Identification and quantitation of oligonucleotides, impurities, and degradation products

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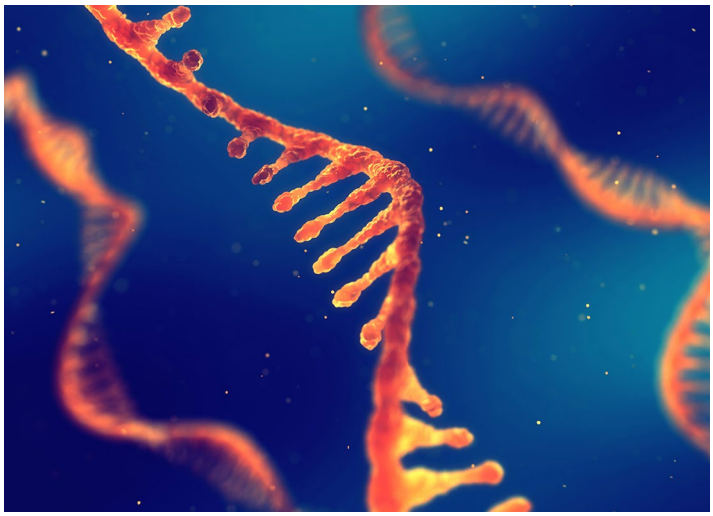


Application benefits

- The capabilities of high-resolution accurate mass (HRAM) and data-dependent tandem mass spectrometry (ddMS^2) enable confident identification, mapping, and relative quantitation of oligonucleotides, impurities, and degradation products in a single experiment.
- Novel software offers a streamlined workflow for the characterization of oligonucleotide impurities and degradation products.
- Comparative data analysis allows quick assessment of oligonucleotide purification methods and in-depth understanding of forced degradations of oligonucleotides.
- HRAM and ddMS^2 provide insight into the structure of high molecular weight (MW) impurities.

Goal

- To develop a sensitive and robust ddMS^2 method for identification, mapping, and relative quantitation of impurities in therapeutic oligonucleotides.
- To demonstrate the benefit of ddMS^2 in three case studies: (1) identification and relative quantitation of impurities in synthetic oligonucleotide samples purified by different methods; (2) characterization of degradation products generated by accelerated stress conditions; and (3) structural elucidation of high MW impurities.

Introduction

Oligonucleotides are synthesized, polymeric sequences of nucleotides (RNA, DNA, and their analogs) that are increasingly being developed as direct therapeutic agents against a wide range of disease conditions. They have attracted increasing attention from the biopharmaceutical industry due to the successes of applying this new modality for the treatment of rare diseases as well as their potential in treating common diseases and even coronavirus disease-2019 (COVID-19).[1-3] Therapeutic oligonucleotides produced by chemical synthesis carry various types of product-related impurities, including deletion sequences ('shortmers'), addition sequences ('longmers'), and the modified full-length species.[4-7] The n–x shortmers, the most common impurities present in synthetic oligonucleotides, are formed due to failed base coupling at the 5' end followed by incomplete capping, which may also result in n–1 impurities with different single deletions.[4] The longmers are mostly the n+1 or n+2 species, while the modified impurities correspond to the full-length product with modifications on its nucleobases or phosphorothioate linkages.[4] Degradation of synthetic oligonucleotides may introduce additional species in the products. Although chemically modified oligonucleotides are normally quite stable, the rate of degradation may be affected by their sequences and the presence of different stressors.[4-6]

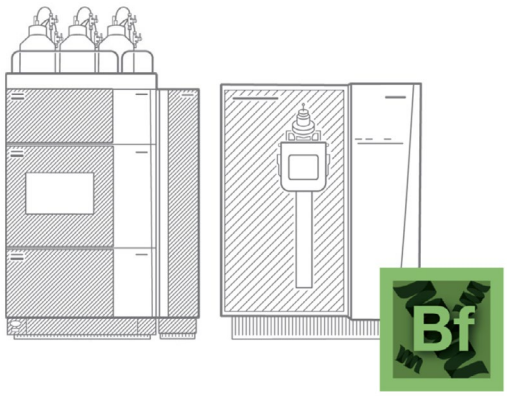
Advanced analytical tools are indispensable for the characterization of various oligonucleotide impurities and degradation products, some of which are present at a very low level. One popular method for oligonucleotide analysis is ion-pair reversed-phase liquid chromatography coupled with mass spectrometry (IP-RP LC-MS).[4-6] The MS1-based LC-MS method offers intact mass confirmation for oligonucleotides and their common impurities, however, it does not provide base-by-base sequence information and localization of modifications. Additionally, it is challenging to apply the MS1-based method for the identification of impurities with modifications and degradation products. By comparison, an HRAM based ddMS^2 method allows confident identification and mapping of unmodified and modified oligonucleotides, as demonstrated in our recent application note.[8] The introduction of powerful Oligonucleotide Analysis tools in Thermo Scientific™ BioPharma Finder™ 4.0 software enables fast processing and annotation of ddMS^2 data, as well as a comparative analysis of multiple raw files simultaneously.

In this application note, the capability of ddMS^2 was extended to the characterization of impurities and degradation products of a synthetic oligonucleotide using a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system, a Thermo Scientific™ DNAPac™ RP column, and BioPharma Finder software. The optimization of ddMS^2 was performed using a strategy described previously.[8] The optimal ddMS^2 method was then applied to identification, mapping, and relative quantitation of oligonucleotide impurities in samples purified with different methods or treated under different stress conditions. The power of HRAM and ddMS^2 for structural characterization of high MW impurities will also be highlighted.

Experimental

Equipment

- Thermo Scientific Orbitrap Exploris 240 mass spectrometer (P/N BRE725535)
- Thermo Scientific Vanquish Horizon UHPLC system (P/N 5400.0105)



Software

- Thermo Scientific BioPharma Finder 4.0 software (P/N OPTON-30988)

Columns

- Thermo Scientific DNAPac RP column (4 µm, 2.1 × 50 mm, P/N 088924)

Vials and closures

- Thermo Scientific™ 11 mm Autosampler Snap-It Caps (P/N C4011-50B)
- Thermo Scientific™ 11 mm Plastic Crimp/Snap Top Autosampler Vials (P/N C4011-13)
- Eppendorf™ DNA LoBind Microcentrifuge Tubes (P/N 022431005)

Solvents

- Thermo Scientific™ UHPLC-MS Water (P/N W81)
- Thermo Scientific™ UHPLC-MS Methanol (P/N A456-1)
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), >99.0%, TCI Chemicals (P/N H0424)
- *N,N*-Diisopropylethylamine (DIPEA), >99.0%, TCI Chemicals (P/N D1599)
- Solvent A: 2% (~190 mM) HFIP and 0.1% (~5.7 mM) DIPEA in water (pH 7.8)
- Solvent B: 0.075% (~7.1 mM) HFIP and 0.0375% (~2.1 mM) DIPEA in methanol

Oligonucleotide samples

A lyophilized DNA 21mer (CAG TCG ATT GTA CTG TAC TTA) was purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA) in the following two forms by choosing different purification methods available from IDT.

A. 21mer purified with standard desalting, and

B. 21mer purified using HPLC

No further purification was performed in house prior to LC-MS analysis. The 21mer sample described in this application note, unless otherwise mentioned as HPLC purified, is referred to as Sample A.

A stock solution of 1 mg/mL was prepared for two 21mer samples of different purity. This solution was diluted to 100 µg/mL before LC-MS analysis.

Forced degradation

In a heat stress study, the 21mer (1 mg/mL) was heated at 80 °C for 1, 2, 4, 6, and 24 hours. The samples were let to cool to room temperature and then diluted to 100 µg/mL for LC-MS analysis.

The oxidative stress was performed by incubating the 21mer (1 mg/mL) with 5% H_2O_2 at room temperature for 1, 2, 4, 6, and 24 hours. The samples were diluted to 100 µg/mL before LC-MS analysis.

Ion-pair reversed-phase liquid chromatography

Oligonucleotide separations were performed with a DNAPac RP column using a Vanquish Horizon UHPLC system. The autosampler was held at 5 °C while the column was maintained at 60 °C with the column oven Thermostatting Mode set to Still Air. The solvents were prepared in the original UHPLC solvent bottles to minimize salt contamination. The LC gradient used in this study is shown in Figure 1.

Time (min)	%B
0	10
0.5	10
4	22
10.5	35
11.7	80
13.5	80
13.6	10
15	10

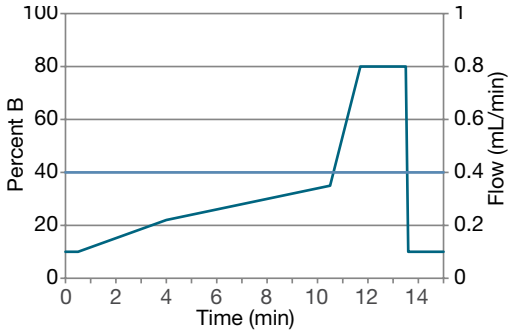


Figure 1. LC gradient for oligonucleotide separation at a flow rate of 0.4 mL/min

Mass spectrometry

The Orbitrap Exploris 240 mass spectrometer was operated with Thermo Scientific™ Xcalibur™ 4.4 software and controlled by Orbitrap Exploris Series 2.0 instrument control software. Instrument calibration was performed using Thermo Scientific™ Pierce™ FlexMix™ calibration solution. Data acquisition was performed in negative ion mode. The ddMS^2 methods were based on templates provided with the Orbitrap Exploris instrument control software (ICSW). Tables 1 and 2 list the global and scan parameters of the ddMS^2 methods, respectively.

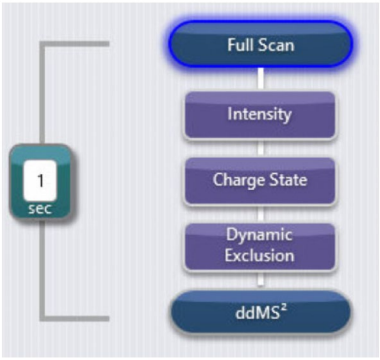
Table 1. Ion source properties and global method settings of ddMS² methods

Ion source properties	Value
Ion source type	H-ESI
Spray voltage	Static
Negative ion (V)	2,500
Sheath gas (Arb)	40
Aux gas (Arb)	20
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	300
Method settings	**Value**
Application mode	Peptide
Method duration (min)	15
Expected LC peak width (s)	6
Advanced peak determination	Checked
Default charge state	1
Internal mass calibration	Off

Table 2. Full Scan only and Full Scan/ddMS² method settings

Full scan only	
Full scan	**Value**
	
Orbitrap resolution	120,000
Scan range (*m/z*)	550–2,000
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	100
Max. injection time mode	Custom
Max. injection time (ms)	100
Microscans	1
Data type	Profile
Polarity	Negative

Table 2 (continued). Full Scan only and Full Scan/ddMS² method settings

Full Scan/ddMS²	
Full scan	**Value**
Orbitrap resolution	60,000
Scan range (*m/z*)	550–2,000
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	100
Max. injection time mode	Custom
Max. injection time (ms)	100
Microscans	1
Data type	Profile
Polarity	Negative
Intensity	**Value**
Intensity threshold	5.0e3
Charge state	**Value**
Include charge state(s)	1–20
Dynamic exclusion	**Value**
Dynamic exclusion mode	Auto
	
ddMS²	**Value**
Isolation window (*m/z*)	2
Isolation offset	Off
Collison energy mode	Stepped
Collision energy type	Normalized
HCD collision energies (%)	18,20,22[I]
Orbitrap resolution	30,000
Scan range mode	Auto
AGC target	Standard
Max. injection time mode	Custom
Max. injection time (ms)	200
Microscans	1
Data type	Profile
Data dependent mode	Cycle time
Time between master scans (s)	1

[I]Stepped NCE was varied from 10-12-14 to 20-22-24 with an incremental value of 1. A stepped NCE of 18-20-22 was used here for impurity identification, mapping, and relative quantitation in one experiment, as described in detail below.

Oligonucleotide analysis in BioPharma Finder software

The details about ddMS^2 method optimization and data analysis using BioPharma Finder software were described in an application note published recently.[8] Briefly, the identification and mapping of 21mer and its impurities were performed in the Oligonucleotide Analysis workflow within BioPharma Finder software. The main identification parameters include: Use MS/MS = Use All MS/MS, Mass Accuracy = 5 ppm, and minimum confidence = 0.80. The task of "Find All Ions in the Run" under Component Detection was chosen for most of the data analysis. The summed peak area of all charge states of 21mer was obtained by choosing the task of "Find All Masses in the Run".

Accurate masses of high MW impurities were obtained by performing the deconvolution of MS1 data using the Intact Mass Analysis workflow within BioPharma Finder software. The Xtract (Isotopically Resolved) deconvolution algorithm was used together with the Sliding Windows algorithm for generating source spectra in the retention time (RT) range above the full-length product (7.5–9.5 min). The output mass and charge state ranges were set 5,000 to 20,000 and 3 to 30, respectively. The sequence matching mass tolerance was set to 5 ppm.

BioPharma Finder software provides relative quantitation results of impurities in the modification results table under the Modification Summary section. The components used for % abundance calculation can be modified in the components table. The % abundance of the impurities checked in the modification result table are visualized in a modification plot. Some of the plots in this application note were generated in a spreadsheet using the export function from the modification results table.

Nomenclature of impurities

In this application note, the conventional "n-x" nomenclature is applied to deletion sequences produced from incomplete base coupling at the 5' end. In BioPharma Finder software, the impurities are assigned as "x-y" where x and y represent the position numbers of the first and last bases, respectively. The "x-y" species may carry -OH or -PO_4H at 5' end. For example, "A2-A21 [A2+Dephosphorylation]" assigned in the BioPharma Finder result stands for the n-1 impurity of 21mer with -OH at 5' end, while "A2-A21 [Nonspecific]" corresponds to that with 5'-PO_4H. Unless otherwise mentioned, the "n-x" impurities in this application are referred to as those with 5'-OH.

Results and discussion

HRAM mass spectrometry of 21mer

The Vanquish Horizon UHPLC system coupled with a DNAPac RP column provides robust and reproducible separation of oligonucleotides and impurities. Figure 2 displays a representative mass spectrum of 21mer. A resolution setting of 120,000 at *m/z* 200 provided baseline separation of the 21mer isotopes, thereby allowing accurate mass measurement of this intact oligonucleotide (~1 ppm). The inset in Figure 2 highlights the low salt adduct (< 2%) under the experimental conditions employed in this study. To minimize the level of salt adducts, it is recommended to carefully choose the ion-paring reagents and LC solvents.

Optimization of the stepped NCE for 21mer and impurities

The optimal NCE varies depending on the oligonucleotide sequence, size, modification, and charge state, and hence needs to be carefully determined. As described previously,[8] the ability to compare multiple raw files in BioPharma Finder software led to a quick survey and determination of the optimal stepped NCE value or range for an oligo. The same strategy for optimizing stepped NCE was applied to 21mer in this study.

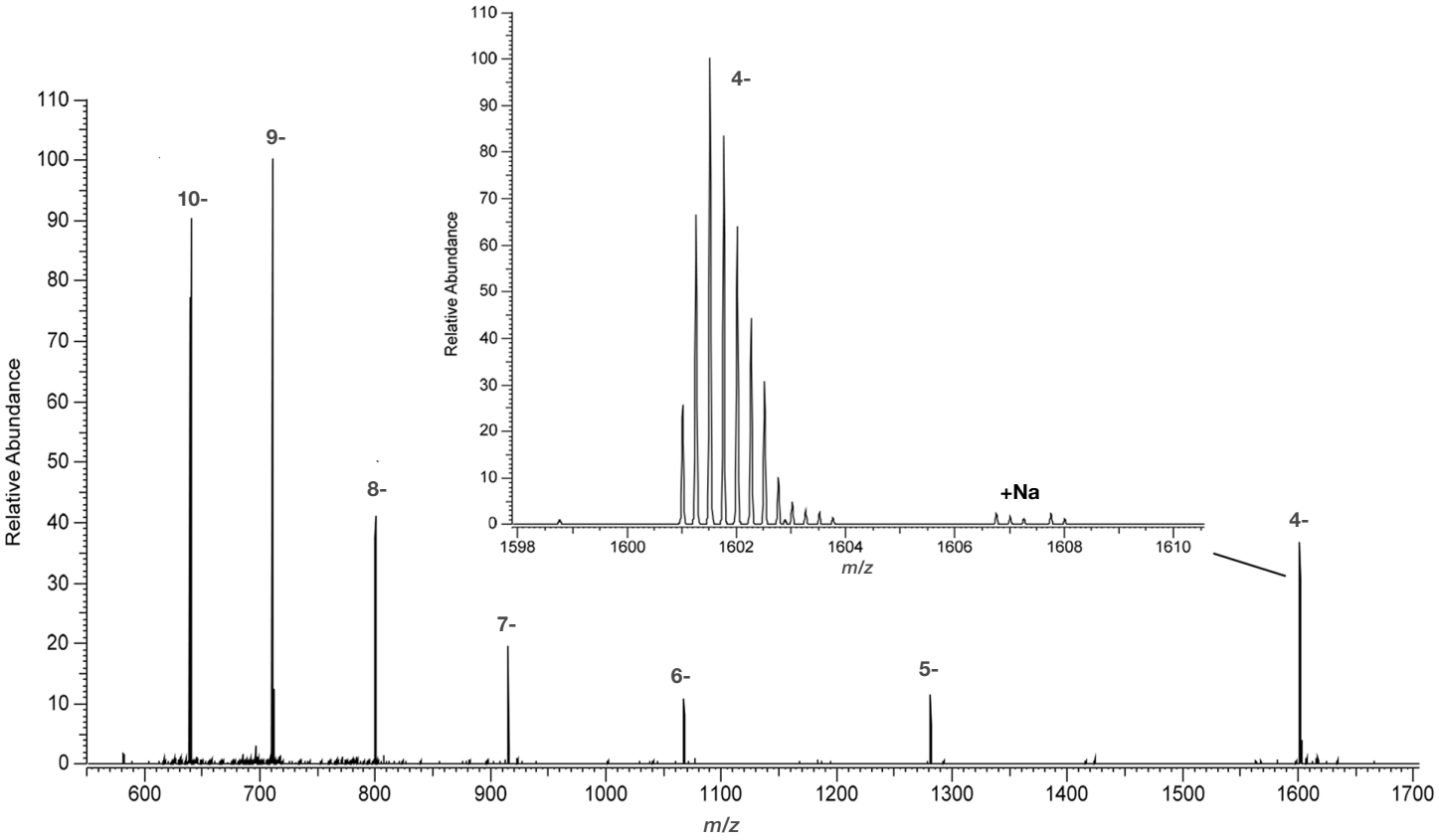


Figure 2. Mass spectrum of 21mer acquired at a resolution setting of 120,000 (at *m/z* 200). The inset shows the isotopic envelope of the 4- and its sodium adduct present at a very low level (< 2%).

Figure 3 displays fragment cover maps and annotated MS^2 spectra of the 9- of 21mer acquired using two different stepped NCEs. While a stepped NCE of 10-12-14 provided insufficient fragmentation and hence incomplete coverage of 21mer (Figure 3a and 3b), a stepped NCE of 14-16-18 gave 100% coverage for this oligo.

The average structural resolution (ASR) is a score that evaluates the completeness of fragment coverage of an oligonucleotide. An ASR of 1.0 indicates a full fragment coverage of an oligonucleotide sequence. The larger the ASR is than 1.0, the less complete the fragment coverage map is. Figure 4 shows ASR scores measured for eleven $ddMS^2$ raw files of 21mer (9-) and a selected impurity (n-7, 3-) acquired at stepped NCEs ranging from 10-12-14 to 20-22-24. While a wide range of stepped NCEs (11-13-15 to 20-22-24) provided complete or nearly complete fragment coverage for the 9- of 21mer (ASR = 1.0-1.2; Figure 4a), higher stepped NCEs (NCE ≥ 18-20-22) worked better for the 3- of the n-7 species (ASR = 1.3-1.5; Figure 4b). A stepped NCE of 18-20-22 was determined to give good coverage for 21mer and its impurities; hence it was used for studies described in the following sections. However, if large impurities (e.g., n-1) are of interest, it is recommended to use stepped NCEs optimal for the full-length product, which is between 12-14-16 and 16-18-20 for the 21mer studied here (Figure 4a).

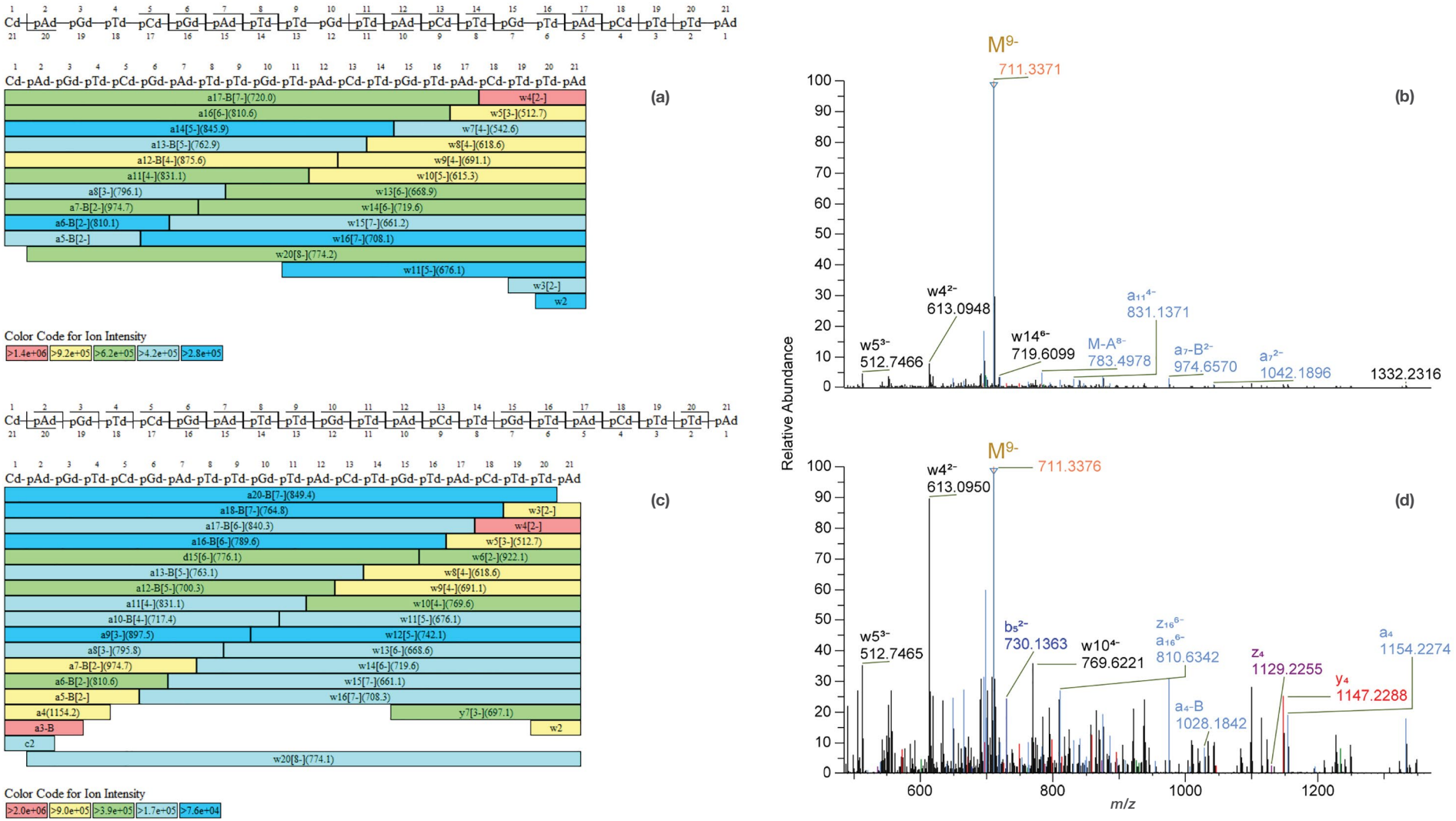


Figure 3. Fragment coverage maps and annotated MS² spectra of the 9- of 21mer acquired at stepped NCEs of 10-12-14 (a-b) and 14-16-18 (c-d)

(a) 21mer, 9-

No.	Condition	MS Area	Delta (ppm)	Confidence Score	Average Structural Resolution	ID Type
1	NCE101214	1.10E+08	-1.36	100.0	1.4	MS2
2	NCE111315	1.08E+08	-1.28	100.0	1.2	MS2
3	NCE121416	9.97E+07	-1.06	100.0	1.0	MS2
4	NCE131517	1.01E+08	-1.13	100.0	1.0	MS2
5	NCE141618	1.34E+08	-1.06	100.0	1.0	MS2
6	NCE151719	1.40E+08	-0.52	100.0	1.0	MS2
7	NCE161820	1.38E+08	-0.52	100.0	1.0	MS2
8	NCE171921	1.39E+08	-0.60	100.0	1.1	MS2
9	NCE182022	1.34E+08	-0.52	100.0	1.2	MS2
10	NCE192123	1.41E+08	-0.83	100.0	1.1	MS2
11	NCE202224	1.32E+08	-0.60	100.0	1.2	MS2

(b) n-7, 3-

No.	Condition	MS Area	Delta (ppm)	Confidence Score	Average Structural Resolution	ID Type
1	NCE101214	1.13E+05	0.63	100.0	2.0	MS2
2	NCE111315	1.11E+05	-0.18	100.0	2.0	MS2
3	NCE121416	1.12E+05	0.63	100.0	2.0	MS2
4	NCE131517	1.10E+05	0.97	100.0	2.0	MS2
5	NCE141618	1.14E+05	1.20	100.0	1.8	MS2
6	NCE151719	1.14E+05	0.63	100.0	1.8	MS2
7	NCE161820	1.14E+05	1.09	100.0	2.0	MS2
8	NCE171921	1.15E+05	0.40	100.0	1.6	MS2
9	NCE182022	1.14E+05	1.32	100.0	1.4	MS2
10	NCE192123	1.15E+05	0.97	100.0	1.4	MS2
11	NCE202224	1.13E+05	0.74	100.0	1.3	MS2

Figure 4. Comparative analysis of raw files acquired using 11 different stepped NCE settings for (a) 21mer (9-) and (b) n-7 (3-). Shaded in green, yellow, and red are ASRs of 1.0-1.2, 1.3-1.5, and >1.5, respectively.

Identification, mapping, and relative quantitation of impurities of 21mer purified using a standard desalting vs. an HPLC method

The ddMS^2 method not only allows quick confirmation of oligonucleotide sequence and localization of modifications (if any), but it can also confidently identify very low abundant impurities that are not discernible in a chromatogram. Figures 5a and 5b display total ion chromatograms (TICs) of two 21mer samples (desalting and HPLC) with shaded colors highlighting the impurities identified in each sample. The inset of Figure 5a shows selected ion chromatogram (SIC) (top) and zoomed TIC of n-16 present at a very low level (~0.015%, NL: 6.9E+4). However, this impurity was confidently identified by its high-quality MS^2 spectrum (Figure 5c) and a complete fragment coverage map (Figure 5d).

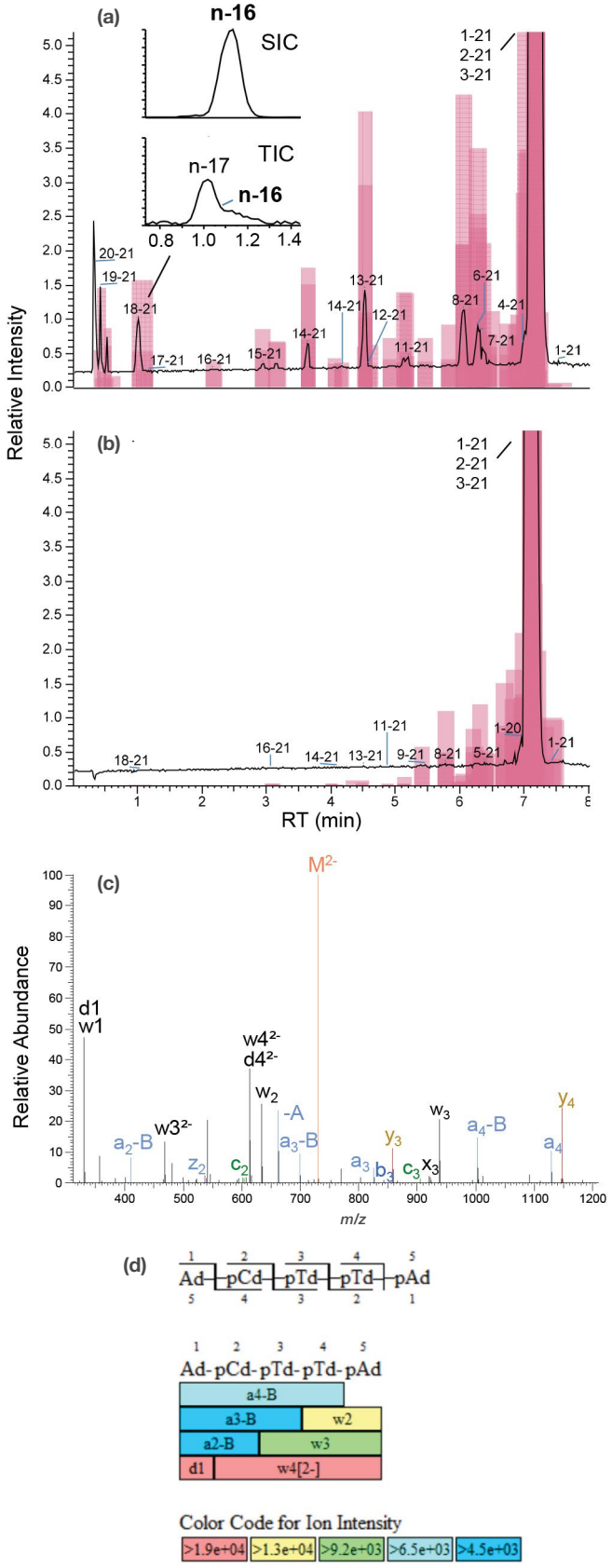


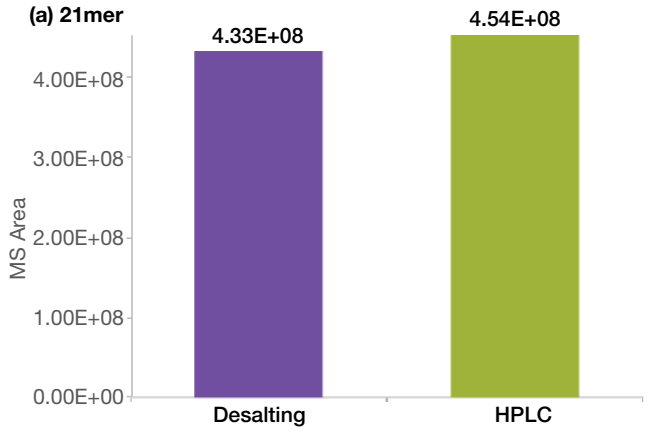
Figure 5. TICs of 21mer purified using (a) standard desalting vs. (b) HPLC method with the shaded representation of oligonucleotide and impurities identified. The inset in (a) displays the zoom-in of TIC (bottom) and SIC (top) of n-16, a very low abundant (~0.015%) impurity in 21mer. (c) MS^2 spectrum and (d) fragment coverage map of n-16 confirming the identity of this low-level impurity. Note: Not all impurities identified are shown in Figures 5a and 5b.

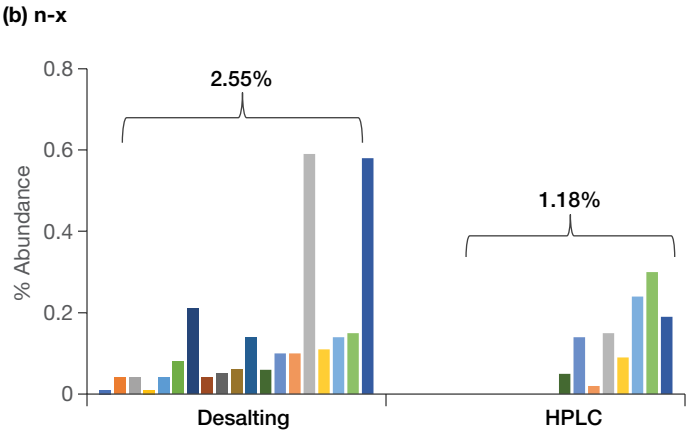
Figure 6 compares the MS area of 21mer and % abundances of its n-x impurities in two samples purified with the desalting or HPLC method. While the total peak area of the full-length product (21mer) remained nearly the same in two samples (Figure 6a), a drastic decrease in % abundance of n-x impurities (from 2.55% to 1.18%), particularly the shorter ones, was measured in the HPLC-purified sample as compared to the desalting sample (Figure 6b and 6c), as anticipated. A similar trend was observed for n-x impurities with phosphate at 5' end (data not shown). These results showcase the ease of using BioPharma Finder software for relative quantitation of oligonucleotide impurities and quick assessment of sample purity.

Forced degradations of 21mer

Degradation of DNA can occur at high rates *in vivo* or under physiological conditions.[6] To understand degradation pathways of synthetic oligonucleotides, the effects of acid, base, oxidative, thermal, and photolytic stresses on these molecules have been extensively studied.[4-6] In this work, thermal and oxidative stresses on 21mer were investigated.

Figure 7 illustrates that heating 21mer for an extended period (up to 24 hours) resulted in significant degradation of this oligonucleotide. A drastic decrease in the abundance of 21mer was observed between 2 to 4 hours of heat stress. At 6 hours, abundant degradation products in various lengths were detected. Extending the heat stress to 24 hours led to nearly complete degradation of 21mer and larger impurities.





(c) n-x

Impurity	Desalting	HPLC	Impurity	Desalting	HPLC
n-1	0.58	0.19	n-11	0.05	0
n-2	0.15	0.30	n-12	0.04	0
n-3	0.14	0.24	n-13	0.21	0
n-4	0.11	0.09	n-14	0.08	0
n-5	0.59	0.15	n-15	0.04	0
n-6	0.10	0.02	n-16	0.01	0
n-7	0.10	0.14	n-17	0.04	0
n-8	0.06	0.05	n-18	0.04	0
n-9	0.14	0	n-19	0.01	0
n-10	0.06	0	**Total**	**2.55**	**1.18**

Figure 6. (a) Total MS area of all detected charge states (4- to 11-) of 21mer in two samples purified with the desalting or HPLC method. (b) Modification plots and (c) % abundance table of n-x impurities of 21mer in the desalting vs. HPLC samples. This table was created using the spreadsheet export functionality in BioPharma Finder software.

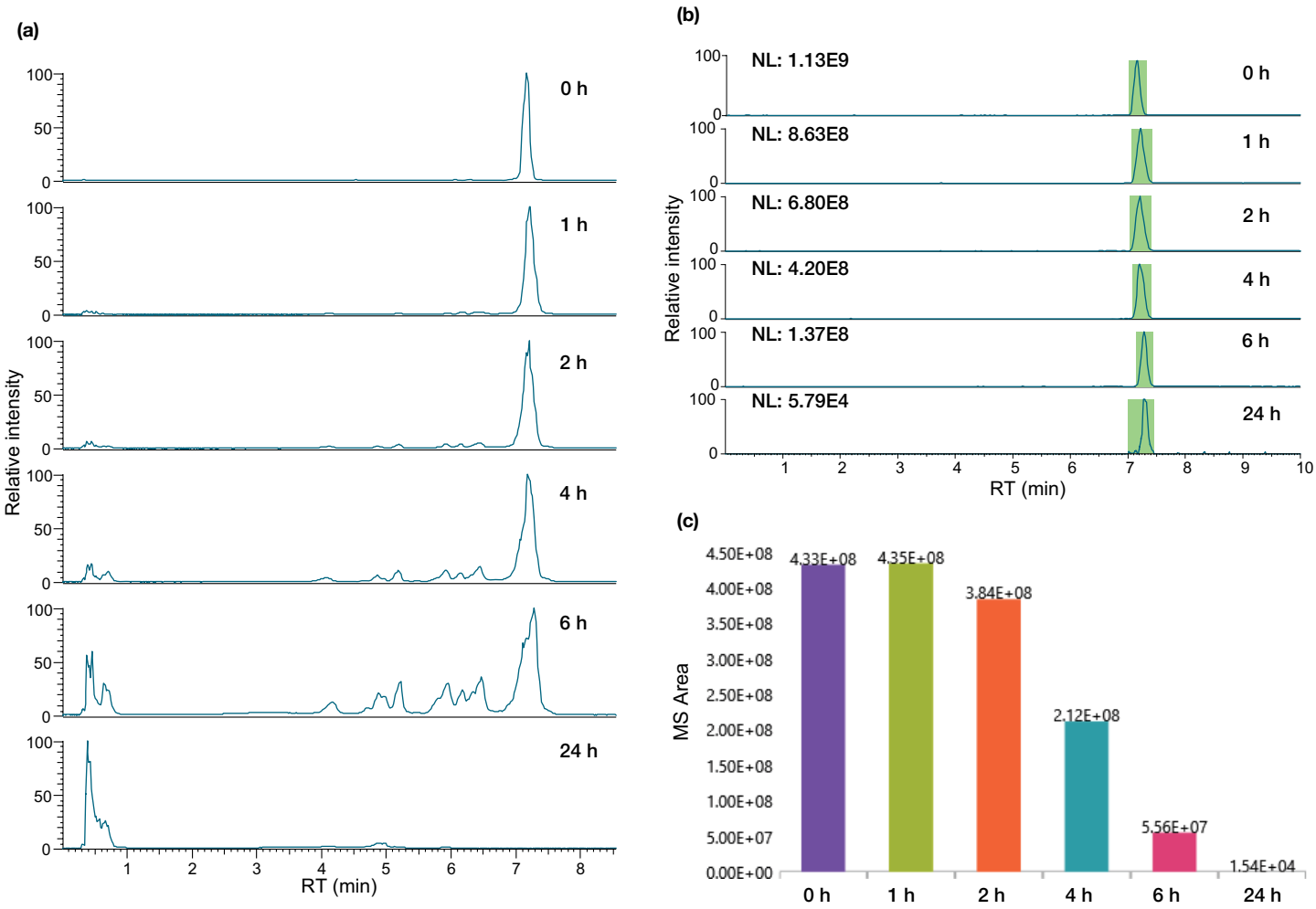


Figure 7. (a) TICs and (b) SIC of the 21mer samples heated at 80 °C for various time points. (c) Total MS area of all charge states of 21mer in the control and heat stress samples.

The ddMS^2 results of heat stress samples revealed different behaviors for different impurities (Figure 8). While the % abundance of n-1 gradually decreased, the ratio of n-5 increased at 1 h and then steadily declined (Figure 8a). By contrast, a significant increase in % abundance of small impurities (e.g., n-18) was seen at 24 h (Figure 8a). However, the main changes upon heat degradation of 21mer were significant increases in % abundances of n-x impurities with 5'-PO_4H (Figure 8b), base loss products (Figure 8c), and the impurities associated with depyrimidination or depurination (Figure 8d).

In contrast to heat stress, oxidative stress did not result in a significant change in TICs of 21mer (Figure 9a). The level of n-x impurities increased slightly upon H_2O_2 treatment but then remained stable over time (Figure 9b). Similarly, the MS area of 21mer (Figure 9c) did not show a significant change (Figure 9c). However, the relative abundance of oxidized 21mer increased dramatically in the 24 h course of oxidative stress, as anticipated. Taken together, the results of forced degradation showcased the power of using ddMS^2 for identification and relative quantitation of impurities in a single experiment as well as for gaining insight into the degradation pathways of oligonucleotides.

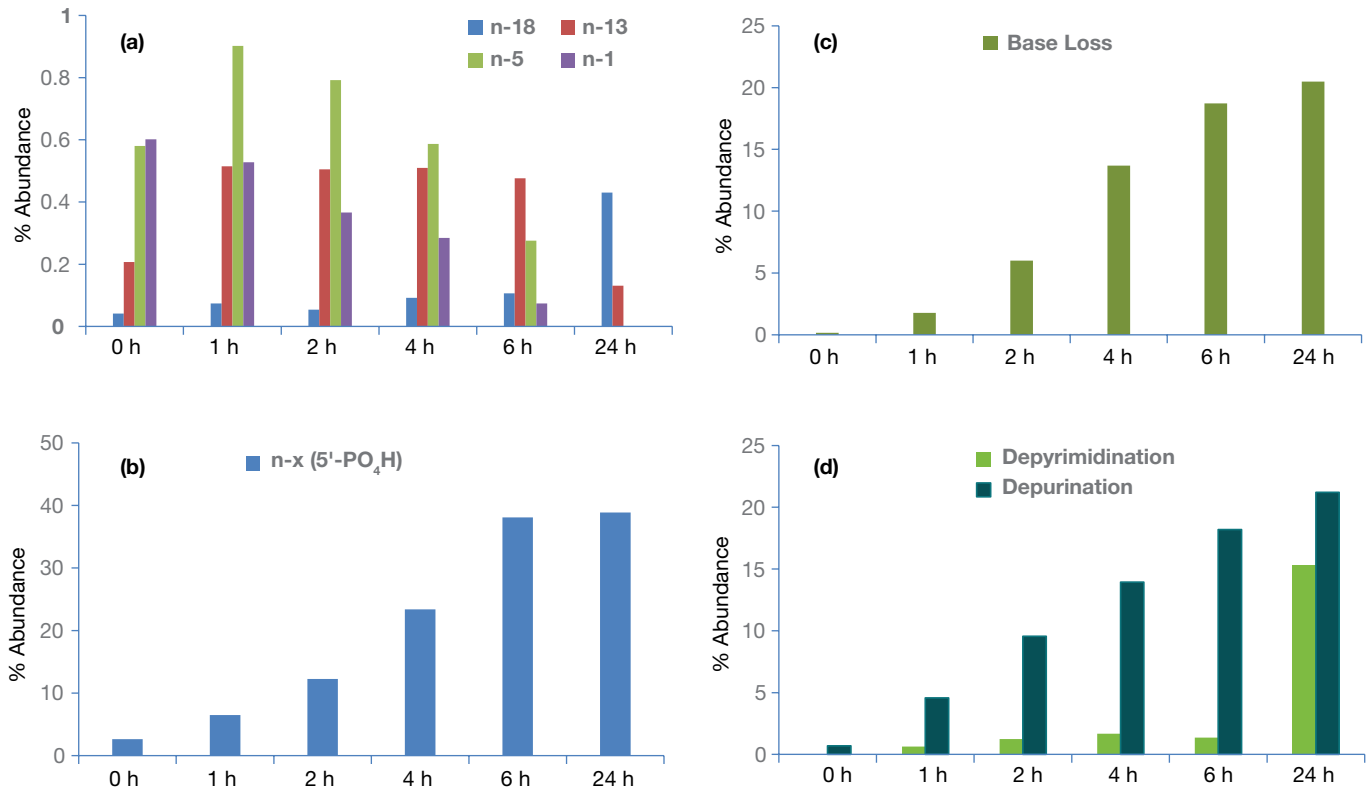


Figure 8. % Abundances of (a) selected n-x (5'-OH) impurities and total % abundances of (b) n-x (5'-PO_4H) impurities, (c) base loss products, and (d) products associated with deprimidination and depurination in the control (0 h) and heat stress (1–24 h) samples

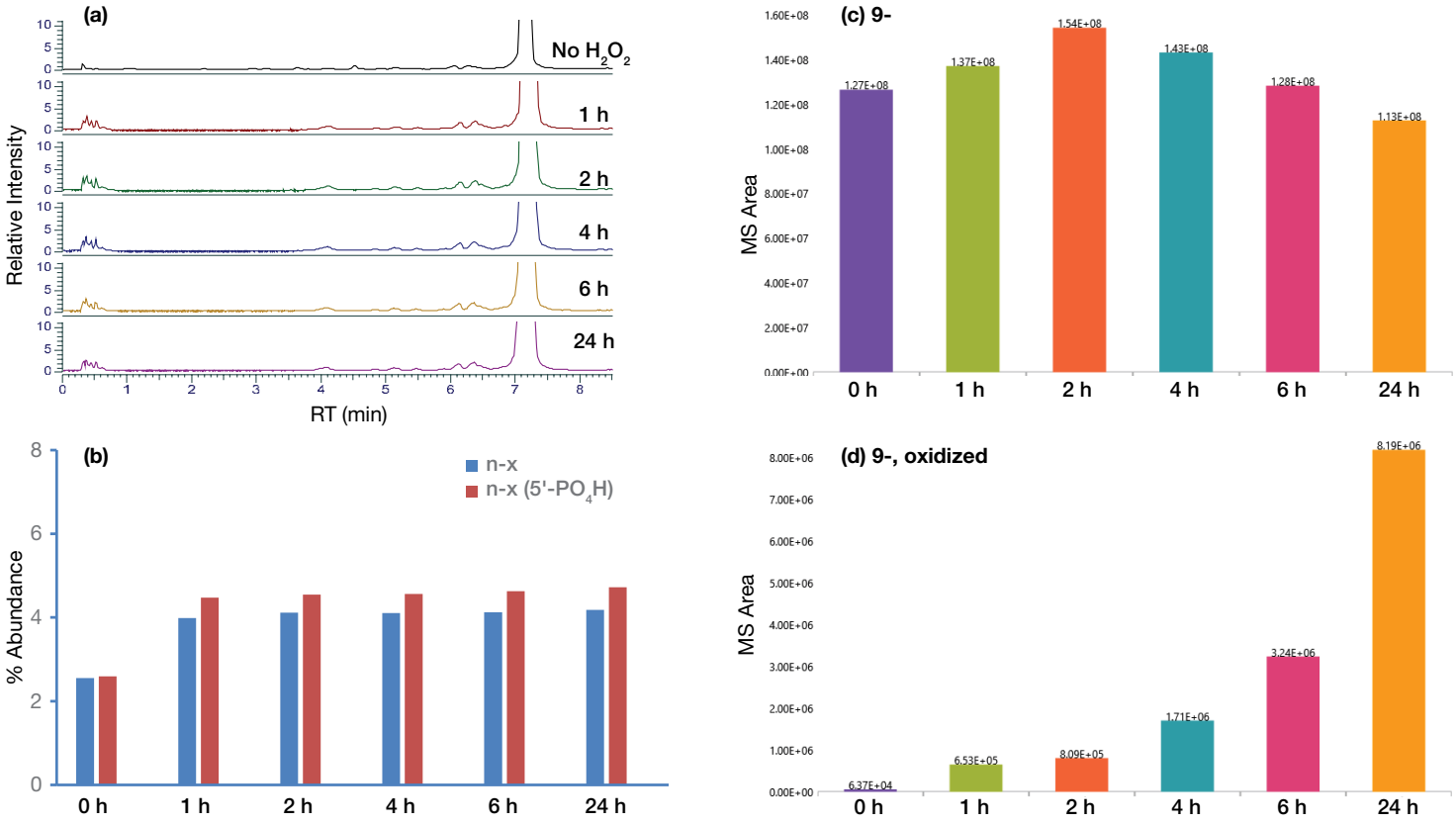


Figure 9. (a) TICs of the 21mer control (no H_2O_2 treatment) and 21mer samples treated by 5% H_2O_2 for 1 h, 2 h, 4 h, 6 h, and 24 h; (b) % Abundances of n-x impurities and MS area of (c) non-oxidized and (d) oxidized 9- in the control and oxidative stress samples

High MW impurities of 21mer

In the 21mer sample purified with the desalting method, a series of high MW impurities were detected at RTs later than that of the full-length product. The main high MW species was eluted out at ~9 min (Figure 10a). Interestingly, these MW species were completely absent in the HPLC sample (Figure 10b), showing the effectiveness of HPLC for the removal of these species. The chromatographic peak at RT ~ 9 min corresponds to two major charge state envelopes, one of which is displayed in Figure 10c. The HRAM capability of the Orbitrap Exploris 240 mass spectrometer allowed accurate mass measurement of these high MW impurities. The deconvolution of MS1 data using the Sliding Windows algorithm in the Intact Mass Analysis workflow within BioPharma Finder software revealed a series of peaks whose MWs match perfectly with the total masses of a 21mer plus its n-x impurities, i.e., "M + (n-x)" (Figure 10d). The mass errors measured for the eight largest impurities were all less than 1 ppm (Table 3). It should be mentioned that similar high MW species have been observed for oligonucleotides with A at 3' end.[9] In that study, a branched structure, where the 3'-OH of n-x is linked to the exocyclic amino group of the 3'-A of 21mer, was proposed for these high MW impurities. However, no MS^2 data of high MW impurities was acquired.

Table 3. Mass errors for selected high MW species. M = 21mer

Species	Exp. MW	Theo. MW	Error (ppm)
M + n-1	12261.033	12261.044	0.9
M + n-2	11956.994	11956.998	0.4
M + n-3	11667.947	11667.952	0.4
M + n-4	11354.898	11354.894	0.3
M + n-5	11050.853	11050.848	0.4
M + n-6	10721.802	10721.796	0.6
M + n-7	10417.755	10417.750	0.5
M + n-8	10128.712	10128.703	0.9

In this study, $ddMS^2$ was employed to facilitate structural elucidation of these high MW impurities. A stepped NCE of 11-13-15 was used to acquire $ddMS^2$ data of high MW impurities. Figure 11 displays fragment coverage maps of the "M + (n-1)" impurity obtained by searching its $ddMS^2$ data against a 41 nt sequence with 3'- (Figure 11a) or 5'-end (Figure 11b) of n-1 placed to the 3' end of 21mer. Interestingly, mapping the former sequence did not identify any MS^2 fragments (e.g., w_1–w_{20}) covering the 3' end of the 41 nt sequence (Figure 11a). By contrast, a series of *w* and *x* fragments were identified using the 41 nt sequence

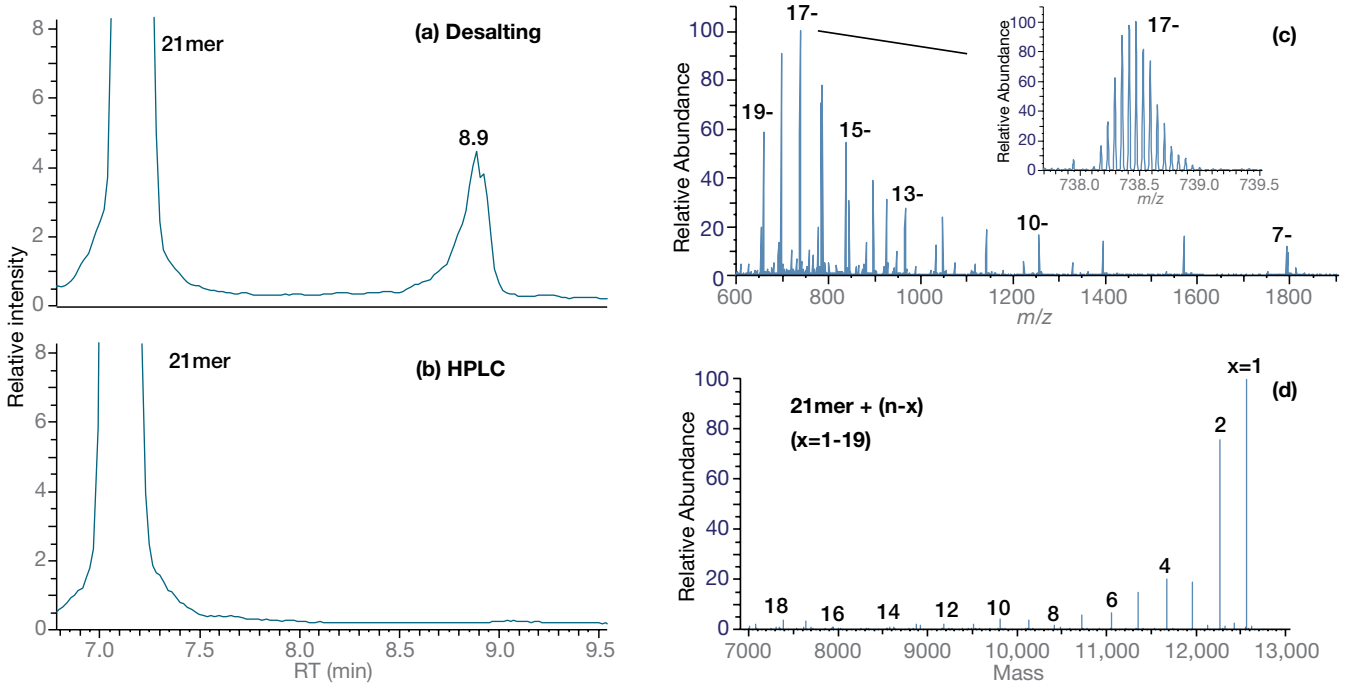
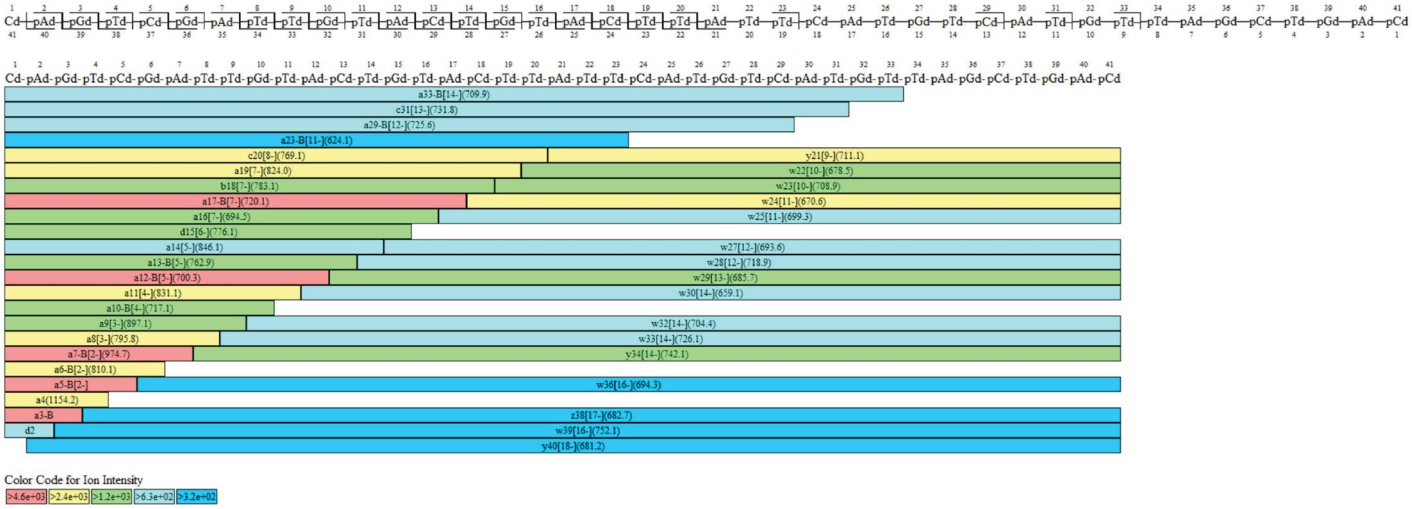


Figure 10. Zoom-ins of TICs of 21mer purified with (a) desalting or (b) HPLC method showing high MW species eluting at ~9 min; (c) mass spectrum of a high MW species with inset showing the isotopically resolved envelope of its 17-charge state; (d) deconvolution spectrum of high MW species assigned as "21mer + (n-x)" with x = 1-19

where the 5’ end of n-1 was placed to the 3’ end of 21mer (Figure 11b). Similar ddMS^2 results were also observed for other “M + (n-x)” impurities (data not shown). The two chains (21mer and n-x) likely form a similar branched structure described before[9]. However, our ddMS^2 data indicate the linkage occurring on the 5’ side of n-x instead of its 3’ end, as proposed in the previous study[9]. These results show that ddMS^2 can provide additional insight into the structure of high MW impurities that may not be obtained by MS1 alone. Further structural elucidation of high MW impurities will be the subject of future study.

(a) M + 3’ (n-1)



(b) M + 5’ (n-1)

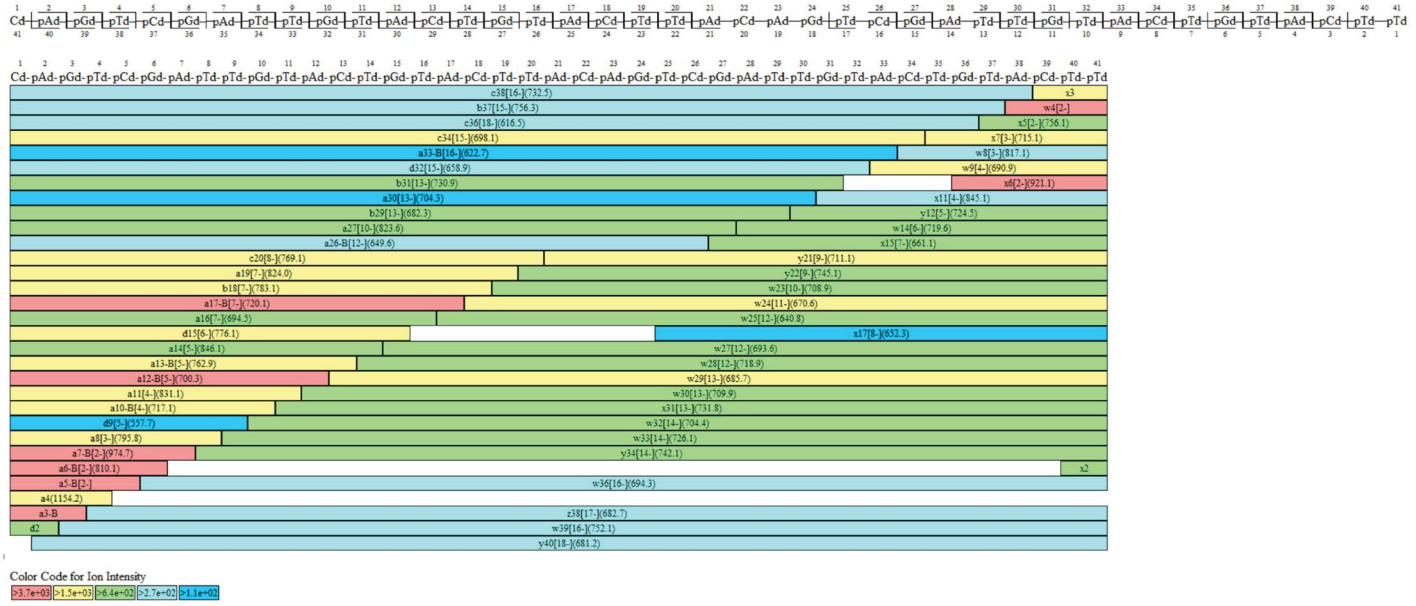


Figure 11. Fragment coverage maps of (a) “M + 3’ (n-1)” and (b) “M + 5’ (n-1)”, in which the 3’ and 5’ ends of n-1 are placed to the 3’ end of M (21mer), respectively, to the sequences for oligonucleotide mapping in BioPharma Finder software

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Conclusion

In summary, the ddMS^2 approach described in this application note enables confident identification, mapping, and relative quantitation of oligonucleotides and their impurities in a single experiment. The high confident identification can be obtained for very low abundant impurities that are not discernible at the chromatographic level. The three case studies presented here demonstrate the ability of ddMS^2 for quick assessment of oligonucleotide purity, comparison of different purification methods, and in-depth understanding of forced degradations of oligonucleotides. Additionally, the capabilities of HRAM and ddMS^2 offered by the Orbitrap Exploris 240 mass spectrometer provide insight into the structure of high MW impurities, which cannot be obtained using the MS1 method alone. The new Oligonucleotide Analysis workflow introduced in BioPharma Finder software provides a streamlined process from sequence creation and oligonucleotide mapping to relative quantitation of impurities and result reviewing. An array of tools for comparative analysis offered by BioPharma Finder software allow easy optimization of ddMS^2 and comparisons of data from different studies, including impurity analysis and time-course degradation.

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