for Cas9 to cleave double stranded DNA: a highly variable 17–20 nt crispr RNA (crRNA) responsible for genome targeting and a longer, highly conserved (80–100 nt) tracer RNA (tracrRNA) which serves as a binding scaffold

for Cas9. For CRISPR-related applications, the two RNA molecules can be fused together into a single guide RNA (sgRNA) with a typical length of 100 nucleotides. sgRNAs are typically produced through solid-phase synthesis[3] and their purity is typically assessed using LC-MS assays. Synthesizing such long oligonucleotides is challenging because it requires a very high yield in each coupling step. For example, even for a 99.9% coupling step efficiency for every synthesis step, the expected yield of a full-length product (FLP) is only about 60% for a 100-mer oligonucleotide.[5] Obviously, the number of oligonucleotide impurities increases as the length of the FLP increases, bringing significant challenges for purifying sgRNAs. A recent report[9] investigated several chromatographic columns for optimum separation of sgRNAs and their impurities.

The workflow proposed here is used for fast, automated characterization of sgRNAs and their impurities. Separations of the 100-mer sgRNA oligonucleotide and its impurities were performed on three different Premier C_{18} chemistries with identical column dimensions (2.1 mm ID x 100 mm length) and same particle size (1.7 µm), to find the optimum chromatographic conditions for separation of these analytes. As the oligonucleotide mixture contains components with closely related structures (see Table 1), several chromatographic parameters were optimized (including gradient length/profile, flow rate, column temperature) to avoid full (complete) chromatographic peak coelutions as much as possible. Three TUV chromatograms recorded on two Premier OST columns containing C_{18} particles with 130 and 300 Å pores, and on a Premier CSH (charged surface hybrid) Column with 130 Å pores are displayed in Figure 5. As shown in this figure, when using identical experimental conditions, the best chromatographic selectivity for the sgRNA impurities was obtained on the Premier CSH Column (p/n: 186009461 <https://www.waters.com/nextgen/global/shop/columns/186009461-acquity-premier-csh-c18-column-17--m-21-x-100-mm-1-pk.html>). This observation is evident for an impurity eluting just before the parent 100-mer oligonucleotide, which is just starting to be resolved on the CSH Column. Upon a closer inspection of the MS-acquired data, the separation of this impurity from its major counterpart was accomplished to an even greater extent. Figure 6 shows the overlaid extracted mass chromatograms recorded for the same charge states ($[M-29H]^{-29}$) of both oligonucleotides. A great portion of the 100-mer impurity does not co-elute with the most abundant oligonucleotide. This result is rather surprising, because the impurity differs only by about 16 Da from the major component. The impurity was created by a PS to PO conversion (desulfurization). Literature reports indicate that oxidative desulfurization of oligonucleotides could be occurring during oligonucleotide synthesis,[10] during oligonucleotide solubilization/formulation[11] or it might be related to an in-source ionization artifact.[12] Chromatographic separation enables us to distinguish between these three possibilities, because the in-source created artifact signal would have fully coeluted with the parent oligonucleotide. The chromatogram from Figure 5 clearly demonstrates that the oligonucleotide impurity either originated from the sample (it was already present in some amount), or it was created in solution after dissolving

the solid sgRNA oligonucleotide in DI water. To better understand the origin of this impurity further oligo degradation studies are needed, but these studies were not pursued here. In addition, the results clearly demonstrate that the BioAccord ESI-MS source does not create this type of artifacts.

Oligonucleotide peak label	Oligonucleotide peak length	Type of oligonucleotide modification, Delta mass (Da)	Elemental composition	Accurage average molecular weight	Mass accuracy (ppm)
Impurity 1	100-mer	- 16 Da (PS to PO conversion, desulfurization)	C959 H1183 N376 O692 P99 S5	32275.4857	-0.7
PARENT OLIGO - 2	100-mer	no modification	**C959 H1183 N376 O691 P99 S6**	**32291.5513**	-2.1
Impurity 3	100-mer	+53 Da (CNET, cyanoethyl, C_3H_3N added)	C962 H1186 N377 O691 P99 S6	32344.6139	-2.7
Impurity 4	100-mer	+70 Da (IBT, isobutyryl, C_4H_6O added)	C963 H1189 N376 O692 P99 S6	32361.6411	16.3
Impurity 5	100-mer	+80 Da (extra phosphate, HPO_3 added)	C959 H1184 N376 O694 P100 S6	32371.5312	-11.0

Table 1. Oligonucleotide impurities identified in the 100-mer sgRNA oligonucleotide.

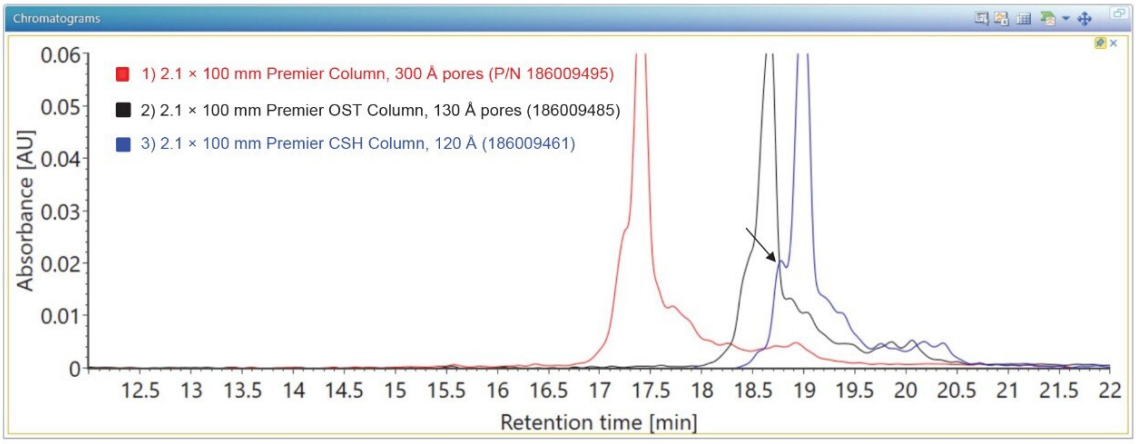


Figure 5. TUV chromatograms showing the separation of the 100-mer sgRNA and its impurities on three Premier Columns with the same dimensions (2.1 x 100 mm) and packed with three different types of C_{18} 1.7 µm particles: 130 Å pores for the OST Premier Column (black trace), 300 Å pores for the Premier PST column (red trace) and 130 Å pores for the CSH (charged surface hybrid) column (blue trace). An oligonucleotide impurity identified as the 100-mer oligo containing only a single modification (PS to PO conversion, ~ 16 Da modification) is clearly resolved much better by the CSH Column (see the black arrow sign) compared to the other two columns. The separations were performed under identical experimental conditions.

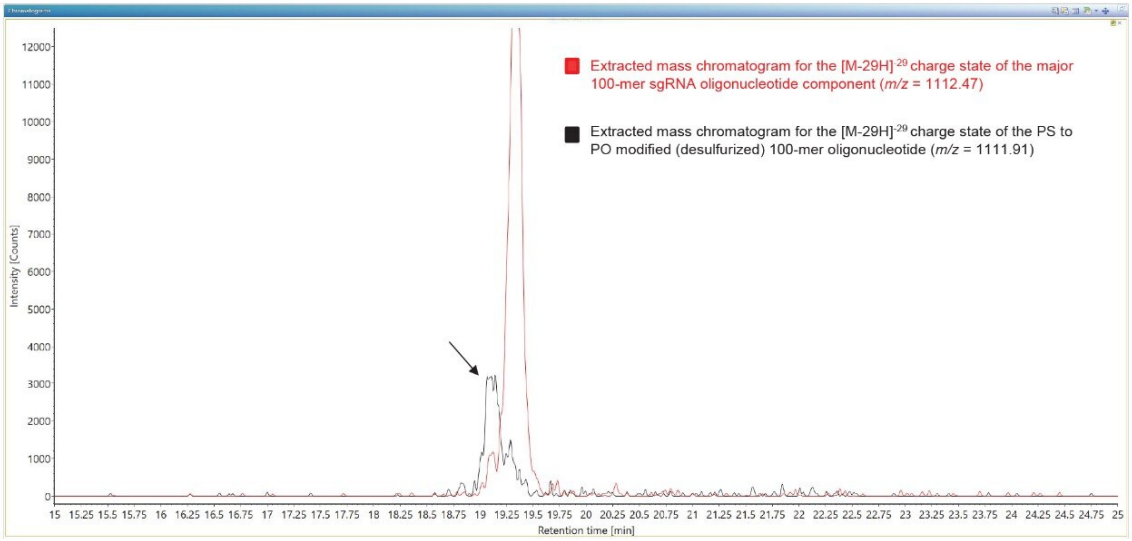


Figure 6. Overlaid extracted mass chromatograms recorded for an abundant charge state ([M-29H]$^{-29}$) present in the major 100-mer sgRNA oligonucleotide (m/z = 1112.47, red trace), as well as in the most abundant impurity present in this sample, the PS to PO modified 100-mer (m/z = 1111.91, black trace). Both mass chromatograms were extracted using a tolerance of 0.1 Da.

A more detailed view of the TUV chromatogram recorded on the CSH Column is presented in Figure 7. This chromatogram clearly demonstrates that baseline separation for all the closely related sgRNA 100-mer impurities is very challenging. In addition, it is very likely to have several coeluting impurities hidden under the UV signal of the main peak. These two observations underly the necessity to use the ESI-MS spectra recorded for all the oligonucleotide impurities for oligo purity calculations, rather than their corresponding peak areas from the TUV chromatogram. While in the case of shorter synthetic oligonucleotides (20- to 25-mers) and their impurities, the sensitivities of the UV and MS channels were very comparable,[8] in the case of sgRNAs, the ESI-MS signal does not match the UV sensitivity. As a result, it is very challenging to reach similar detection limits (about 0.2% purity levels) for sgRNAs compared to shorter oligos (see Figure 2 and reference 8). Four oligonucleotide impurities surrounding the parent 100-mer (and labeled as peaks 1,3,4, and 5) are distinguishable in the UV trace from Figure 6.

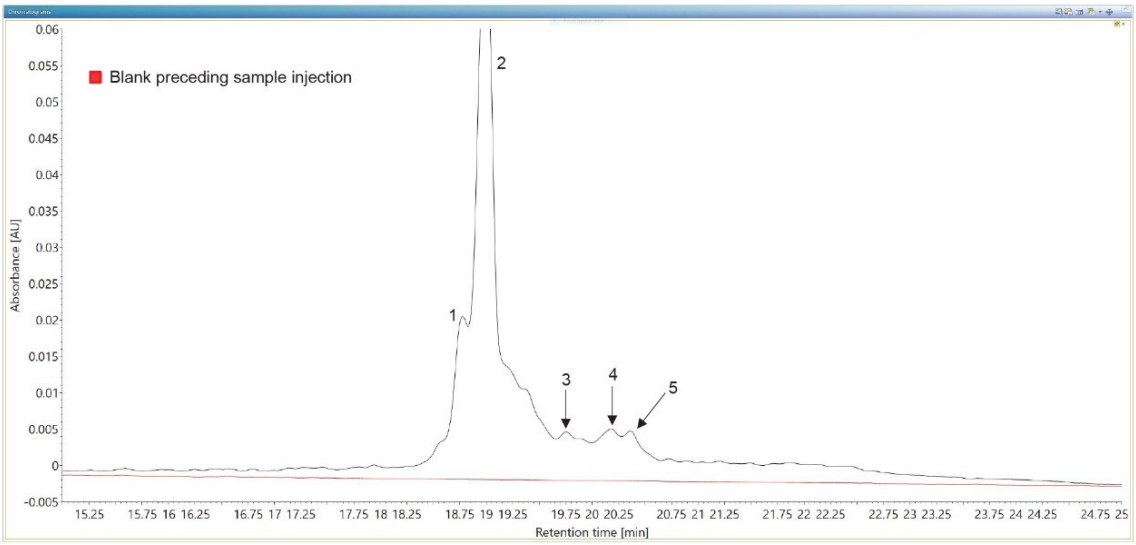


Figure 7. TUV chromatogram showing the separation of oligonucleotide impurities from a 100-mer sgRNA oligonucleotide. The sample was analyzed on a 2.1 x 100 Premier CSH Column using a 25-min gradient described in the Experimental section. The red trace corresponds to the blank preceding sample injection.

The ESI-MS spectrum recorded for the parent 100-mer sgRNA oligonucleotide is displayed in Figure 8B and the deconvolved spectrum is shown in the panel above (Figure 8A). Consistent spectra, with the same wide distribution of charge states (17–40), were obtained for this oligonucleotide on all three Premier columns and a wide charge state distribution was observed for all oligonucleotide impurities as well (data not shown). The ESI-source parameters (ESI voltage, cone voltage, source temperature, desolvation temperature) were optimized to increase the sensitivity of the ESI-MS signal and the reduce the levels of sodium adducts. The optimum column temperature for reduced background in ESI-MS spectra was 50 °C, confirming previous observations.[13] It is also worth mentioning that the choice of the ion pairing reagent, the amount and chemical purity of all the other reagents, as well as the mobile phase preparation procedures are critical in generating cleaner, low-adduct ESI-MS spectra which facilitates detection of low-abundance oligonucleotide impurities. When taking a closer look at two abundant charge states (33 and 34) detected for the desulfurized impurity and the major sgRNA shown in Figure 9, it is obvious that the ESI-MS signals of these closely related species were well resolved chromatographically as indicated by their extracted mass chromatograms displayed in Figure 6. The spectra from Figure 8 were produced by combining ESI-MS spectra from relatively “pure” regions of each of the two

corresponding chromatographic peaks displayed in Figure 6. These ESI-MS spectra provide another confirmation that it is possible to separate, at least to some extent, the desulfurized impurity(ies) from the parent oligonucleotide using the CSH column.

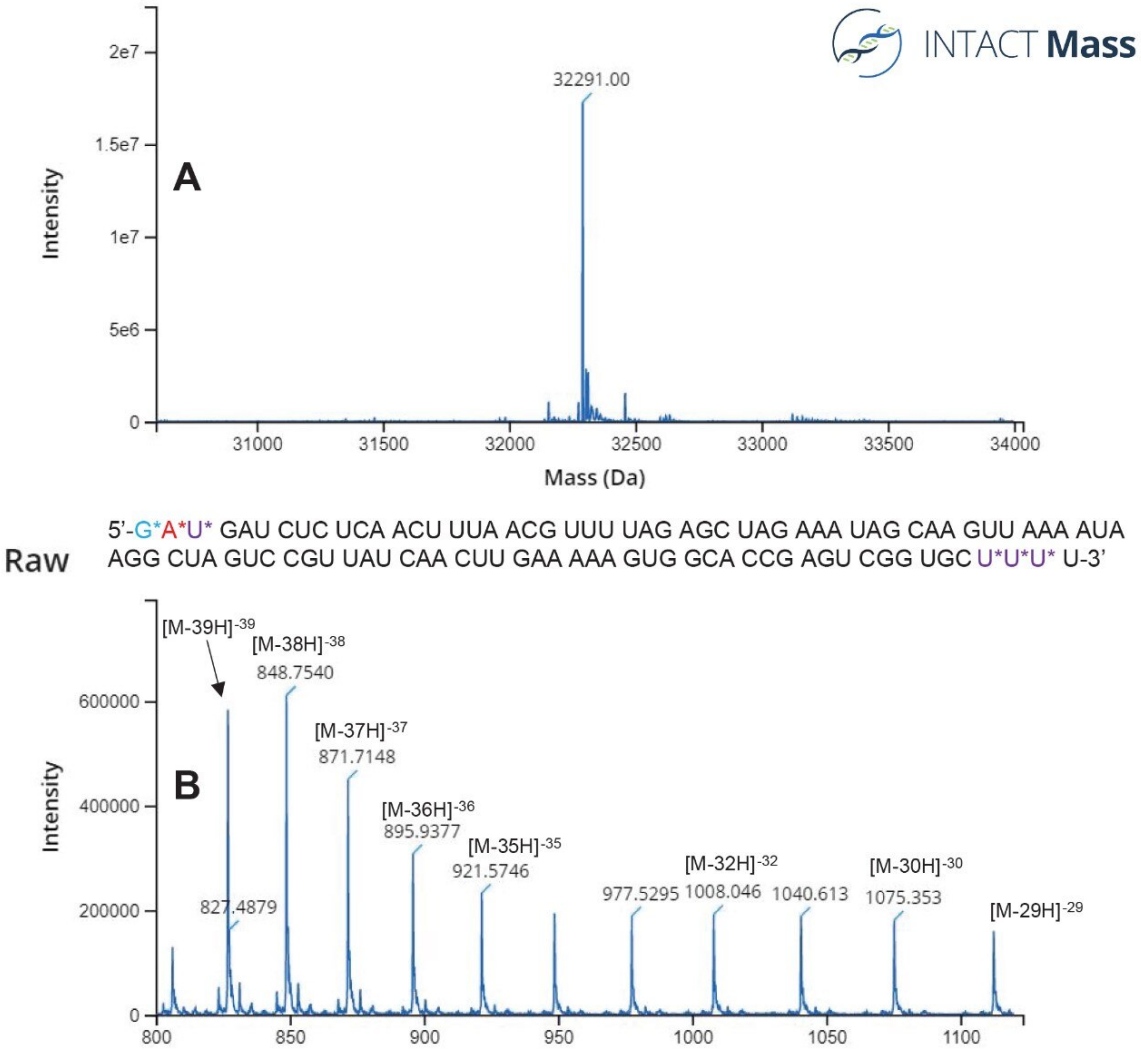


Figure 8. INTACT Mass spectra recorded for the most abundant 100-mer sgRNA oligonucleotide: (A) MaxEnt1 charge deconvolved spectrum; (B) raw ESI-MS spectrum.

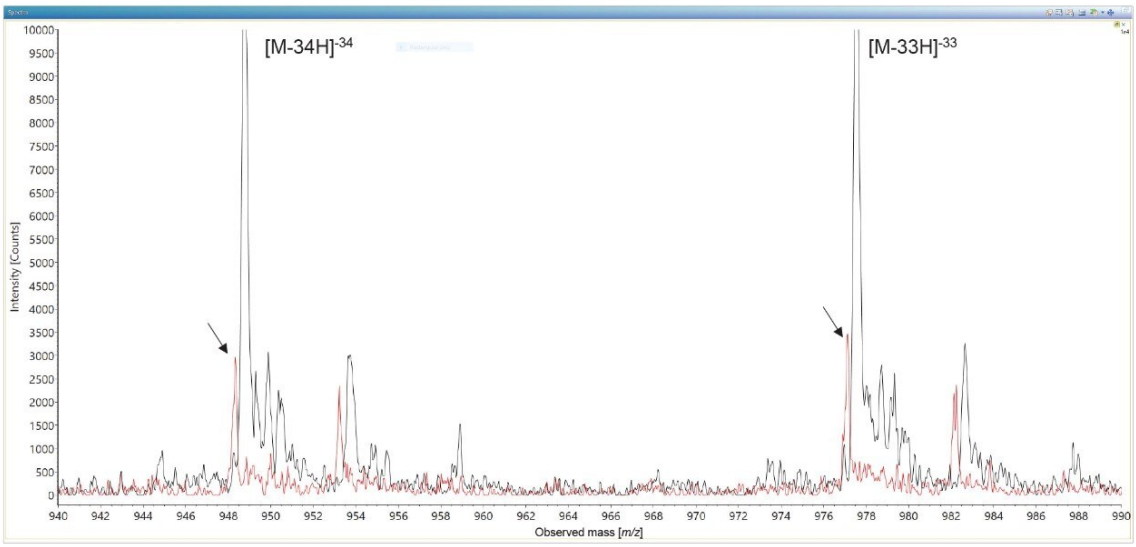


Figure 9. Detailed view of the overlapping ESI-MS spectra of the parent 100-mer sgRNA oligonucleotide (black trace) and its desulfurized analogue (red trace), showing two abundant charge states: $[M-33H]^{-33}$ and $[M-34H]^{-34}$. The two arrows indicate the charge states produced by the PS to PO converted oligonucleotide impurity. As indicated in this spectrum, these two closely related molecular entities can be separated chromatographically.

As shown in the chromatogram from Figure 7, getting an adequate chromatographic separation for complete resolution of all impurities present in large oligonucleotides (***e.g.,*** sgRNA) is not feasible,[4,5,13–15] therefore another quantification approach should replace the traditional quantification of oligonucleotide impurities based on their UV peak areas. In such situations, the ESI-MS ion counts can be used instead of the UV peak areas for oligonucleotide purity calculations.[14,16] The INTACT Mass Application[17] performs automatic deconvolution of all detected oligonucleotide impurities (above a user specified threshold) regardless of their retention times or chromatographic profile. This approach (automated deconvolution non-RT based) is especially well suited for the analysis of co-eluting oligonucleotide impurities distributed around a parent oligonucleotide species. All the ESI-MS spectra belonging to oligonucleotide impurities eluting between 15 and 25 minutes in the chromatogram shown in Figure 7 were automatically deconvolved using the MaxEnt1 algorithm,[18] with the software optimizing the deconvolution settings. In the next step, the deconvolved spectra were searched automatically against a comprehensive and customizable library containing a variety of common and less common oligonucleotide modifications. Currently there are 197 oligonucleotide modifications available. Custom (user defined)

oligonucleotide modifications can be easily added to this list. A screenshot showing the oligonucleotide modifications selected for the analysis of the 100-mer sgRNA oligonucleotide is displayed in Figure 10. The deconvolved spectral results from the automatically generated report are shown in Figure 11. As indicated in this results report, four oligonucleotide impurities were identified with mass accuracies better than 20 ppm, while the MS-calculated abundance of the lowest detected impurity (containing an extra phosphate moiety) was about 1%. A detailed description of all putatively assigned impurities is presented in Table 1. Impurities detected in this sample were previously described in the literature, including the -16 Da PS to PO desulfurized oligonucleotide,[10,13] the +53 Da CNET (cyanoethyl) modification,[14,15] the +70 Da IBT (isobutyryl) modification,[19] and the +80 Da extra phosphorylated oligonucleotide.[20]

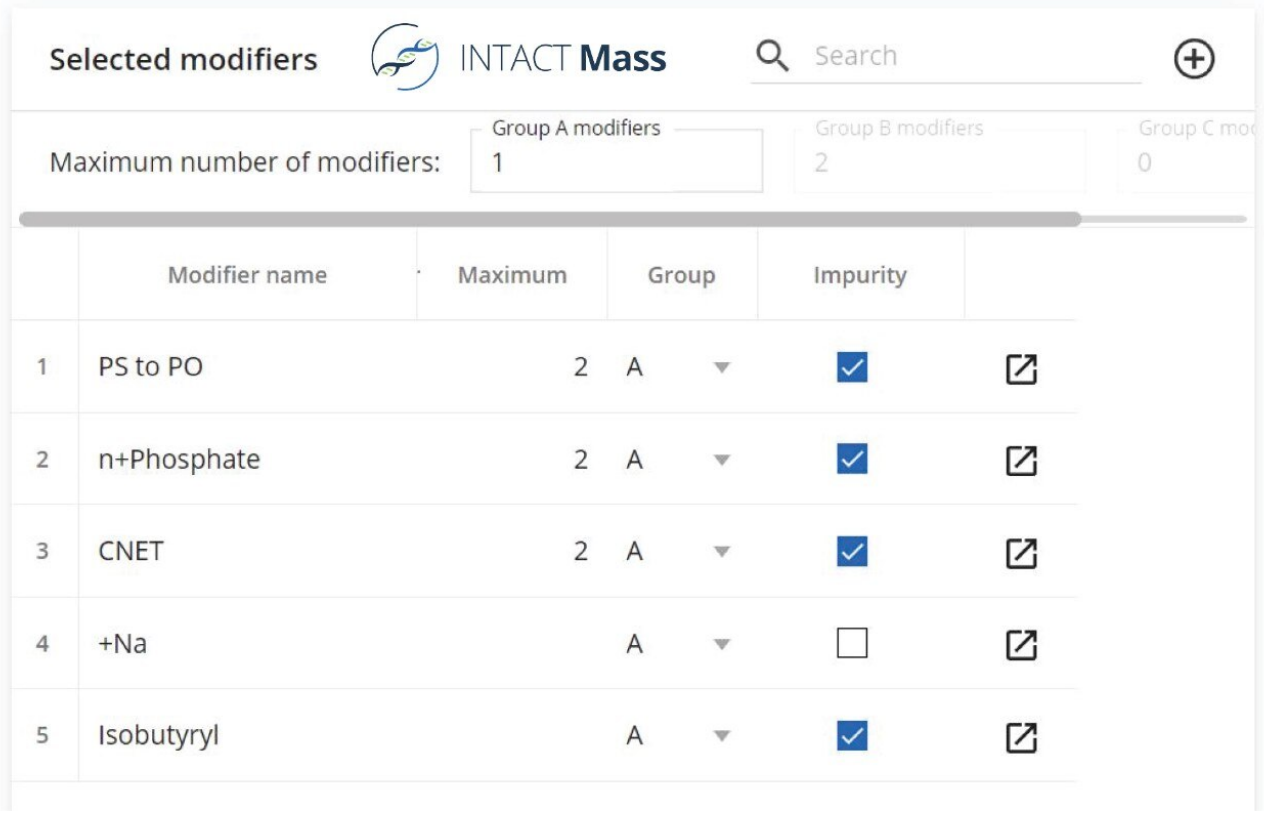
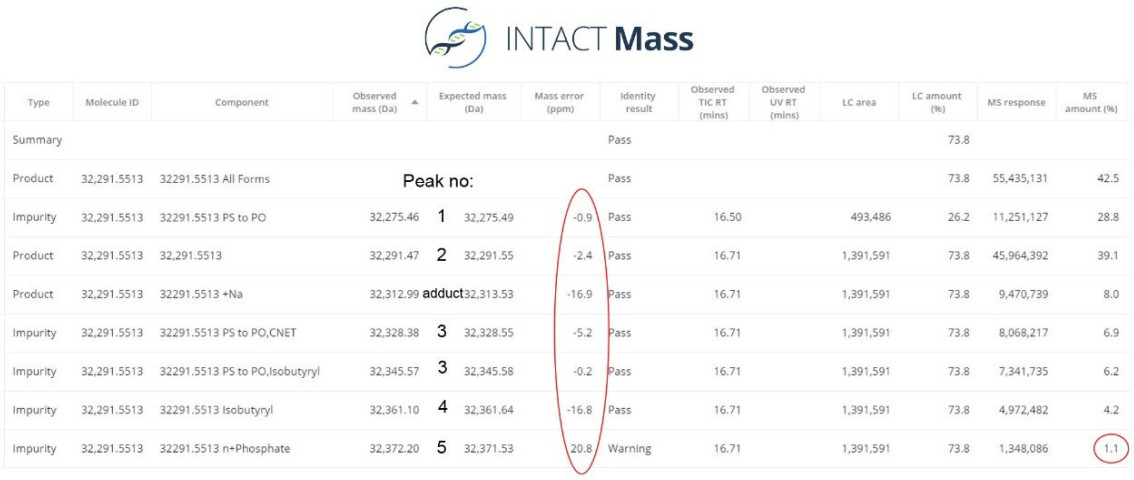


Figure 10. INTACT Mass screenshot showing the oligonucleotide modifications selected for the analysis of the 100-mer sgRNA oligonucleotide.



Type	Molecule ID	Component	Observed mass (Da)	Peak no:	Expected mass (Da)	Mass error (ppm)	Identity result	Observed TIC RT (mins)	Observed UV RT (mins)	LC area	LC amount (%)	MS response	MS amount (%)
Summary							Pass				73.8		
Product	32,291.5513	32291.5513 All Forms					Pass				73.8	55,435,131	42.5
Impurity	32,291.5513	32291.5513 PS to PO	32,275.46	1	32,275.49	-0.9	Pass	16.50		493,486	26.2	11,251,127	28.8
Product	32,291.5513	32,291.5513	32,291.47	2	32,291.55	-2.4	Pass	16.71		1,391,591	73.8	45,964,392	39.1
Product	32,291.5513	32291.5513 +Na	32,312.99	adduct	32,313.53	-16.9	Pass	16.71		1,391,591	73.8	9,470,739	8.0
Impurity	32,291.5513	32291.5513 PS to PO,CNET	32,328.38	3	32,328.55	-5.2	Pass	16.71		1,391,591	73.8	8,068,217	6.9
Impurity	32,291.5513	32291.5513 PS to PO,Isobutyryl	32,345.57	3	32,345.58	-0.2	Pass	16.71		1,391,591	73.8	7,341,735	6.2
Impurity	32,291.5513	32291.5513 Isobutyryl	32,361.10	4	32,361.64	-16.8	Pass	16.71		1,391,591	73.8	4,972,482	4.2
Impurity	32,291.5513	32291.5513 n+Phosphate	32,372.20	5	32,371.53	20.8	Warning	16.71		1,391,591	73.8	1,348,086	1.1

Figure 11. Section of the INTACT Mass report displaying the processing results obtained for the analysis of the 100-mer sgRNA oligonucleotide. Four closely related impurities, all 100-mers, were identified with mass accuracies under 20 ppm. Impurity abundances were calculated based on their ESI-MS response and the lowest detected impurity was an extra phosphorylated 100-mer oligonucleotide present at 1%.

Conclusion

- The compliance-ready waters_connect INTACT Mass Application performs automated deconvolution of oligonucleotide spectra across the entire chromatographic space, providing fast intact mass confirmation of FLP (full length product) and impurity assignments as well as the required metrics (mass accuracy and abundance) to support impurity analysis.
- The waters_connect INTACT Mass Application is shown to provide better than 20 ppm mass accuracy for intact mass confirmation of both shorter siRNA and longer sgRNA oligonucleotides analyzed with an IP-RP LC-MS assay.
- The workflow described here provided purity information for sample components, down to about 1% abundance levels.
- The BioAccord System with ACQUITY Premier and the Premier CSH Column are ideally suited for detailed

intact mass confirmation and purity of therapeutic oligonucleotides for gene silencing and single guide oligonucleotides (sgRNA) for gene editing applications.

References

1. Sharma VK, Watts JK, Oligonucleotide Therapeutics: Chemistry, Delivery, and Clinical Progress, *Future Med Chem*, 2015, 7(16), 2221–2242.
2. Roberts TK, Langer R, Wood MJA, Advances in Oligonucleotide Drug Delivery, *Nat Reviews*, 2020, 19, 673–694.
3. Obika S, Sekine M – Editors of Synthesis of Therapeutic Oligonucleotides, 1st edition, Springer, 2018.
4. Sutton JM, Guimaraes GJ, Annavarapu V, van Dongen WD, Bartlett MG, Current State of Oligonucleotide Characterization Using Liquid Chromatography-Mass Spectrometry: Insight Into Critical Issues, *J ASMS*, 2020, 31, 1775–1782.
5. Pourshahian S, Therapeutic Oligonucleotides, Impurities, Degradants, and Their Characterization by Mass Spectrometry, *Mass Spectrometry Reviews*, 2019, 00, 1–35.
6. Doneanu., *et al,* An Automated Compliance-Ready LC-MS Workflow for Intact Mass Confirmation and Purity Analysis of Oligonucleotides, 2020, Waters Application Note, 720006820.
7. Doneanu., *et al,* Intact Mass Confirmation Analysis on the BioAccord LC-MS System for a Variety of Extensively Modified Oligonucleotides, 2020, Waters Application Note, 720007028.
8. Doneanu., *et al,* Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier, 2021, Waters Application Note, 720007301.
9. Wei B, Wang J, Cadang L, Goyon A, Chen B, Yang F, Zhang K, Development of an Ion Pairing Reversed-Phase Liquid Chromatography Mass Spectrometry Method for Characterization of Clustered Regularly Interspaced Short Palindromic Repeats Guide Ribonucleic Acids, *J Chrom A*, 2022, 1665, 462839, 1–8.
10. Reese CB, Yan H, An Approach to the Desulfurization of Oligonucleotide Phosphorothioates, T*etrahedron Lett,* 2003, 44, 2501–2509.

11. Krotz AH, Mehta R, Hardeee GE, Peroxide-Mediated Desulfurization of Phosphorothioate Oligonucleotides and Its Prevention, *J Pharm Sci,* 2005, 94, 341–348.

12. Wu L, White DE, Ye C, Vogt FG, Therfloth GI, Matsuhashi H, Desulfurization of Phosphorothioate Oligonucleotides via the Sulfur-By-Oxygen Replacement Induced by the Hydroxyl Radical During Negative Electrospray Ionization Mass Spectrometry, *J Mass Spectrom*, 2012, 47, 836–844.

13. Nikcevic I, Wyrzykiewicz TK, Limbach PA, Detecting Low-Level Synthesis Impurities in Modified Phosphorothioate Oligonucleotides Using Liquid Chromatography-High Resolution Mass Spectrometry, *Int. J Mass Spectrom*, 2011, 304, 98–104.

14. Fountain K, Gilar M, Gebler JC, Analysis of Native and Chemically Modified Oligonucleotides by Tandem Ion-Pair Reversed-Phase High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry, *Rapid Commun. Mass Spectrom*, 2003, 17, 646–653.

15. Capaldi D, Teasdale A, Henry S, Akhtar N, den Besten C, Gao-Sheridan S, Kretschmer M, Sharpe N, Andrews B, Burm B, Foy J, Impurities in Oligonucleotide Drug Substances and Drug Products, Impurities, Degradants, and Their Characterization by Mass Spectrometry, *Nucleic Acid Ther*, 2017, 27, 309–322.

16. Rousis SG, Cedillo I, Rentel C, Automated Determination of Early Eluting Impurities Using Ion-Pair Reversed-Phase Liquid Chromatography High Resolution-Mass Spectromtery, *Anal Biochem*, 2020, 595, 113623.

17. Henry Shion., *et al,* INTACT Mass - a Versatile waters_connect Application for Rapid Mass Confirmation and Purity Assessment of Biotherapeutics Suitable for Regulated and Non-Regulated Environments, Waters Application Note, 720007547, 2022.

18. Ferrige AG, Seddon MJ, Green BN, Jarvis SA, Skilling J, Disentangling Electrospray Spectra With Maximum Entropy, *Rapid Commun. Mass Spectrom.*, 1992, 6, 707–711.

19. Rodriguez AA, Cedillo I, McPherson AK, Formation of the N2-acetyl-2,6-diaminopurine Oligonucleotide Impurity Caused by Acetyl Capping *Bioorgan Med Chem Letters*, 2014, 24, 3243–3246.

20. Smith M, Beck T, Quantitation of a Low-Level Coeluting Impurity Present in a Modified Oligonucleotide by both LC-MS and NMR, *J Pharm Biomed Anal*, 2016, 118, 34–40.

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