thermoscientific

APPLICATION NOTE 72820

Oligonucleotide characterization for quality control and increased productivity by single quadrupole mass spectrometer with extended mass range

Authors
Katherine Lovejoy, Sylvia Grosse, Mauro de Pra, Martin Samonig, Frank Steiner; Thermo Fisher Scientific, Germering, Germany

Keywords
Single quadrupole mass spectrometer, ISQ EM, UHPLC, ssDNA, oligonucleotides, quality control, extended mass range, LC-MS, high-performance liquid chromatography, Vanquish Flex UHPLC

Goal

Demonstrate increased productivity in the analysis of synthetic oligonucleotides between 29 and 40 base pairs in length by incorporating the Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer into a UV-based quality control method.

Application benefits

- Expanding a UV quality control method with MS provides mass confirmation and impurity identification.
- Use of common DNA ion pairing reagents facilitates transfer of existing QC methods.

Introduction

Quality control (QC) of oligonucleotide synthesis requires confident confirmation of oligonucleotide mass and rough quantification of yield and impurity levels using a rapid and efficient method. Quantification of yield can easily be performed by UV detection because of the strong DNA absorption at 260 nm. Rough estimation of impurities requires a mass spectrometer, as aborted sequences (N-1) are not usually chromatographically separated from complete sequences (N) during a quick QC method. A mass spectrometer also allows for non-ambiguous confirmation of oligomer identity.

Mass spectrometry (MS) is often considered to be complex and too difficult to use in routine quality control applications. The ISQ EM single quadrupole mass spectrometer is developed for operation by chromatographers. Its full integration into the Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) and the Thermo Scientific™ AutoSpray smart method setup make LC-MS operation and data analysis straightforward and intuitive. The ISQ EM mass spectrometer offers an extended mass range of *m/z* 10–2000, allowing chromatographers to collect data over multiple charge states.

ThermoFisher
SCIENTIFIC

The orthogonal source design provides high levels of instrument robustness, even for challenging conditions of ion pairing chromatography, commonly used to ensure retention of DNA oligomers. This application note describes quality control of oligonucleotides, including mass confirmation and impurity level estimation by mass spectrometry, as well as quantification by UV.

Experimental

Oligomers and reagents

Oligonucleotides (Table 1) were provided by the Genetic Science Division of Thermo Fisher Scientific, Pleasanton, California. Fisher Chemical™ Optima™ Methanol, LC/MS-grade (P/N A454) was used. The ion pairing reagent 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, P/N 105228) and triethylamine (TEA, P/N T0886) were purchased from Sigma-Aldrich. Ultrapure water was produced from a Thermo Scientific™ Barnstead™ GenPure xCAD™ Plus Ultrapure Water Purification System.

Instrumentation

A Thermo Scientific™ Vanquish™ Flex UHPLC system equipped with an ISQ EM single quadrupole mass spectrometer was used for the analysis.

- Thermo Scientific™ Vanquish™ Binary Pump F (P/N VF-P10-A)
- 35 µL mixer (P/N 6044.3870)
- Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F (P/N VF-D40-A)
- 2.5 µL standard stainless steel flow cell (P/N 6077.0360)
- ISQ EM Mass Spectrometer (P/N ISQEM-ESI)

Chromatographic procedure and detection parameters

Reversed-phase ion pairing chromatography was performed using a unique polymeric (divinylbenzene) column chemistry designed for oligonucleotide analysis. Oligomers were eluted with an ion pairing mobile phase commonly used for mass spectrometry, specifically TEA/HFIP. A short quality control-type method that did not yield chromatographic separation of N and N-1 oligomers was used for a 10 mm column (Table 2). CID voltage was optimized by maximizing the peak area associated with the most abundant mass for the -9 charge state. The instrument was tuned once at the beginning of experiments using the operational qualification wizard in Chromeleon CDS.

Preparation of Eluent A

Eluent A was prepared by adding 3.36 g of neat HFIP to 90 mL of water in a 250 mL graduated cylinder. A 112 µL portion of neat TEA was added while stirring vigorously. Water was then added to 100 mL. The eluent was sonicated for 15 minutes and allowed to stand until it reached room temperature before use.

Preparation of samples

Lyophilized, desalted, detritylated samples were reconstituted in DNase-free water to a concentration of 100 µM and stored at -20 °C. Samples for injection were diluted to the concentrations described in the text using micropipettes and water from the purification system.

Preparation of samples for calibration

Samples of 37mer were prepared at 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 µM using micropipettes and water from the purification system.

Chromatography Data System

The ISQ EM mass spectrometer is fully integrated into Chromeleon 7.2.9 CDS, which was used for system operation and subsequent data analysis.

Table 1. Oligomer samples analyzed

Length	Sequence	Average Mass (Da)
40	CTCTCTGACACAATTAAGGGATAAAATCTCTGACGGAATG	12,306.1
37	CAGGAAACAGCTATGACCCGCGCTCACCTCGCCTCTG	11,234.9
31	ATGATATTATGATTAGGAGCCGCGCAGGGAG	9659.6
29	TGAAGGAITGCACTGAAAGGCAGGCTAAT	9024.9

Table 2. Short column, chromatographic conditions and detector settings

Parameter	Setting
Column	Thermo Scientific™ DNAPac™ RP 2.1 × 10 mm, 4 µm (P/N 088925)
Eluents	A: 200 mM HFIP, 8.0 mM TEA, pH 8.0 B: Methanol
Injection volume	1 µL
Analytical gradient	0.5 mL/min flow rate Time (min) %B 0 15 0.5 15 1 60 1.3 60 1.4 15 6 15
Column temperature	50 °C forced air mode, 50 °C active preheater
Autosampler temperature	4 °C
UV settings	λ=260 nm, 10 Hz data collection rate, 0.5 s response time
MS settings	HESI source type, easy ESI source settings for 0.5 mL/min flow rate, specifically: Sweep gas pressure 0.5 psig Sheath gas pressure 49.9 psig Aux gas pressure 5.7 psig Vaporizer temperature 282 °C Ion transfer tube temperature 300 °C
MS Full Scan	Time 0–6 min Mass range *m/z* 600–2000 Dwell time 0.2 s Polarity Negative Source CID voltage 15 V Spectrum type Profile

Results and discussion

With a mass range that extends to *m/z* 2000, the ISQ EM mass spectrometer lends itself to analysis of long oligomers with multiple charge states. This capability was applied to a quality control workflow, in which oligomers of 29–40 nucleotides in length were injected separately and subjected to a step gradient such that each oligomer eluted quickly and at the same retention time as all other oligos. The UV detector was used for quantification and the mass spectrometer was used for mass confirmation and impurity identification. Detection by mass spectrometer of oligonucleotides is commonly performed using the ion pairing reagents TEA and HFIP.[1] Although these reagents are relatively persistent, the orthogonal source design did not require cleaning, even after over 100 injections.

Quantification by UV

Quantification of the 37mer oligonucleotide by UV shows that the concentration has a linear relationship with the absorbance at 260 nm over the entire examined concentration range of 0.05 to 50 µM. This curve is shown in Figure 1. The MS-based calibration curve using the peak area of the XIC of the -9 charge state, including the HFIP adducts and potassium salts listed in Table 5, for concentrations of 1, 2, 5, 10, 20, and 50 µM yielded a coefficient of determination for the linear fit of 0.902, a y-intercept of -4055, and a slope of 1039.5. An additional MS-based calibration curve was calculated using the peak area of a SIM scan corresponding to the average mass of the -9 charge state (*m/z* 1247.9, dwell time = 0.1 s, SIM width = 0.1 amu). The SIM scan-based calibration curve was obtained for concentrations of 0.5, 1, 2, 5, 10, 20, and 50 µM and yielded a coefficient of determination for the linear fit of 0.966, a y-intercept of -443.6, and a slope of 368.1.

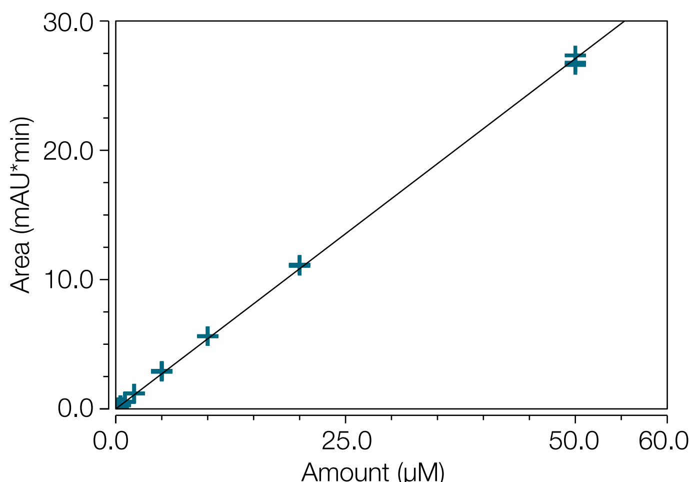

Figure 1. UV calibration curve over the values 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 µM with a coefficient of determination of 0.9996, a y-intercept of 0.099 and a slope of 0.539

Mass confirmation

Injections of 5 pmol of 29mer, 31mer, 37mer, and 40mer oligomers were made in triplicate for the purpose of identification by mass confirmation. The mass confirmation of the charge state -9 for each of these oligomers is shown in Table 3, demonstrates that mass accuracy is excellent over various oligonucleotide lengths at a relatively low mass on column. The observed masses are all within the mass accuracy specification of ≤ ±0.1 Da of the ISQ EM mass spectrometer. The repeatability of the observations is excellent, with a relative standard deviation (RSD) of 0.02% or less.

Multiple charge states were observed for all oligomers examined. For mass confirmation of all charge states with *m/z* 600–2000, injections of 50 pmol of the 37mer oligonucleotide were made in quadruplicate. The charge states for the 37mer are shown in Table 4. The average molecular weight of each oligonucleotide was used to calculate the expected mass of each charge state. The observed masses, except for the -13 charge state, are within the mass accuracy specification. The repeatability of the observations is excellent, with an RSD of 0.02% or less except for the -14 charge state, which had an RSD of 0.07%. The increased instrument error associated with higher charge states explains the slightly worse results for charge states 13, 14, and 15.

The average mass was used for mass confirmation. A single quadrupole mass spectrometer is a low-resolution spectrometer and, at the charge states we considered, peaks from many isotopes converged to the average mass.

Table 3. Mass confirmation for 29mer, 31mer, 37mer, and 40mer using the relatively high-abundance $[M-9H]^{9-}$ charge state

Length	Average Mass (Da)	Average Mass, $[M-9H]^{9-}$	Found Mass, Trial 1, $[M-9H]^{9-}$	Found Mass, Trial 2, $[M-9H]^{9-}$	Found Mass, Trial 3, $[M-9H]^{9-}$	Mean (n = 3) Observed	Mass Accuracy	Std. Dev. (n = 3)	RSD
40	12312.0	1367.0	1367.1	1366.9	1366.9	1367.0	0.0	0.12	0.01%
37	11240.3	1247.9	1247.9	1247.8	1247.8	1247.8	-0.1	0.06	0.00%
31	9664.3	1072.8	1072.9	1073.0	1072.8	1072.9	0.1	0.10	0.01%
29	9024.9	1001.8	1001.6	1001.7	1002.0	1001.8	0.0	0.21	0.02%

Table 4. Charge states calculated and found for 37mer (TIC, scan *m/z* 600–2000, CID 15, 0.2 s dwell time, negative mode, 50 pmol on-column). Found values and standard deviations (Std. Dev.) and RSDs are from the average of four injections.

	Expected and Found Masses for Charge States $[M-xH]^{x-}$									
	6	7	8	9	10	11	12	13	14	15
Expected	1872.4	1604.8	1404.0	1247.9	1123.0	1020.9	935.7	863.6	801.9	748.4
Found	1872.4	1604.8	1403.8	1247.9	1122.9	1020.7	935.7	864.0	802.1	748.6
Mass Accuracy	-0.0	-0.0	-0.2	-0.0	-0.1	-0.2	-0.0	+0.4	+0.2	+0.2
Std. Dev. (n = 4)	0.16	0.20	0.15	0.10	0.15	0.10	0.10	0.21	0.56	0.17
RSD	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.07%	0.02%

Impurity identification by mass spectrometry

Because impurities such as aborted sequences (N-1), depurination products, 2-cyanoethyl modifications, and isobutyryl modifications are not usually chromatographically separated from complete sequences (N) during a quick QC method, identification of impurities and rough estimation of their levels requires a mass spectrometer.

Based on peak area, rough amounts of adducts and impurities relative to the sum of the areas of all adducts identified for the -9 charge state are shown in Table 5. Potential impurities and adducts were identified using peer reviewed literature sources.[1,2]

Impurities identified at low levels (< 10%) included the N-1 aborted sequence. Impurities identified at trace levels (< 5%) included the 2-cyanoethyl impurity, isobutyryl impurity, and the chloral N-mer impurity. To confirm the presence of these trace-level impurities, examination of the extracted ion chromatograms (XICs) is helpful. XICs for the ninth charge state of the 37mer and some impurities are shown in Figure 2. Some peaks are clearly present at 1.05 minutes, including those corresponding to the masses of the main peak, $[M-9H]^{9-}$, the N-1 aborted sequence peak, the single potassium salt peak $[M+K^{+}-10H]^{9-}$, the depurination peak, and the 2-cyanoethyl impurity peak. Some peaks are less prominent, including the peak for the masses corresponding to the chloral impurity and the isobutyryl impurity. The presence of these impurities would need to be confirmed by high-resolution mass spectrometry.

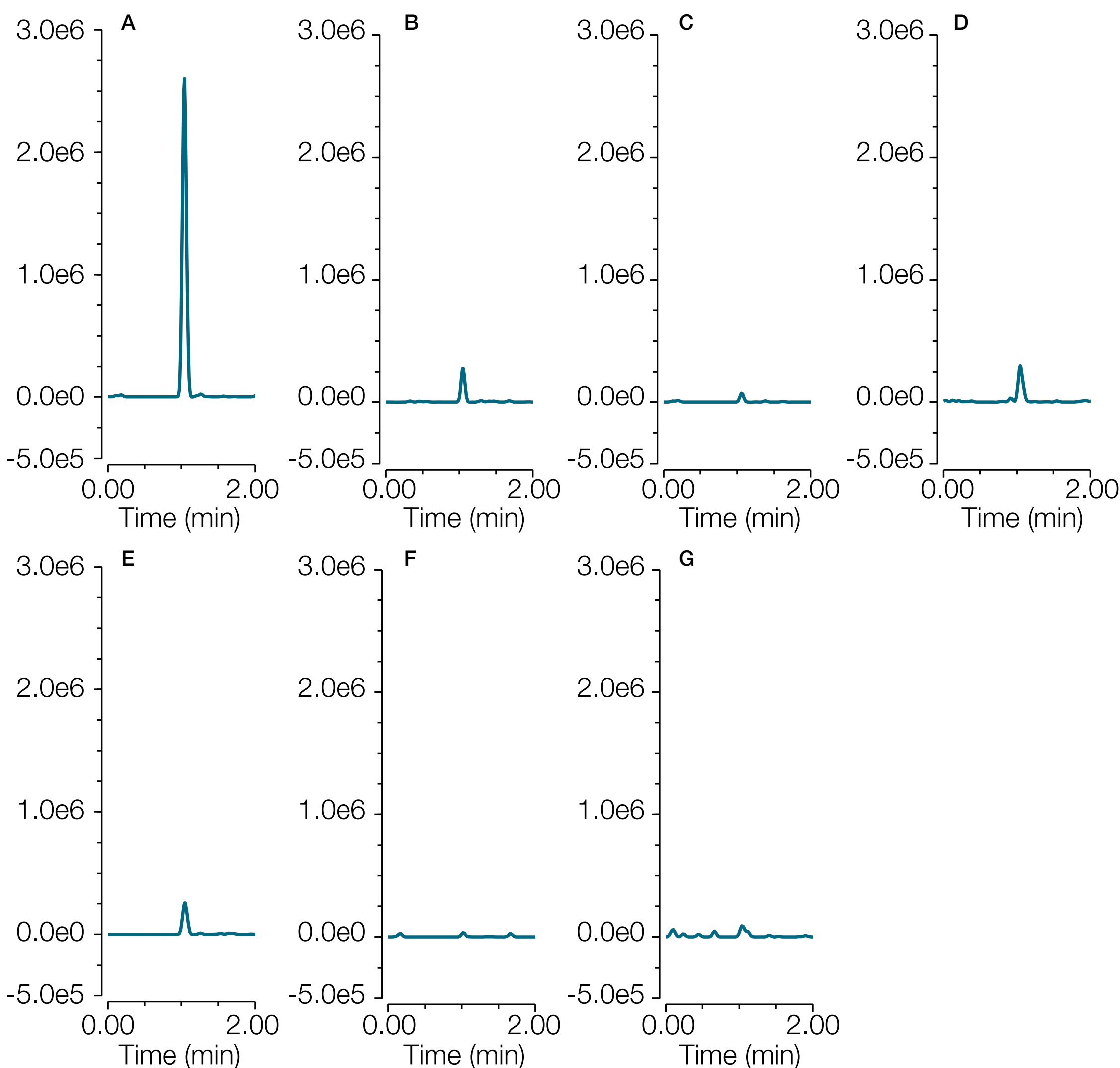

Figure 2. XICs for the $[M-9H]^{9-}$ charge state of the 37mer and some of the identified impurities using a full scan in negative mode from *m/z* 600–2000, a dwell time of 0.2 s, a CID of 15, and smoothing using the Gaussian function and setting of 11 points in Chromeleon CDS. A) $[M-9H]^{9-}$, *m/z* 1247.9. B) The -9 charge state for the aborted sequence, N-1, *m/z* 1211.3. C) $[M+K^{+}-10H]^{9-}$, *m/z* 1252.2. D) The -9 charge state for the depurination resulting in loss of one adenine base, *m/z* 1232.9. E) The -9 charge state for the 2-cyanoethyl impurity, *m/z* 1253.5. F) The -9 charge state for the chloral impurity, *m/z* 1263.8. G) The -9 charge state for the isobutyryl impurity, *m/z* 1255.4.

Table 5. Identification and rough estimation of impurity levels based on the sum of the peak area of all adducts identified for the -9 charge state of the 37mer oligonucleotide

Ion Found	Mass Found	Area Relative to Sum of Area of All Adducts Found (Standard Deviation, n = 3)	Characterization
$[M-9H]^{9-}$	1247.9	21.1% (1.2%)	Base peak
$[M-9H^{+}xHFIP]^{9-}$ (x = 1-5)	1266.6 1285.3 1303.9 1322.6 1341.3	39.3% (3.9%)	Solvent adduct
$[M-9H^{+}xMeOH]^{9-}$ (x = 1–2)	1251.5 1255.0	2.2% (1.7%)	Solvent adduct
$[M-10H+TEA]^{9-}$	1259.2	0.7% (0.6%)	Solvent adduct
$[M+xK^{+}-yH+zHFIP]^{9-}$ x = 1–5 y = 10–14 z = 0–2	1252.2 1270.8 1289.5 1308.2 1326.8 1255.7 1256.4 1275.1 1293.7 1260.6 1264.9 1269.1	14.4% (3.4%)	Potassium counterion
$[M+Na^{+}-10H]^{9-}$	1250.4	2.0% (0.9%)	Sodium counterion
Depurination products	1231.1 1249.8 1234.7 1235.4 1232.9 1251.6 1236.5 1237.2	8.5% (2.1%)	Impurity or possible MS-related decomposition
N-1 and adducts	1211.3 1230.0 1214.9 1215.6 1220.2	6.1% (2.5%)	Impurity, minor
2-Cyanoethyl impurity	1253.5	2.1% (0.4%)	Impurity, trace
Isobutyryl impurity	1255.4	0.8% (0.2%)	Impurity, trace
Chloral N-mer impurity	1263.8	0.6% (0.3%)	Impurity, trace
4,4-Dimethoxytrityl N-mer impurity	1281.2	0.5% (0.3%)	Impurity, probably not present
M with 3’ cyclic phosphate	1254.8	1.6% (1.3%)	Impurity, probably not present
M with 3’ phosphate	1256.8	0.1% (0.1%)	Impurity, probably not present

Depurination, the loss of an adenine or guanine base, results from hydrolysis by the acid used to remove the trityl protecting group from heating or in-source fragmentation during mass spectrometry analysis. Depurination accounted for 8.5% of the sum of peak areas of all adducts found. The XIC for the loss of adenine is shown in Figure 2D. The CID voltage was optimized for this application by maximizing the peak area of the most abundant charge state for $[M\text{-}xH]^{x-}$, but an alternative optimization strategy would be to choose a CID that leads to minimal depurination.

A larger share of peak area was attributable to adducts of 37mer with the solvent additive HFIP than to the 37mer. The ratio of HFIP-adducted to non-adducted 37mer was unresponsive to CID voltage. A negligible amount of methanol and TEA adducts were found. Potassium salts and their HFIP adducts contributed to 14.4% of total peak area and sodium salts were only 2.0% of total peak area. Possible sources of potassium and sodium ions include the eluents, eluent bottles, sample vials, sample buffers, or oligonucleotide production processes.

Benefit of longer equilibration times

Chromatographers frequently determine the time required for system equilibration between injections based on the pump pressure trace. As soon as the pump pressure signal has returned to the starting value, the next injection begins. But when using ion pairing reagents, an equilibration time that is a minimum of three times longer leads to greatly improved peak area reproducibility. As shown in Figure 3, the RSD for the peak area of the full scan of six 1 µL injections (5 µM 37mer sample) improved from 15.4% to 8.1% when the equilibration time was increased from 1 to 4.5 minutes.

Conclusion

The implementation of a mass spectrometer in an existing UV workflow provides mass confirmation and impurity identity information. The extended mass range of the ISQ EM mass spectrometer allows accurate mass confirmation using eight charge states for a 37mer oligomer. The orthogonal source design did not require cleaning through 100 injections of the traditional ion pairing reversed-phase liquid chromatography mobile phase of triethylamine and hexafluoro-2-propanol. A slightly extended equilibration phase between injections greatly improves peak area reproducibility, as compared with a shorter equilibrium phase adjusted only according to the recovery of the pump pressure trace. An ISQ EM MS-based workflow improves productivity over that of a simple UV-based quality control workflow because it provides additional information in the same amount of time via an efficient, easy-to-use interface for MS control and data processing.

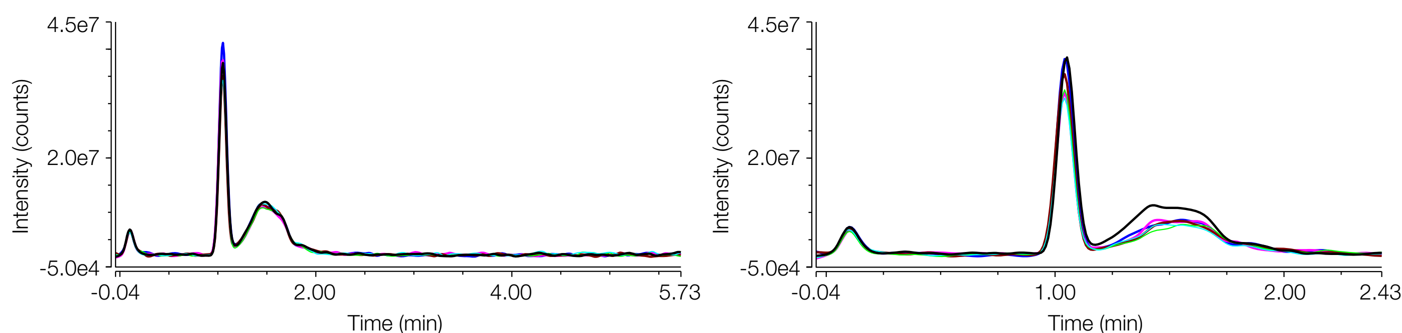

Figure 3. Overlay of six injections with long (left side) and short (right side) equilibration times. The RSD for peak area is 8.1% for the long equilibration time and 15.4% for the short equilibration time. The trace shown is the full scan (*m/z* 600–2000, 0.2 s dwell time, CID 15 V, negative mode).

thermo scientific

References

1. Apffel, A.; Chakel, J.A.; Fischer, S.; Lichtenwalter, K.; Hancock, W.S. Analysis of oligonucleotides by HPLC-electrospray ionization mass spectrometry. *Anal. Chem.* **1997**, *69*, 1320-1325.

2. Nikcevic, I.; Wyrzykiewicz, T.K.; Limbach, P.A. Detecting low-level synthesis impurities in modified phosphorothioate oligonucleotides using liquid chromatography—high resolution mass spectrometry. *Int. J. Mass Spectrom.* **2011**, *304*, 98-104.

Find out more at **thermofisher.com/ISQEM**

ThermoFisher SCIENTIFIC

© 2018 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representatives for details. **AN72820-EN 0918M**