INVESTIGATION OF OLIGONUCLEOTIDE DEAMINATION USING HIGH-RESOLUTION MASS SPECTROMETRY

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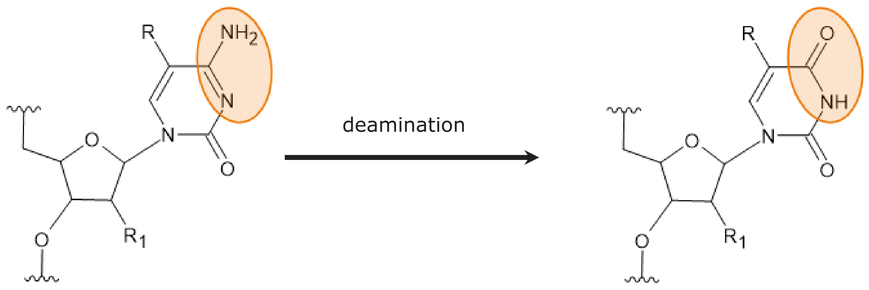
INTRODUCTION

• Oligonucleotide deamination is a degradation reaction of nucleic acids that primarily occurs at cytosine (C) and 5-methylcytosine residues(5-MeC), resulting in the formation of uracil (U) and thymine (T), respectively [1]. This common degradation process can significantly impact the stability and efficacy of therapeutic oligonucleotides [2].

• Deamination can be influenced by various factors, including the oligonucleotide sequence and environmental conditions such as pH and temperature. Understanding and controlling deamination is crucial for the development and storage of oligonucleotide-based therapies [3].

• Deamination leads to impurities that differ from the full-length product (FLP) in sequence, potentially causing off-target effects in oligonucleotide therapeutics. The deaminated degradation products exhibit a loss of NH (−15.0146 Da) and a gain of O (+15.9994 Da), resulting in a mass difference of +0.9848 Da.

• The difference of +1 Da results in overlapping isotope patterns for the deaminated impurity and the full-length product (FLP) as shown in Figure 1.



R = H (Cytosine to Uracil) or CH_3 (5-methyl Cytosine to Thymine)

R_1 = H (DNA), OH (RNA) or other modification

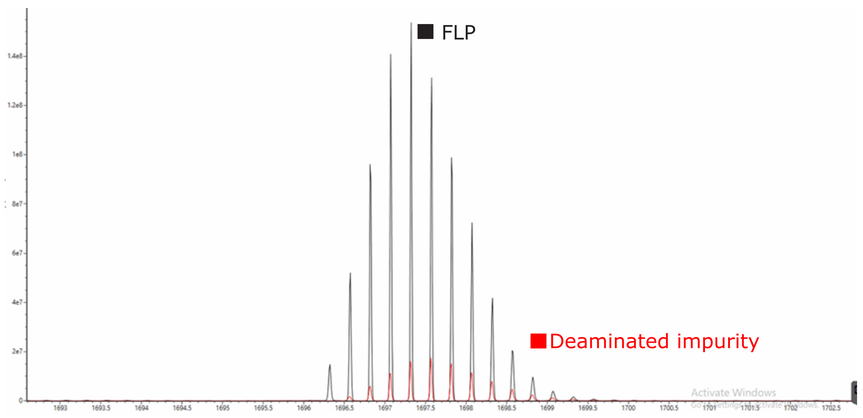


Figure 1. Combined ESI-MS spectra of the FLP and a co-eluting deaminated oligonucleotide impurity. These overlapping isotopic distributions were recorded for the triply charged state ([M-3H] $^{-3}$) oligo precursor on a Xevo G3 QTof instrument.

Figure 2. Xevo™ G3 QTof instrument coupled to the ACQUITY™ Premier UPLC™ BSM (Binary Solvent Manager) System.

Experimental Section

Sample preparation

A antisense phosphorothioated deaminated synthetic oligonucleotide (ASO) impurity was spiked into its pure non-deaminated oligonucleotide (FLP) at six different concentration levels: 0.1, 0.25, 0.5, 0.75, 1.5 and 5.0 %.

LC-MS experimental conditions

An ACQUITY™ Premier UPLC™ BSM System equipped with a 2.1 x 50 mm ACQUITY™ Premier OST Column (P/N 186009484) was used for all oligonucleotide separations. A IP-RP mobile phase containing 5 mM TBuAA (tri-butyl ammonium acetate), 1 µM EDTA in 10% ACN was used as Solvent A, while the composition of Solvent B was 5 mM TBuAA, 1 µM EDTA in 80% ACN. Gradient separations were performed from 60% B to 80% B over 1.45 min with a total runtime of 3.7 min. The column flow rate was 0.25 mL/min and the column temperature was 50°C. UV data was acquired at a fixed wavelength of 260nm.

A Xevo™ G3 QTof mass spectrometer was used for acquiring the ESI-MS data in negative ion mode, over the m/z range of 400-4000, with an acquisition time of 0.5 sec. The capillary voltage was set to 2.0 kV, cone voltage at 60V, source temperature 120°C, desolvation temperature at 450°C, 600 L/h desolvation gas and a collision energy of 6V.

Informatics

Data acquisition and processing was performed using *waters_connect*™ software. ESI-MS spectra of oligonucleotides were centroided with the Centre Spectrum processing option from *waters_connect*™.

METHODS

• The presence of a deaminated degradation product causes a small shift in peak height of each isotope peak, which has been reported to allow estimation of the relative amounts of FLP and deaminated impurity [1]

• According to this publication, the amount of deamination can be derived directly from the isotope peak heights in the form of an *Isotopic Distribution Factor* (IDF), which is calculated using the equation:

$$IDF = \sum (\text{Isotopic peaks 6 to 12}\rangle - \sum (\text{Isotopic Peaks 1 to 4})$$

where:

$\sum$ (Isotopic peaks 6 to 12〉 = Sum of the relative peak height intensities of the isotopic peaks located to the *RIGHT* of the calculated most abundant isotope (P5) of the parent oligonucleotide (P6 to P12)

$\sum$ (Isotopic peaks 1 to 4〉 = Sum of the relative peak height intensities of the isotopic peaks located to the *LEFT* of the calculated most abundant isotope (P5) of the parent oligonucleotide (P1 to P4)

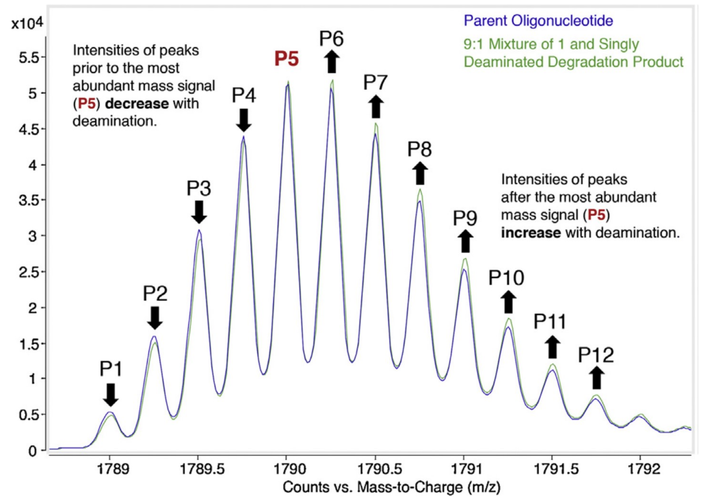


Figure 3. High resolution mass spectra of parent oligonucleotide 1 (blue trace) and 9:1 mixture of a singly deamination degradation product (green trace). The shift in the intensity of the P6-P12 isotopic peaks that accompanies deamination is visible in the green trace profile. Please note that the intensity of the most abundant isotope (labeled P5 above) was not included in the calculations presented in this poster, or in the Ionis publication [1].

RESULTS

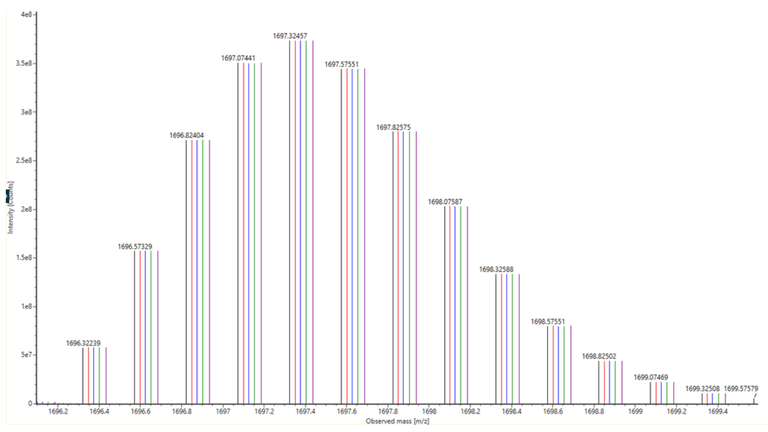


Figure 4. Five combined centroided isotopic distributions replicates recorded on a Xevo G3 QTof instrument. These spectra were obtained from the raw acquired ESI-MS spectra after using the Centre Spectrum processing option from *waters_connect*™.

A

FLP only (control for 0.10% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.56	42.00	72.56	93.85	100.00	223.97	92.22	74.76	54.27	35.61	21.32	11.75	5.94	295.87	71.90
Injection 2	15.49	42.10	72.56	93.89	100.00	224.03	92.20	74.91	54.34	35.65	21.36	11.74	5.96	296.17	72.14
Injection 3	15.47	42.06	72.58	93.85	100.00	223.97	92.25	74.82	54.24	35.65	21.35	11.74	5.97	296.03	72.06
Average	15.51	42.05	72.56	93.86			92.22	74.83	54.29	35.64	21.34	11.74	5.95	Average	72.03
stdev	0.05	0.05	0.01	0.02			0.02	0.08	0.05	0.02	0.02	0.00	0.02	StDev	0.12
%RSD	0.30	0.11	0.02	0.02			0.03	0.10	0.10	0.06	0.10	0.02	0.29	%RSD	0.17

FLP only (control for 0.25% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.57	42.01	72.55	93.87	100.00	223.99	92.22	74.78	54.34	35.62	21.31	11.74	5.95	295.97	71.98
Injection 2	15.48	42.09	72.53	93.83	100.00	223.94	92.23	74.84	54.39	35.57	21.38	11.72	5.98	296.10	72.17
Injection 3	15.50	42.01	72.53	93.87	100.00	223.90	92.13	74.84	54.26	35.63	21.34	11.73	5.99	295.91	72.00
Average	15.51	42.04	72.53	93.86			92.19	74.82	54.33	35.61	21.34	11.73	5.97	Average	72.05
stdev	0.05	0.05	0.01	0.02			0.06	0.03	0.07	0.03	0.03	0.01	0.02	StDev	0.10
%RSD	0.29	0.11	0.01	0.02			0.06	0.04	0.12	0.10	0.15	0.08	0.28	%RSD	0.14

FLP only (control for 0.50% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.54	42.01	72.46	93.71	100.00	223.72	92.09	74.71	54.26	35.59	21.27	11.69	5.95	295.57	71.85
Injection 2	15.52	42.08	72.52	93.89	100.00	224.00	92.18	74.82	54.27	35.66	21.30	11.72	6.01	295.96	71.97
Injection 3	15.48	42.00	72.51	93.76	100.00	223.76	92.16	74.75	54.19	35.60	21.29	11.72	5.95	295.66	71.90
Average	15.51	42.03	72.49	93.79			92.14	74.76	54.24	35.62	21.29	11.71	5.97	Average	71.90
stdev	0.03	0.04	0.03	0.09			0.05	0.05	0.05	0.04	0.02	0.01	0.03	StDev	0.06
%RSD	0.18	0.10	0.05	0.10			0.05	0.07	0.09	0.11	0.07	0.12	0.52	%RSD	0.08

FLP only (control for 0.75% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.45	42.03	72.43	93.73	100.00	223.64	92.10	74.74	54.18	35.60	21.31	11.70	5.97	295.59	71.96
Injection 2	15.56	41.99	72.47	93.82	100.00	223.84	92.16	74.75	54.20	35.62	21.29	11.72	5.95	295.70	71.85
Injection 3	15.47	42.01	72.38	93.72	100.00	223.58	92.14	74.73	54.27	35.65	21.32	11.72	5.96	295.79	72.21
Average	15.50	42.01	72.43	93.75			92.13	74.74	54.22	35.62	21.31	11.71	5.96	Average	72.01
stdev	0.06	0.02	0.04	0.05			0.03	0.01	0.05	0.03	0.01	0.01	0.01	StDev	0.15
%RSD	0.38	0.04	0.06	0.06			0.04	0.01	0.08	0.07	0.06	0.08	0.18	%RSD	0.21

FLP only (control for 1.50% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.52	41.92	72.41	93.72	100.00	223.57	92.07	74.65	54.14	35.54	21.33	11.69	5.95	295.37	71.80
Injection 2	15.54	41.99	72.36	93.73	100.00	223.62	92.06	74.72	54.22	35.59	21.30	11.72	5.96	295.57	71.95
Injection 3	15.58	41.92	72.38	93.75	100.00	223.62	92.08	74.71	54.17	35.66	21.28	11.74	5.95	295.59	71.96
Average	15.55	41.94	72.38	93.73			92.07	74.69	54.18	35.60	21.30	11.72	5.95	Average	71.90
stdev	0.03	0.04	0.03	0.02			0.01	0.04	0.04	0.06	0.02	0.03	0.01	StDev	0.09
%RSD	0.21	0.10	0.04	0.02			0.01	0.05	0.08	0.16	0.11	0.21	0.11	%RSD	0.13

FLP only (control for 5.00% deamination)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.60	41.91	72.36	93.65	100.00	223.52	92.11	74.64	54.19	35.59	21.31	11.72	6.00	295.57	72.05
Injection 2	15.43	41.90	72.41	93.77	100.00	223.51	92.08	74.73	54.13	35.59	21.28	11.69	5.95	295.44	71.93
Injection 3	15.55	41.97	72.42	93.77	100.00	223.70	92.08	74.66	54.19	35.51	21.29	11.71	5.96	295.39	71.88
Average	15.53	41.93	72.40	93.73			92.09	74.68	54.17	35.57	21.29	11.71	5.97	Average	71.89
stdev	0.08	0.04	0.03	0.07			0.02	0.05	0.03	0.04	0.01	0.02	0.03	StDev	0.19
%RSD	0.53	0.08	0.05	0.07			0.02	0.07	0.06	0.12	0.06	0.16	0.43	%RSD	0.26

B

FLP and impurity (0.10% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.58	42.02	72.61	93.86	100.00	224.06	92.23	74.87	54.48	35.66	21.35	11.74	5.97	296.30	72.23
Injection 2	15.55	42.06	72.62	93.83	100.00	224.06	92.24	74.93	54.37	35.60	21.35	11.72	5.96	296.16	72.10
Injection 3	15.46	42.07	72.57	93.82	100.00	223.91	92.31	74.93	54.36	35.73	21.37	11.70	5.96	296.36	72.45
Average	15.53	42.05	72.60	93.84			92.26	74.91	54.40	35.66	21.36	11.72	5.96	Average	72.26
stdev	0.06	0.03	0.03	0.02			0.04	0.03	0.07	0.07	0.01	0.02	0.01	StDev	0.18
%RSD	0.41	0.06	0.04	0.03			0.05	0.05	0.12	0.19	0.05	0.15	0.16	%RSD	0.25

FLP and impurity (0.50% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.47	42.00	72.51	93.77	100.00	223.75	92.27	74.95	54.42	35.69	21.44	11.74	5.98	296.48	72.73
Injection 2	15.58	42.04	72.59	93.76	100.00	223.97	92.27	74.93	54.44	35.71	21.41	11.76	5.98	296.50	72.53
Injection 3	15.40	41.92	72.39	93.66	100.00	223.38	92.15	74.90	54.33	35.63	21.36	11.74	5.95	296.06	72.68
Average	15.48	41.99	72.50	93.73			92.23	74.93	54.40	35.68	21.40	11.74	5.97	Average	72.65
stdev	0.09	0.06	0.10	0.06			0.07	0.02	0.06	0.04	0.04	0.02	0.02	StDev	0.10
%RSD	0.56	0.15	0.14	0.06			0.08	0.03	0.11	0.11	0.18	0.14	0.26	%RSD	0.14

FLP and impurity (0.25% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.49	41.90	72.37	93.77	100.00	223.54	92.26	75.00	54.40	35.77	21.43	11.77	5.99	296.63	73.09
Injection 2	15.37	41.87	72.37	93.63	100.00	223.24	92.21	74.95	54.39	35.66	21.41	11.78	6.00	296.42	73.17
Injection 3	15.48	41.90	72.37	93.65	100.00	223.40	92.18	74.93	54.38	35.71	21.37	11.76	5.96	296.29	72.89
Average	15.45	41.89	72.37	93.69			92.22	74.96	54.39	35.71	21.40	11.77	5.99	Average	73.05
stdev	0.07	0.02	0.00	0.08			0.04	0.03	0.01	0.05	0.03	0.01	0.02	StDev	0.15
%RSD	0.43	0.04	0.00	0.08			0.05	0.05	0.02	0.15	0.14	0.09	0.37	%RSD	0.20

FLP and impurity (0.75% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.37	41.86	72.27	93.68	100.00	223.18	92.27	75.06	54.44	35.80	21.45	11.80	6.02	296.84	73.66
Injection 2	15.43	41.78	72.25	93.63	100.00	223.11	92.24	74.87	54.43	35.72	21.44	11.74	6.00	296.44	73.34
Injection 3	15.33	41.83	72.26	93.64	100.00	223.07	92.19	74.98	54.43	35.78	21.41	11.79	6.01	296.58	73.52
Average	15.38	41.83	72.26	93.65			92.23	74.97	54.43	35.76	21.43	11.78	6.01	Average	73.50
stdev	0.05	0.04	0.01	0.02			0.04	0.10	0.01	0.04	0.02	0.03	0.01	StDev	0.16
%RSD	0.34	0.09	0.02	0.03			0.05	0.13	0.01	0.11	0.10	0.27	0.17	%RSD	0.22

FLP and impurity (1.50% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	15.24	41.57	71.98	93.46	100.00	222.25	92.38	75.15	54.65	35.93	21.56	11.91	6.06	297.64	75.40
Injection 2	15.32	41.59	72.02	93.54	100.00	222.47	92.40	75.08	54.68	35.93	21.57	11.89	6.09	297.64	75.17
Injection 3	15.29	41.53	71.95	93.59	100.00	222.36	92.37	75.17	54.60	35.90	21.52	11.88	6.04	297.48	75.12
Average	15.28	41.56	71.98	93.53			92.39	75.13	54.64	35.92	21.55	11.89	6.06	Average	75.23
stdev	0.04	0.03	0.03	0.06			0.02	0.05	0.04	0.02	0.03	0.01	0.03	StDev	0.15
%RSD	0.26	0.08	0.04	0.07			0.02	0.07	0.08	0.05	0.12	0.11	0.45	%RSD	0.19

FLP and impurity (5.00% deamination spike)

	1	2	3	4	5	SUM(1-4)	6	7	8	9	10	11	12	Sum(6-12)	IDF
Injection 1	14.75	40.62	70.97	92.99	100.00	219.33	92.99	76.01	55.57	36.79	22.21	12.24	6.28	302.10	82.77
Injection 2	14.80	40.63	70.92	92.84	100.00	219.18	92.92	75.94	55.59	36.78	22.20	12.30	6.31	302.03	82.85
Injection 3	14.74	40.60	70.91	92.84	100.00	219.08	92.91	75.98	55.60	36.77	22.20	12.29	6.31	302.07	82.88
Average	14.76	40.62	70.93	92.89			92.94	75.98	55.59	36.78	22.21	12.28	6.30	Average	82.87
stdev	0.03	0.02	0.03	0.09			0.05	0.04	0.01	0.01	0.01	0.04	0.01	StDev	0.11
%RSD	0.23	0.04	0.04	0.09			0.05	0.05	0.03	0.03	0.04	0.29	0.23	%RSD	0.13

Table I. IDFs calculated for three injection replicates for controls (FLP ASO oligo only, Table A) as well as spiked samples (deaminated impurity spiked into the FLP, Table B). The measured average IDF values are circled in red.

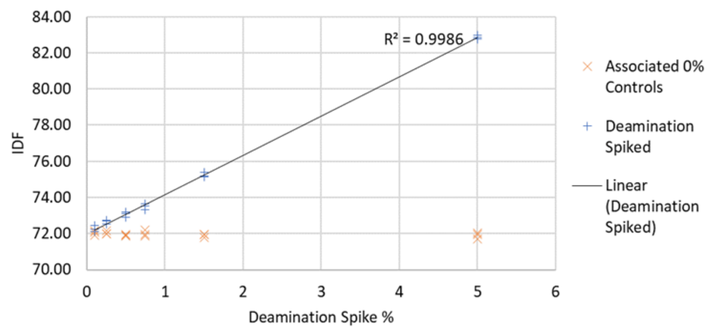


Figure 5. Linearity of the LC-MS assay developed for measuring the spiked levels of the deaminated oligonucleotide impurity in the concentration range of 0.10 to 5.00%. The associated non-spiked controls produced very reproducible IDF values.

CONCLUSIONS

• The Xevo™ G3 QTof was demonstrated to acquire ESI-MS spectra with very repeatable isotopic patterns, suitable for calculating IDF values, for accurate estimation of the relative amounts of FLP and deaminated impurity

• The measured IDF values were consistent across the analytical runs, demonstrating the robustness of the Xevo™ G3 QTof for performing deamination analysis and for achieving very reliable results in a relatively short timeframe (~ 150 min for measuring six spiked samples and their corresponding controls)

• The LC-MS assay provided excellent linearity over the concentration range investigated (0.10 to 5.00%).

REFERENCES:

1. Rentel C, DaCosta J, Roussis S, Chan J, Capaldi DC, Mai B *J Pharm Biomed Anal*, 2019, 173, 56-61.

2. Capaldi, DC "Stress testing of oligonucleotides", book chapter in: Baertski SW, Alsante KM, Reed RA (Editors) of *Pharmaceutical Stress Testing*, second edition, Informa Healthcare, London ,UK, 2011, 391-425.

3. *International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)*; Guideline Q1A(R2) Stability Testing of New Drug Substances and Products, February, 2003.

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