TECHNICAL NOTE 73670

Quality control of oligonucleotides with a single quadrupole mass spectrometer

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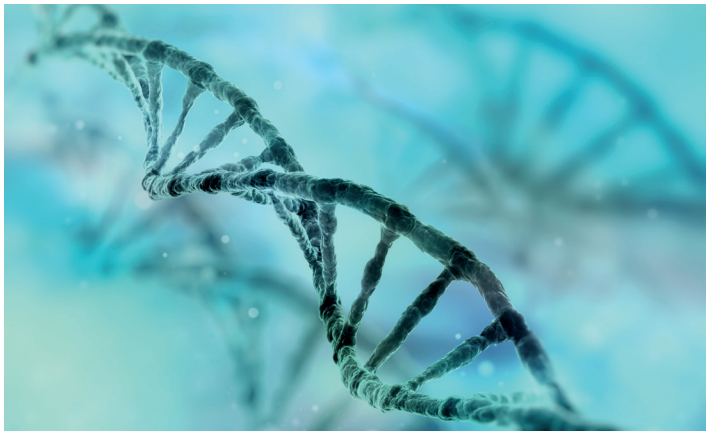

Goal

Show the benefit of mass spectrometric analysis with a single quadrupole mass detector for oligonucleotide quality control.

Introduction

Quality control of oligonucleotides requires mass confirmation and determination of sample purity and yield. Quantification can easily be done by UV detection because oligonucleotides show strong absorption at 260 nm. However, analyte confirmation requires mass spectrometric detection. Quality control also needs a fast and robust method to assure high throughput. This can be achieved by coupling a Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer to a HPLC system, such as a Thermo Scientific™ Vanquish™ Flex Binary UHPLC System. The ISQ EM mass detector is designed for routine applications and operation by chromatographers. It is fully integrated into the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), which now includes intact protein deconvolution engine and oligonucleotide analysis capabilities for ISQ EM data.

Two workflows for data analysis are presented in the following. Both use the peak area of UV and MS detection for determining the relative abundance of the analyte within the sample. However, the first workflow determines the identity of the analyte by its most intense *m/z* species. The second one uses deconvolution of the mass spectrum for mass determination and analyte identification.

Experimental

Sample material was provided by Thermo Fisher Scientific GeneArt, Regensburg, Germany (Table 1). The sample was dissolved in water at a concentration of 10 µM.

Table 1. Overview of analyte

Analyte	Molecular weight (average, Da)	Sequence
Single stranded DNA, 52 mer	16,278.60	ATC ACG ACA CTG CAT AAT CTC CTG CTC CAT CAG GAA GGA GCT AAA ATG GCA GT

Chromatographic separation was performed using LC-MS grade solvents (Table 2) on a Vanquish Flex Binary system coupled to an ISQ EM single quadrupole mass detector (Table 3). Acquisition was done in HESI mode with negative polarity. Method settings are listed in Table 4.

Table 2. Solvents and additives

Reagent	Grade	Supplier	Part number
Acetonitrile	Optima™ LC-MS	Fisher Chemical	A955-212
1,1,1-3,3,3-hexa-fluoro-iso-propanol (HFIP)	≥99% purity	Sigma-Aldrich	105228
Methanol	Optima™ LC-MS	Fisher Chemical	A456-212
Triethylamine (TEA)	≥99% purity	Sigma-Aldrich	T0886
Water	Ultra-Pure, 18.2 MΩ at 25 ˚C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

Table 3. Vanquish Flex Binary UHPLC system modules

Module	Part number
Vanquish System Base Horizon / Flex	VF-S01-A
Vanquish Binary Pump F (with 35 µL mixer set)	VF-P10-A (6044.3870)
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H	VH-C10-A
Vanquish Variable Wavelength Detector F (2.5 µL SST flow cell)	VF-D40-A (6077.0360)
ISQ EM single quadrupole mass detector	ISQEM-ESI

Table 4. LC-MS conditions

Parameter	Value
Mobile phase	A – 100% water, 0.1% TEA, 2% HFIP B – 100% MeOH, 0.1% TEA, 2% HFIP
Analytical column	Thermo Scientific™ Hypersil GOLD™, 2.1 × 50 mm, 1.9 µm (P/N 25002-052130)
Gradient	0–2.2 min: 10–90% B 2.2–2.5 min: 90–95% B 2.5–3.0 min: 95% B 3.0–5.0 min: 10% B
Flow rate	0.4 mL/min
Column temperature	40 ˚C, Forced Air Mode Active Pre-heater
Injection volume	2 µL
UV detection	260 nm, 100 Hz

Table 4 continued. LC-MS conditions

Parameter	Value
Source settings	
Vaporizer temperature	300 ˚C
Ion transfer tube temperature	350 ˚C
Source voltage	-3,000 V
Sheath gas pressure	75 psig
Aux gas pressure	7.5 psig
Sweep gas pressure	0 psig
Full scan	
Mass range	600–2,000 *m/z*
Dwell/scan time	0.5 s
Polarity	Negative
Spectrum type	Profile
Source CID voltage	0 V

The ISQ EM mass detector is fully integrated into Chromeleon software. Data acquisition and analysis were performed with Chromeleon software and its updated Intact Protein Deconvolution engine (Table 5).

Table 5. Intact Protein Deconvolution settings

Parameter	Value
Peak retention window	1.250–1.360 min
Algorithm	ReSpect™
Output mass range	10,000–20,000
Deconvoluted spectra display mode	Isotopic profile
Model mass range	10,000–100,000
Peak model	Nucleotide
Resolution	Raw file specific
Charge carrier	H^+
Charge high	20
Charge low	10
High number adjacent charges	10
Low number adjacent charges	4
Intensity threshold scale	0.01
Min peak significance	1
Negative charge	True
Noise compensation	True
Noise rejection	95
Number of peak models	1
Peak model width scale	1
Quality score threshold	0
Relative abundance threshold	0
Target peak mass	16,000
Target peak shape left	2
Target peak shape right	2

Results and discussion

In a previous publication on oligonucleotide analysis for quality control, multiple adducts were detected when default ISQ EM source settings were applied.[1] This may have reduced the detection sensitivity. Therefore, source settings were optimized to reduce adduct formation. A first round of optimization was done using the source settings sliders available under the Easy Mode in the Chromeleon Instrument Method Editor (data not shown).[2] Afterwards the source settings were further improved using Advanced Mode. To allow optimization without the need to create multiple method files, custom variables for the following method parameters were used: vaporizer temperature, transfer tube temperature, sheath gas, auxiliary gas, and spray voltage (Figure 1 and Table 6). The sequence of optimization is indicated in Table 6.

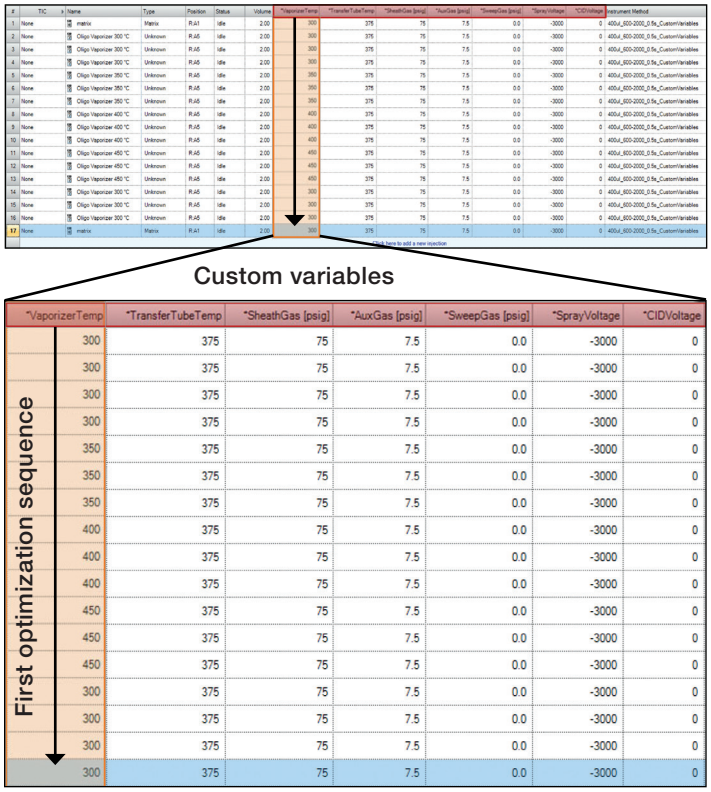

Figure 1. Using custom variables for method optimization. In Chromeleon CDS, custom variables can be defined. Subsequently, selected method parameters are not set in the instrument method but in the sequence table. This allows for faster method optimization without the need to create multiple instrument methods. In this experiment, multiple custom injection variables were used for iterative optimization of source parameter settings. Custom variables are denoted by an asterisk before the parameter name in the sequence table.

Table 6. Optimization of source parameters

Order	Source parameter	Optimization range	Steps
1	Vaporizer temperature	300 to 450 °C	50 °C
2	Transfer tube temperature	300 to 400 °C	25 °C
3	Sheath gas (Auxiliary gas)	50 to 80 psig (5 to 8 psig; 10% of sheath gas)	5 psig (0.5 psig)
4	Spray voltage	-500 to -3,000 V	500 V

The improved source settings resulted in greatly reduced levels of adduct formation and greater signal intensity (Figure 2). These parameters were used for subsequent analysis (Table 4). In addition, the effect of CID voltage was tested. Since adding a CID voltage neither improved signal intensity nor reduced the presence of adducts, no CID voltage was applied. The scan time of 0.5 s was selected to have enough scans over the peak while maximizing the scan time.

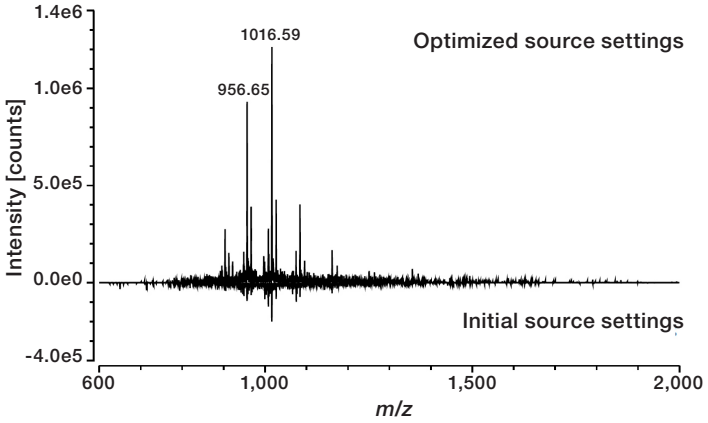

Figure 2. Optimization of source parameters. The apex spectra (bunched over 11 spectra) before (bottom) and after (top) optimization are shown as a mirror plot. After optimization the signal intensity was two to three times higher than before optimization.

The oligonucleotide sample was then measured in quadruplicate. Retention time and signal area for UV and MS detection showed high precision with a relative standard deviation of less than 0.05% for the retention time and less than 4% for signal area with both detection types (Table 7 and Figure 3).

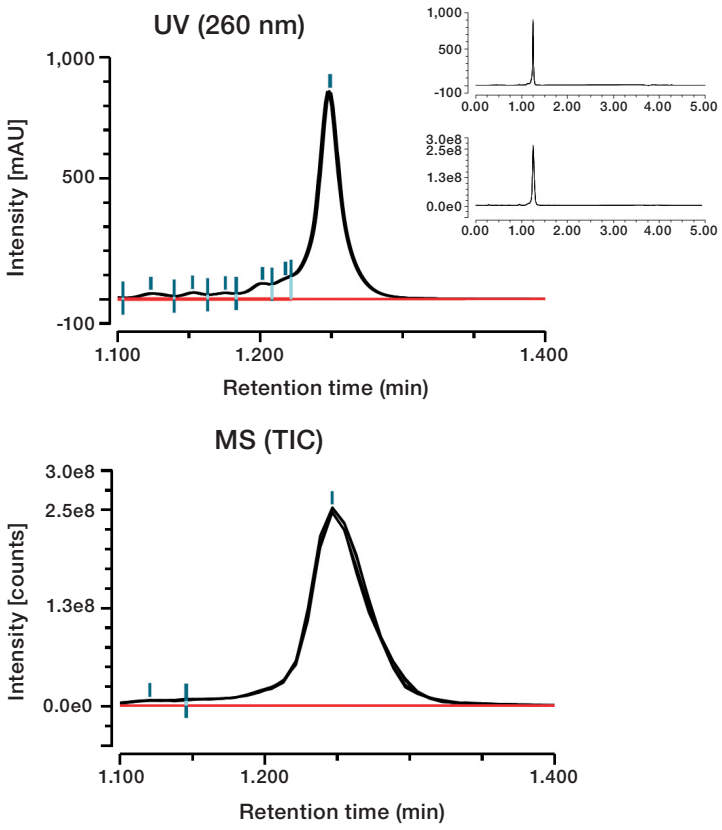

Figure 3. Chromatographic overlay of quadruplicate injections. The UV chromatograms for the analyte peak are shown at the top, the MS chromatograms at the bottom. The full chromatograms are shown as an insert at the top right. For both detection types, the consecutive injections showed high reproducibility for retention time and signal area.

Table 7. Chromatographic overview

Sample	UV (260 nm)			MS (TIC)		
	Retention time (min)	Signal area (mAU * min)	Relative area (%)	Retention time (min)	Signal area (counts * min)	Relative area (%)
#1	1.249	18.74	79.89	1.249	1.30E+07	92.69
#2	1.249	18.75	79.92	1.249	1.30E+07	93.60
#3	1.248	18.83	80.05	1.248	1.20E+07	89.79
#4	1.249	19.74	84.2	1.248	1.30E+07	97.11
Average	1.249	19.02	81.02	1.249	1.28E+07	93.30
Standard deviation	0.0005	0.49	2.12	0.00058	5.00E+05	3.02
Relative standard deviation	0.04%	2.55%	2.62%	0.05%	3.92%	3.23%

The apex mass spectra (bunched over 11 spectra) showed multiple distinct peaks which are the charge states 12 to 18 of the deprotonated species and its HFIP (molecular weight: 168.00 Da) adducts (Figure 4 and Table 8).

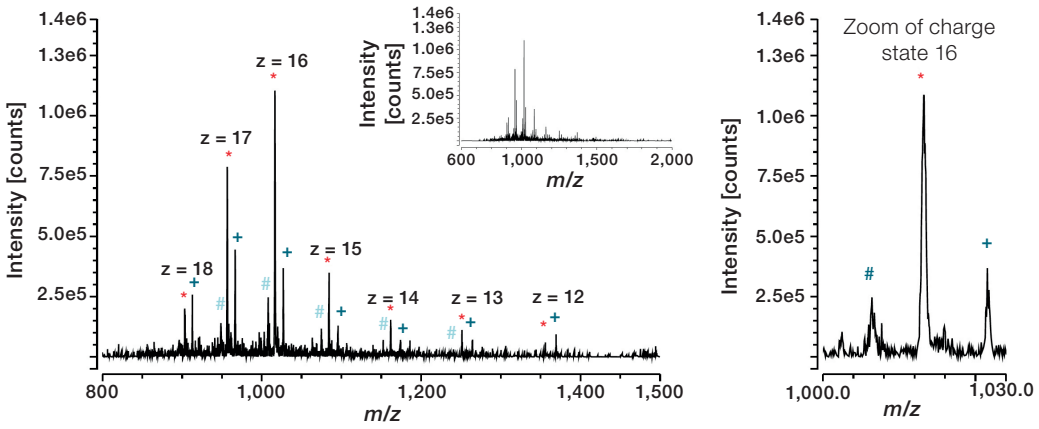

Figure 4. Mass spectrum of the analyte. The mass spectrum clearly showed the series of deprotonated charge states z = 12 to 18 (marked with *), and at lower intensity a series of respective HFIP adducts (marked with +). For the most intense charge state (z = 16), an *m/z* of 1008.04 was additionally observed (marked with #). This could be a loss of a single base, e.g., adenine, guanine, thymine, or cytosine.

Table 8. Observed masses of the analyte

Charge state (z)	Theoretical *m/z*		Observed *m/z*		Mass deviation *(m/z)*	
	Deprotonated analyte[1]	HFIP adduct[2]	Deprotonated analyte[1]	HFIP adduct[2]	Deprotonated analyte[1]	HFIP adduct[2]
12	1355.55	1369.55	1355.87	1369.4	0.32	-0.15
13	1251.20	1264.12	1251.38	1264.3	0.18	0.18
14	1161.76	1173.76	1161.8	1173.13	0.04	-0.63
15	1084.24	1095.44	1084.49	1095.49	0.25	0.05
16	1016.41	1026.91	1016.52	1026.92	0.11	0.01
17	956.56	966.45	956.65	966.6	0.09	0.15
18	903.37	912.70	903.28	912.89	-0.09	0.19

[1][M-zH]$^{-z}$; [2][M+HFIP-zH)]$^{-z}$

The four most intense charge states of the deprotonated analyte (z = 14 to 18) showed an *m/z* deviation from theoretical *m/z* of approximately 0.1 *m/z*, with z = 15 showing a deviation of 0.25 *m/z*. Hence, the deviations were sufficiently accurate for analyte confirmation by *m/z* species detection (using 0.2 *m/z* mass deviation as detection window). The most intense signal (z = 16; *m/z* 1016.41) was selected as quantitation ion and the four others as confirmation ions in the processing method. The identity of the analyte peak could then be verified by mass spectrometric detection. Usually, a quantitation ion and one or two confirmation ions are enough for confirmation; in this case, it was shown that a greater number of confirmation ions are possible with Chromeleon CDS.

Sample purity was assessed through UV and MS detection. In both cases the results vary slightly (Table 7). For MS detection the relative signal area is above 90% indicating a clean sample, while for UV detection it is around 80%. This discrepancy is caused by contamination eluting shortly before the analyte that was detected by UV but did not yield a substantial MS signal (Figure 3). So, combining MS detection and UV detection assured that sample purity was not overestimated.

Another way of confirming the identity of the analyte is through deconvolution. This can be used even if only the mass of the oligonucleotide analyte is known while the *m/z* species of different charge states that are suitable for mass confirmation are unknown. While specific *m/z* species were used for identity confirmation before, now the complete mass spectrum of a peak is used for analyte confirmation. The *m/z* peaks are picked by the software algorithm and the molecular mass of the analyte is inferred from them automatically. In Chromeleon 7.3 CDS, the Intact Protein Deconvolution engine can deconvolute oligonucleotide data acquired on an ISQ EM mass detector. For determining the sample purity, the workflow for oligonucleotide quality control would be the same as described before, while deconvolution is used for analyte confirmation. The applied method settings are described in Table 5.

Three main masses were detected by deconvolution of the mass spectra of the analyte peak (Figure 5 and Table 9). The most intense was 16,280.19 Da, which matches the theoretical mass of the analyte with a mass deviation of 1.59 Da. The second most intense was 16,446.60 Da, which matches the theoretical mass of the HFIP adduct with a mass deviation of 1.39 Da. Its relative intensity was 37.95% compared to the most intense peak. A third mass (16,143.19 Da) with 13.17% relative abundance was also reported. However, the mass deviations of the individual charge states were high, so the mass assignment was with low confidence (data not shown). The mass deviation for the analyte and its adduct were within the expected range. Since the detected charge states were 10 to 19 and the mass accuracy of the ISQ EM is 0.1 *m/z*, the expected mass deviation should be between 1 and 2 Da. Thus, the identity of the oligonucleotide analyte could be also be verified through deconvolution.

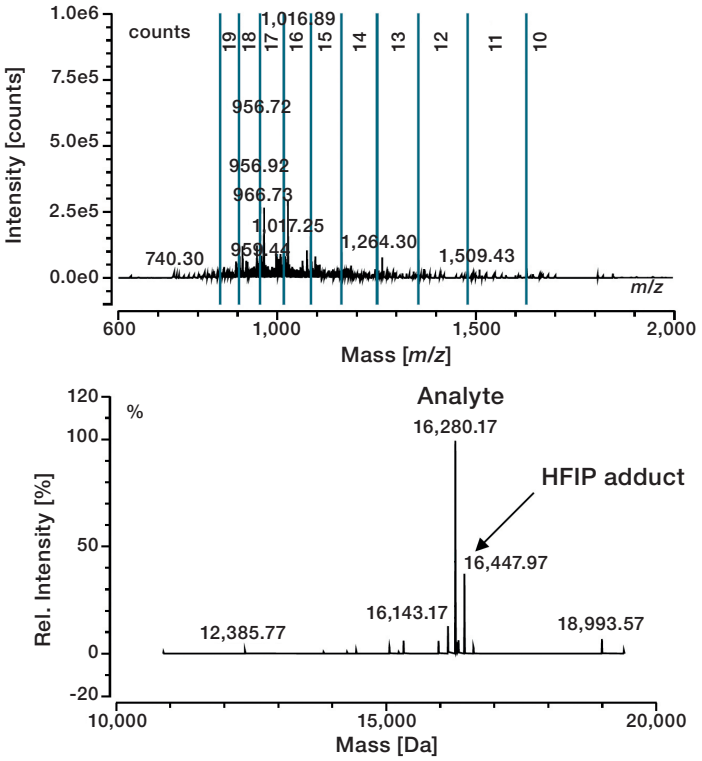

Figure 5. Deconvolution of analyte. At the top the mass spectrum of the peak and in blue the detected charge states are shown. At the bottom the deconvoluted masses are shown. Two main masses, which belong to the analyte (16280.19 Da) and the HFIP adduct (16446.60 Da), are present. In addition, other masses were inferred. However, their relative abundance was 13% or less and the confidence score for the masses was low.

Table 9. Deconvolution results

Compound	Theoretical mass (Da)	Observed mass (Da)	Mass deviation (Da)	Relative abundance in spectrum (%)
Analyte	16,278.60	16,280.19	1.59	100.00
HFIP adduct	16,446.60	16,447.99	1.39	37.95
Unknown		16,143.19		13.17

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Conclusion

It was shown that oligonucleotide analysis for quality control is possible and straightforward with the ISQ EM and Chromeleon 7.3 CDS. Two analysis workflows were shown with an identical determination of sample purity but a differing method of analyte identity confirmation. First, direct mass confirmation through MS peak confirmation provided an easier approach, but the *m/z* species suitable for confirmation need to be known before data analysis. Secondly, the oligonucleotide identity was determined by deconvolution, providing an approach if only the mass of the analyte is known. Both workflows provided accurate mass confirmation results.

References

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