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APPLICATION SOLUTIONS FOR OLIGONUCLEOTIDES

RECENT IMPACT OF WATERS SOLUTIONS IN BIOTHERAPEUTICS

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APPLICATION SOLUTIONS FOR OLIGONUCLEOTIDES

UPLC ANALYSIS OF SYNTHETIC OLIGONUCLEOTIDES

Oligonucleotides are polymeric sequences of nucleotides (RNA, DNA, and their analogs). Oligonucleotides are utilized in a broad array of applications, including basic molecular research employing primers and siRNAs, health diagnostics and pharmacogenomic studies using microarrays and fluorogenic probes, and pharmaceutical therapeutics utilizing antisense oligos, aptamers, and miRNAs. They are often modified to enhance their mechanistic functionality, hybridization specificity and efficiency, or nuclease resistance.

Oligonucleotides are produced by automated solid-phase synthetic processes. Typical lengths range from 20 to 80 nucleotides, and synthesis scales vary from nanograms to kilograms, depending on the application. Although the oligonucleotide synthesis process is highly efficient, sequence truncated oligos and other process-related modifications and impurities are often artifacts of synthesis. It is important to analyze synthetic products to determine if oligonucleotide purity is sufficient or if further purification is required. Intermediate and final analysis of the synthesis products are also useful for Quality by Design studies and process control.

The solid-phase chemical synthesis process and molecular diversity of oligonucleotides across the application spectrum have made analytical characterization highly challenging. The Waters® ACQUITY UltraPerformance LC® (UPLC®) System brings a new level of chromatographic performance, resolution, sensitivity, and throughput to oligonucleotide analysis that enables laboratories to be more productive.

The Waters UPLC® Oligonucleotide Analysis Solution:

- ACQUITY UPLC System
- Oligonucleotide Separation Technology Columns (OST)
- Oasis® HLB solid-phase extraction (SPE) cartridges and plates
- Flexible detection capabilities, with high-sensitivity ACQUITY UPLC tunable UV (TUV), photodiode array (PDA), fluorescence (FLD), and mass spectrometry (MS)
- Robust software for processing and managing data

With Waters as your biopharmaceutical laboratory technology partner, it is possible to easily achieve the characterization capability your lab requires. The UPLC Oligonucleotide Analysis Solution is a comprehensive system solution that delivers the clear information you need, so you will know as much as possible about your oligonucleotides.

INTELLIGENTLY DESIGNED FOR OPTIMUM PERFORMANCE

The UPLC Oligonucleotide Analysis Solution, based on our award-winning ACQUITY UPLC System separation technology and new OST Columns, provides the ultimate in high-resolution chromatographic performance. And the system offers flexible detection options – such as tunable UV, photodiode array, fluorescence, and mass spectrometry – that are all optimized for UPLC.

Waters considered every aspect of oligonucleotide analysis to create a complete system solution, including:

- Separation chemistries
- Instrumentation
- Software
- Methodology
- Usability
- Documentation
- Application support
- Remote services

EXPANDING THE BOUNDARIES OF RESOLUTION AND SENSITIVITY

UPLC delivers significant improvements in oligonucleotide analysis when compared to HPLC. Exceptional resolution is possible through the use of UPLC's small particles, which result in dramatically narrower and sharper peaks. Just as significant, the surface chemistry of our second-generation Ethylene-Bridged Hybrid (BEH Technology™) OST Columns provide exceptional peak shape for a wide range of oligonucleotide properties.

This remarkable column performance can only be achieved with the optimized instrumentation of the ACQUITY UPLC System, which features reduced system volumes, minimal detector band-broadening, and accelerated data acquisition rates that are required to preserve high-efficiency separations – while maximizing sensitivity.

DESIGNED FOR REPRODUCIBILITY AND RELIABILITY

The ACQUITY UPLC System's advanced control of flow rate and precise gradient combines with stable column chemistry to provide highly reproducible chromatography. Long-term assay stability is ensured with columns that are quality-control tested specifically for oligonucleotide analysis.

Qualitative and quantitative analysis of impurities and synthesis artifacts

All elements of the UPLC Oligonucleotide Analysis Solution contribute to the successful qualitative and quantitative analysis of oligonucleotide variants, trace impurities, and truncated sequences. With this combination, oligonucleotides can be detected and measured with the greatest of confidence.

- UPLC-based fluidic performance ensures retention time reproducibility
- High-resolution UPLC separations of both large and minor peaks enable accurate integration
- OST Columns deliver exceptional chromatographic resolution and undistorted peak shape at extreme molar ratios

The system's advanced optical and mass detection technologies have a wide dynamic range, ensuring that oligonucleotides and impurities at extreme molar ratios are detected, even at the lowest limits of quantitation.

UPLC/MS FOR OLIGONUCLEOTIDES

The analysis of oligonucleotides by LC coupled with mass spectrometry is becoming a common practice. Many applications require the identification and analysis of oligonucleotides, modifications, variants, and process-related impurities at extremely low concentrations. With versatile configurations that can match your application requirements, Waters' portfolio of MS technologies provides the most appropriate solution for your laboratory's needs.

The ACQUITY® SQD combines the resolution, sensitivity, and speed of UPLC technology with single quadrupole MS detection. Designed specifically for chromatographers performing UPLC/MS analysis, it offers a simple yet powerful solution for multi-tasking, fast-paced, or routine laboratory environments – with robust and reliable performance, and walk-up operation for any expertise level.

The ACQUITY UPLC System's ability to produce unmatched resolution, combined with the spectral quality available with the ACQUITY SQD, enables easy identification of compounds in applications, including:

- Peak purity and impurity profiling
- Synthesis confirmation
- Product deformulation

The LCT Premier™ XE benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer delivers high sensitivity, resolution, and exact mass measurements for LC/MS oligonucleotide analyses. Its fast data acquisition rates and automated workflow features match the requirements for MS detection under UPLC conditions, including:

- High MS resolution for the selectivity needed to separate analyte spectra from isobaric interferences and background chemical noise
- High sensitivity for achieving very low detection limits
- High linear dynamic range, which allows experiments to be carried out across a range of concentration levels
- Exact mass MS measurements, which give elemental composition information that can be used to identify analyte compounds; you can obtain exact mass on molecular and fragment ions, simplifying the process of spectrum interpretation

The Q-Tof Premier,™ a quadrupole, orthogonal acceleration time-of-flight (oa-TOF) tandem mass spectrometer, provides enhanced levels of flexibility, sensitivity, specificity, and speed of MS data acquisition. It enables automated exact mass measurement of precursor and fragment ions to yield the highest confidence in structural elucidation and databank search results. Featuring T-Wave™ (Travelling Wave) Technology, the Q-Tof Premier offers significant enhancements in mass measurement accuracy, dynamic range, sensitivity with Enhanced Duty Cycle, and speed.

UPLC/MS/MS FOR OLIGONUCLEOTIDES

The Waters SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System offers unique, enabling functionality designed for researchers working at the limits of conventional mass spectrometry capabilities who need to further characterize and define their oligonucleotides.

Combining high-efficiency ion mobility-based measurements and separations with quadrupole time-of-flight (TOF) mass spectrometry, the SYNAPT HDMS System enables the analysis of oligonucleotides differentiated by size and shape, as well as mass, to deliver increased specificity and sample definition beyond that achievable by conventional mass spectrometers. This unique capability delivers enhanced sensitivity and mass accuracy, making MS/MS oligonucleotide sequencing possible through the unambiguous assignment of fragment ions.

Offering both TOF and HDMS operation modes, the SYNAPT HDMS expands your capabilities beyond conventional MS:

- Triwave™ Technology provides access to the unique benefits of ion mobility spectrometry (IMS), conformational studies, reducing sample complexity, and enhanced fragmentation
- Time-aligned parallel (TAP) fragmentation provides comprehensive structural (MS/MS/MS) information in a single experiment
- High duty cycle (HDC) mode enables significant sensitivity enhancements over a wide m/z range

OLIGONUCLEOTIDE SEPARATION TECHNOLOGY COLUMNS

Oligonucleotide Separation Technology (OST) Columns effectively isolate and analyze DNA, RNA, or modified and chimeric oligonucleotides. In addition, Oasis® HLB solid-phase extraction (SPE) cartridges and plates are ideally suited for sample desalting prior to MS or LC/MS analysis.

OST Columns contain second-generation hybrid-silica BEH Technology particles functionalized with C18. The analysis of detritylated synthetic oligonucleotide samples is based on the well-established method of ion-pair, reversed-phase chromatography.

OST chemistry delivers exceptional sample resolution and superior column life. In addition, Waters manufacturing and quality control testing procedures help ensure consistent batch-to-batch and column-to-column performance regardless of specific application demands.

The availability of 1.7 µm UPLC technology and 2.5 µm HPLC particles in a portfolio of optimized column dimensions gives you the flexibility to meet oligonucleotide analysis and laboratory-scale isolation needs.

- Scalable reversed-phase columns for lab-scale purifications
- Increased sample throughput with maintained component resolution with UPLC technology
- LC/TUV, LC/FLD, and LC/MS methods for enhanced quantitative and qualitative analyses
- Exceptional column life using Waters' patented BEH Technology particles

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UPLC SYNAPT MS/MS METHOD FOR STRUCTURAL CHARACTERIZATION OF siRNA OLIGONUCLEOTIDES

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INTRODUCTION

RNA interference (RNAi) mechanism plays a fundamental role in post-transcriptional gene silencing. With a knowledge of sequence, gene silencing experiments are now routinely performed using ~21 nucleotide (nt) long synthetic RNAi probes, which are also being developed as therapeutics.

Synthetic RNAi oligonucleotides (siRNA) need to be purified to avoid an off-target silencing of undesirable genes. RNAi drugs need to be well-characterized to satisfy regulatory requirements and minimize possible adverse implications for safety and efficacy.

The high-resolution chromatographic capabilities of Waters® UltraPerformance LC® (UPLC®) Technology coupled with mass spectrometry analysis provides a powerful tool for the analysis of biopharmaceutical drugs such as siRNA oligonucleotides. An important part of oligonucleotide characterization is sequencing, which can be performed using a selective enzyme or chemical. Methods employing MS/MS fragmentation are more generic and faster, and can be used for modified oligonucleotides, which are often resistant to enzymatic cleavage. In order to obtain structural information for the whole 21 nt RNAi, the molecules should be efficiently ionized and produce sequence-related ions during MS/MS using proper mass accuracy and resolution.

This application note presents the use of an exact mass UPLC/MS/MS analytical method for structural characterization of 21-nt-long RNA. The method is highly useful for confirmatory sequencing of siRNA base therapeutic compounds.

EXPERIMENTAL

Sample preparation

Complementary RNA strands of 21 nt length, upper strand 5' -UCG UCA AGC GAU UAC AAG GTT -3', and lower strand 5' -CCU UGU AAU CGC UUG ACG ATT -3', were reconstituted separately in 0.1 M triethylamine acetate (TEAA). A distribution of synthetic byproducts was detected along with the upper and lower strands by UPLC/MS. The sample concentration used for the UPLC/MS analysis was 30 pmol/µL.

Method

UPLC/MS analysis was performed as described previously.[1] The Waters® ACQUITY UPLC® System was used with an Oligonucleotide Separation Technology (OST) C_{18} Column, 1.7 µm, 2.1 x 50 mm (PN 186003949), for analysis. Mass spectrometry parameters were chosen in order to achieve maximum declustering of TEA adducts without compromising the intensity of the precursor ion. The Waters SYNAPT™ HDMS™ Mass Spectrometer was operated in time-of-flight (TOF) MS mode.

Collision-induced dissociation for selected ions was performed with a collision energy ramp from 25 to 55 V. The extent of MS/MS fragmentation was manipulated by selecting appropriate charge states (-3 to -6) and varying the collision energy ramp. Product ion spectra were acquired over the range of 500 to 7000 m/z at a rate of 1 scan/sec. External calibration in negative ion mode was performed with cesium iodide. MaxEnt™ 3 Software was used for spectral deconvolution prior to data analysis.

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18} 1.7 µm, 2.1 x 50 mm
Column temp:	60 °C
Injection volume:	5 µL
Flow rate:	0.2 mL/min
Mobile phase A:	15 mM TEA, 400 mM HFIP
Mobile phase B:	50% A, 50% methanol
Gradient:	20 to 40% B in 10 min

MS conditions

MS system:	Waters SYNAPT HDMS System
Capillary:	2.7 V
Sampling cone:	31 V
Extraction cone:	3 V
Source temp.:	120 °C
Desolvation temp.:	300 °C
Desolvation gas flow:	500 L/h
Trap collision energy:	6 V
Transfer collision energy:	4 V
Mass resolution:	~ 9,000 in V mode (FWHH)
LockMass:	CsI 10 mg/mL (water-isopropanol, 1:1), 5 µL/min flow rate, 1 sec scan time, 30 sec frequency, set mass 1685.765 m/z ($Cs_6I_7^-$)

RESULTS AND DISCUSSION

The collision energy profile affects the extent of MS/MS fragmentation; energy values need to be adjusted for the specific analyte of interest. Typically, the most abundant charge state is selected for MS/MS fragmentation, which for the 21-nt-long species is between -5 and -3 in the 1500 to 2000 m/z range. A collision energy ramp from 25 to 45 V was suitable to generate structurally-useful fragments for 21 nt RNA.

In general, in order to obtain reasonable signal-to-noise (S/N) for MS/MS fragmentation, a minimum signal of 500 ion counts is desirable for the main component or a contaminant in a total ion chromatogram (TIC).

Complementary RNA 21 nt strands were separately injected onto the UPLC/MS/MS system. The chromatogram revealed shorter oligonucleotide peaks (products of 5' hydrolysis) that are well-resolved from the original target 21 nt (Figure 1). Components were assigned based on their exact mass measurement, providing partial sequence verification.[1]

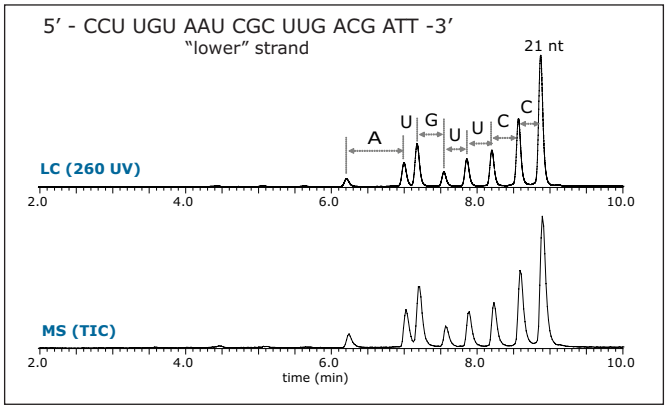

Figure 1. Fast UPLC/MS chromatograms of the lower RNA 21 nt strand.

In order to verify the structure of the whole oligonucleotide, the precursor ion, $[M-4H]^{4-}$, from the 21 nt lower strand was isolated and fragmented (Figure 2). The predominant characteristic fragments were complementary *c* and *y*-ions with low intensity *[a-B]* and *w* ions (nomenclature is shown in Figure 3). These sequence ions resulting from 5'-P-O cleavage are prevalent among the MS/MS fragments of RNA oligonucleotides, in contrast to those produced from DNA, which are almost exclusively *[a-B]* and *w* ions from 3'-C-O cleavage. This is due to the absence of the 2'-hydroxyl group in DNA molecule.[2]

Internal fragments, produced by double cleavage of the backbone as well as a neutral loss of cytosine from *c*-ion (Table 1) were significantly less abundant and did not contribute to structurally-useful peak assignments. MS/MS analysis of the upper 21 nt RNA strand through ramping of collision energy from 30 to 50 V yielded similar results (Figure 2).

ion fragment	theoretical m/z	observed m/z	ppm	ion fragment	theoretical m/z	observed m/z	ppm
$[a_2\text{-}B]^-$	418.066	418.065	2.4	c_{14}^{3-}	1467.164	1467.150	9.5
y_2^-	545.129	545.127	3.7	y_5^-	1524.270	1524.269	0.7
c_2^-	609.075	609.074	1.6	y_{10}^{2-}	1557.227	1557.200	17.3
w_2^-	625.095	625.094	1.6	c_5^-	1566.173	1566.174	-0.6
$[c_3\text{-Cyt}]^-$	804.057	804.057	0.0	w_{10}^{2-}	1597.210	1597.198	7.5
y_3^-	874.181	874.183	-2.3	c_{16}^{3-}	1691.863	1691.863	0.0
c_3^-	915.100	915.102	-2.2	y_{11}^{2-}	1729.751	1729.747	2.3
c_7^{2-}	1100.122	1100.114	7.3	c_{11}^{2-}	1742.705	1742.709	-2.3
$[c_4\text{-Cyt}]^-$	1110.083	1110.085	-1.8	w_{11}^{2-}	1769.734	1769.729	2.8
y_4^-	1219.229	1219.231	-1.6	y_6^-	1853.323	1853.330	-3.8
c_8^{2-}	1264.648	1264.639	7.1	y_{12}^{2-}	1882.271	1882.255	8.5
w_4^-	1299.195	1299.196	-0.8	c_{12}^{2-}	1895.225	1895.229	-2.1
$[a_5\text{-}B]^-$	1335.157	1335.157	0.0	w_6^-	1933.289	1933.289	0.0
c_9^{2-}	1417.660	1417.647	9.2	y_{13}^{2-}	2035.284	2035.298	-6.9
w_9^{2-}	1444.690	1444.683	4.8	c_{13}^{2-}	2048.238	2048.242	-2.0
$[c_5\text{-Cyt}]^-$	1455.130	1455.131	-0.7	c_7^-	2201.251	2201.272	-9.5

Table 1. Mass accuracy of assigned ion fragments of the lower RNA strand. RMS = 5.5 ppm.

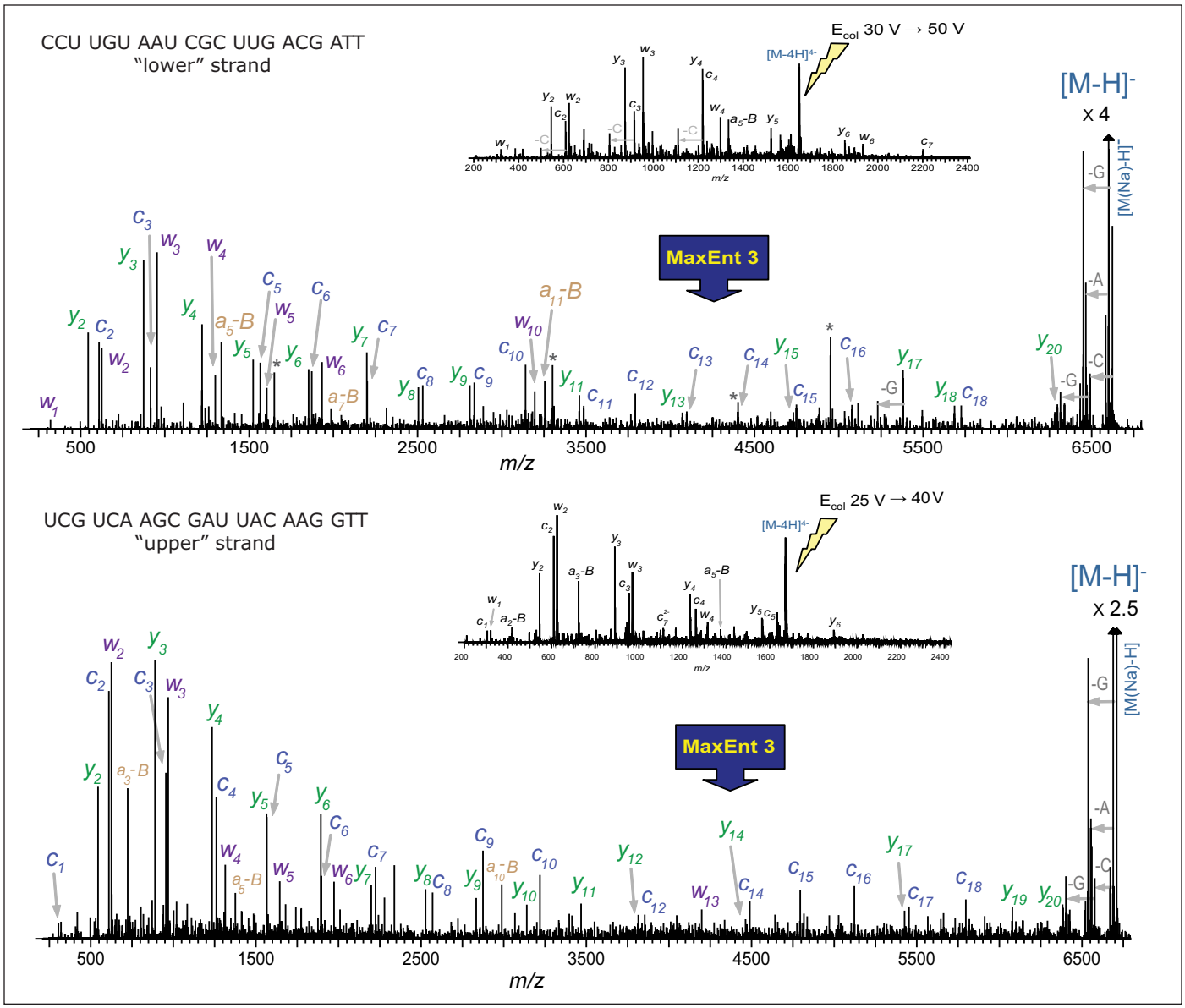

Figure 2. MaxEnt3 deconvoluted MS/MS spectra of 21 nt RNA oligonucleotides. Asterisk denotes harmonic peak (deconvolution artifacts).

Of all the fragment ions in the MS/MS spectra of $[M-4H]^{4-}$, 21 nt RNA were represented in several charge states (Table 1). Deconvolution of the MS/MS spectrum to singly-charged ions was performed using MaxEnt 3 software and significantly reduced the spectral complexity, simplifying the MS/MS data interpretation.

The deconvoluted spectra was sufficient to interpret the entire siRNA sequence (Figure 2). A few peaks corresponding to A, G, and C gas-phase nucleobase losses from the 21 nt molecular ion were also detected. The LockMass calibration, yielding a mass accuracy below 10 ppm, is highly useful for manual data interpretation (Table 1). Mass resolution of triple quadrupole mass spectrometers and ion trap instruments may not be sufficient for unambiguous assignment of characteristic fragments and distinguishing among multiply-charged peaks and other products of oligonucleotides' complex fragmentation.

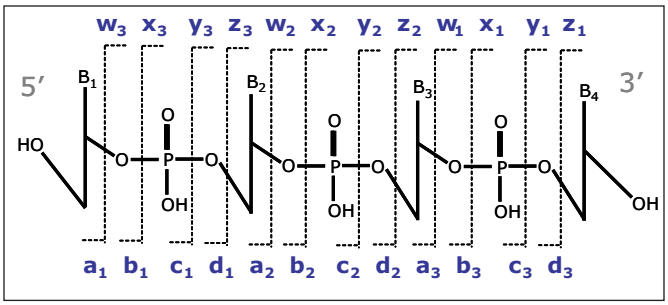

Figure 3. Nomenclature of oligonucleotide fragmentation.[3]

CONCLUSIONS

A reliable sequencing method via exact mass UPLC/MS/MS was developed for the structural characterization of siRNA oligonucleotides.

- The high mass accuracy and resolution of the SYNAPT HDMS System allows for unambiguous assignments of the fragment ions.
- This robust MS/MS method generates characteristic ion fragments that cover an extensive mass range, sufficient for interpretation of a full 21 nt siRNA sequence.
- MaxEnt 3 deconvolution software greatly reduces the complexity of MS/MS spectra and simplifies the sequence elucidation.

This efficient confirmatory sequencing method is very useful for sequence verification of therapeutic oligonucleotides required by the U.S. FDA for biotherapeutic compounds. The method is potentially applicable also for *de novo* sequencing of unknown impurities. It is expected that this MS/MS approach is suitable for chemically-modified oligonucleotides that are difficult to digest with exonucleases (ladder sequencing).

The automation of MS/MS enables the analysis time to be reduced by hours, if not days, and to decrease the cost of sample analysis. In addition, UPLC enables fast separation of RNA species of interest; multiple oligonucleotide strands can be analyzed by MS and MS/MS in a single analysis. The ability of UPLC to resolve target oligonucleotides from their truncated products is highly desirable for siRNA metabolism studies.

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RNAi DUPLEX ANALYSIS AND PURIFICATION

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INTRODUCTION

RNA interference (RNAi) is emerging as a new class of biopharmaceutical therapeutics for temporarily silencing genes and preventing protein translation. siRNA is a double stranded version of RNAi, which binds to RNA-inducing silencing complex (RISC). After cleavage of sense RNAi, part the RISC complex is activated, binds to a specific mRNA target, and, by cleaving, it interferes with protein production. This method of gene silencing is currently being utilized in a variety of animal studies and is receiving increased attention as a potential therapeutic strategy for humans.

A main challenge in developing therapeutics for humans remains the assurance of RNAi purity. The presence of certain related impurities may lead to unwanted, and potentially detrimental, off-target gene silencing. Major sources of impurities in siRNA duplexes originate from the complementary RNA strands' synthesis (failure synthesis by-products).

Duplex RNAi is prepared from complementary single-stranded RNA (ssRNA) sequences. Both single RNA strands typically require purification prior to hybridization and annealing. Annealing should be performed using equimolar amounts of RNA, since the excess of non-hybridized ssRNA in the target duplex is undesirable and often associated with a decrease in siRNA therapeutic potency.

In this application note, we outline a method utilizing the Waters ACQUITY UPLC® System with Oligonucleotide Separation Technology (OST) Columns for the simultaneous annealing and purification of RNAi duplexes in a single step. This method allows for sequential injection of complementary ssRNA molecules, which tightly focus on the column, anneal, and elute as a duplex.

The duplex can be collected by appropriate heart-cutting of the main peak, which yields a highly pure and stoichiometric duplex in a single step, as well as dramatically reduces the time and reagents needed to prepare the duplexes. Reduction in time and reagents coupled with the high purity of our method significantly lowers siRNA purification costs and increases production throughput.

EXPERIMENTAL AND DISCUSSION

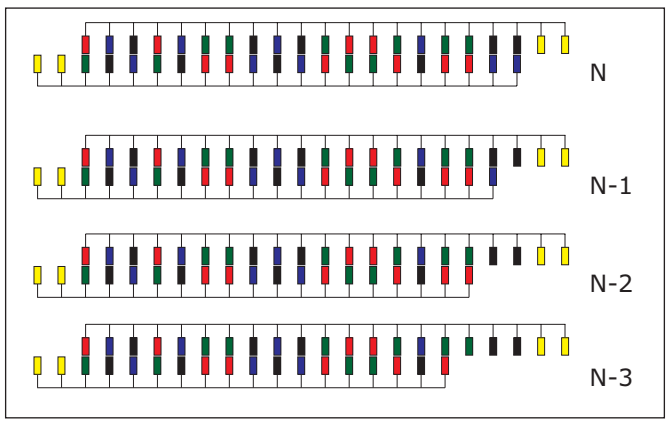
Sample

RNAi complementary strands (5' -UCG UCA AGC GAU UAC AAG GTT -3' and 5' -CCU UGU AAU CGC UUG ACG ATT -3') were purchased from Integrated DNA Technologies and reconstituted in 0.1 M triethylammonium acetate (TEAA), which was purchased as a 2 M solution from Fluka and diluted in 18 MΩ water to yield concentrations of approximately 2 nmol/µL.

An aliquot of one set of complementary strands was purified as ssRNA[1] and one strand was partially digested with the exonuclease phosphodiesterase II to generate a 5' truncated ladder or RNAi.

Mismatch RNAi separations via UPLC

We determined the ability of UPLC® to resolve the desirable siRNA duplex from its truncated forms (that were formed due to presence of failure synthetic RNA strands in the annealing mixture). In order to demonstrate UPLC's resolving performance for RNA duplexes, we utilized purified upper RNAi strand and partially digested lower strand. Upon annealing, a ladder of siRNA duplexes was formed with partially 5' truncated lower RNAi strands, as shown in Scheme 1.



Scheme 1. Duplexes formed by annealing full length upper RNAi strand with partially tryncated lower RNA strand. For UPLC/MS analysis of the duplexes, see Figure 1.

Analytical LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18}
	2.1 x 50 mm, 1.7 µm (P/N 186003949)
Column temp.:	20 °C
Flow rate:	0.2 mL/min
Mobile phase A:	25 mM HAA, pH 7.0
Mobile phase B:	100% Acetonitrile
Gradient:	30% to 40% B in 10.0 min
	(1% ACN/min)
Detection:	PDA Detector, 260 nm
	SQ, 600 to 2000 Da

MS conditions

MS system:	Waters SQ Detector
Mode:	ES-
Capillary:	3.0 kV
Cone:	28.0 V
Extractor:	3.0 V
RF:	0.1 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Cone gas flow:	31 L/h
Desolvation gas flow:	700 L/h

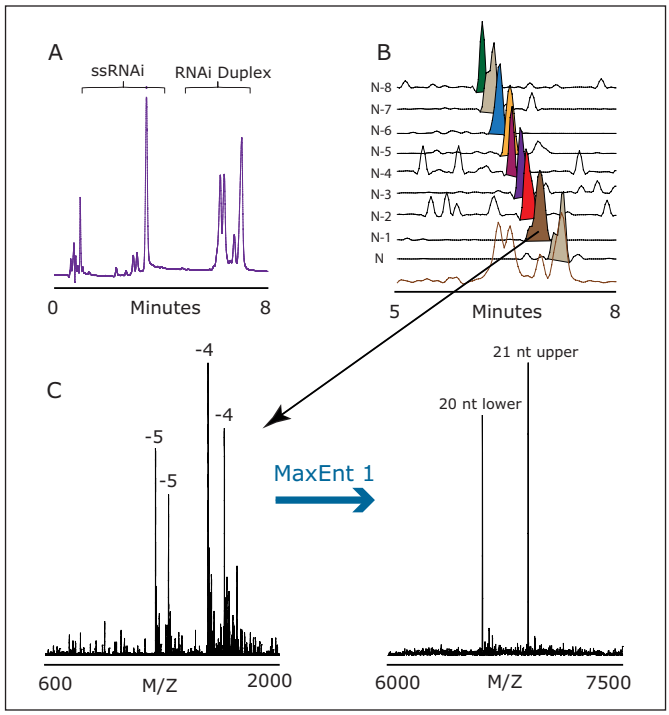


Figure 1. UPLC analysis of RNAi duplex mixture.

Panel A: UPLC PDA Detector 260 trace of full-length upper strand and truncated lower strand.
Panel B: SQ Detector TIC and SIC for UPLC analysis of RNAi duplexes.
Panel C: Representative MS spectrum for RNAi duplex with MaxEnt1 deconvolution.

Figure 1 shows the chromatographic results from the ACQUITY UPLC System and OST Column technology, which successfully resolved truncated siRNA duplexes from full-length duplex and single-stranded RNAi species. The separation was performed at 20 °C to maintain siRNA in a duplex form.

Using MS-compatible mobile phase comprised of hexylammonium acetate, we identified each eluting duplex peak by the corresponding mass of complementary RNAi strands and confirmed the elution order of the impurities. Extracted selected ion chromatograms, shown in Figure 1, indicate that retention time correlates with the length of the truncated complementary strand. The full length siRNA duplex eluted after the partially truncated duplexes.

Scaling to RNAi duplex purification

Based on the ACQUITY UPLC System's ability to provide impressive resolution of RNAi duplexes, we determined its utility for semi-preparative purification of siRNA duplexes as prepared from crude mixtures of ssRNA.

To accomplish this, we first mixed complementary ssRNA stoichiometrically and annealed the resulting mixture by heating the sample to 90 °C followed by cooling slowly to room temperature. This mixture was then separated via HPLC on an analytical-scale column and the appropriate duplex fraction was collected, as shown in Figure 2.

Purification LC conditions

LC system:	Waters Alliance® HPLC Bioseparations System
Column:	Waters XBridge™ OST BEH C_{18} 4.6 x 50 mm, 2.5 µm
Column temp.:	20 °C
Flow rate:	1.0 mL/min
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	20% ACN in A
Gradient:	25% to 75% B in 30 min
Detection:	PDA, 260 nm

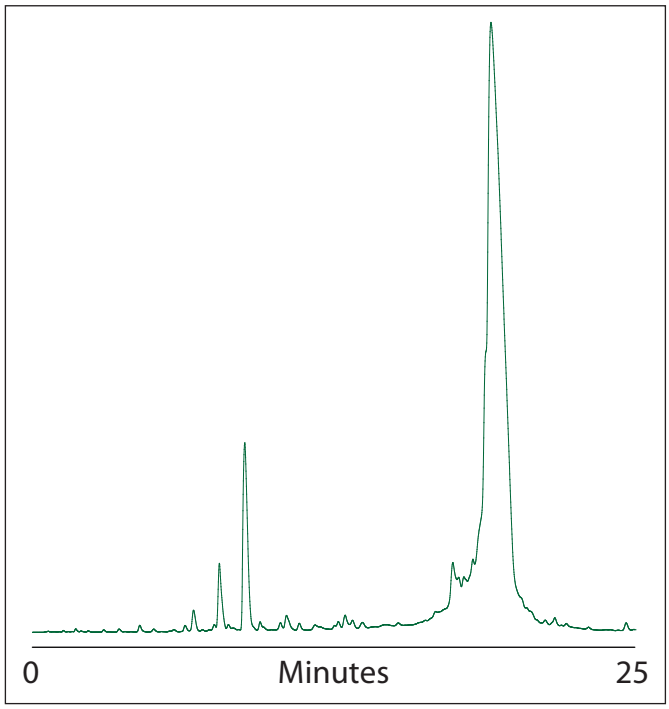


Figure 2. HPLC purification of RNAi prepared by separate annealing of complementary single stranded RNA.

We found quantitative conversion to the desired siRNA duplex with good resolution of the main product from both single-stranded and duplex impurities. Analysis of the collected fraction was obtained using UPLC with the PDA and SQ detectors; MS detection indicated 98% purity of collected siRNA. No single-stranded contaminants were detected by UPLC analysis (data not shown).

To further evaluate the utility of our method, we investigated the possibility of on-column annealing of crude complementary ssRNA. To accomplish this, we prepared solutions of each crude complementary strand in 0.1 M TEAA and verified the concentration by injection of a small amount of each solution.

Since each strand is of the same length, with similar extinction coefficients, the use of integrated peak area was found to be sufficient for calculating the desirable injection volumes to introduce an approximately stoichiometric amount of RNA on column.

The first RNA strand was injected onto the column under initial gradient conditions. Immediately after, the second complementary strand was injected and the gradient elution was initiated.

The chromatogram in Figure 3 reveals that both strands anneal nearly quantitatively on-column and are eluted as duplex siRNA. This is probably due to tight spatial focusing of complementary strands on the head of the column. Small excess of one single-stranded RNAi eluted prior to the main peak, as expected. Truncated RNA duplexes were resolved from the target siRNA.

Figure 3 illustrates the siRNA purification with on-column annealing on three different mass loads. The method for siRNA purification was scaled up to ~85 nmol using analytical column.

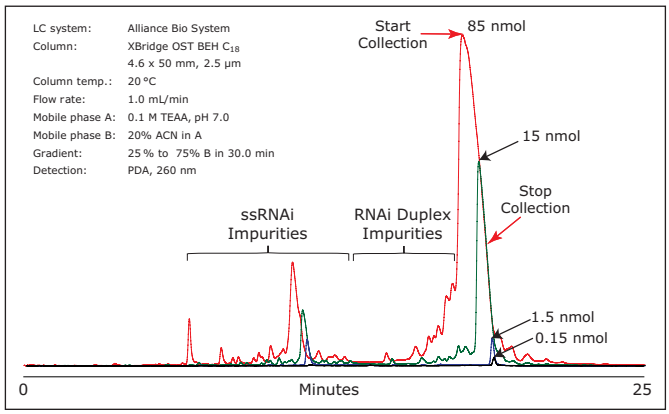


Figure 3. HPLC traces for the purification of RNAi duplexes generated by on-column annealing of ssRNA injected sequentially. Collection window indicated for 85 nmol injection only were generally collected from peak apex to 30% of the peak height.

The on-column annealing siRNA purification methods represent a significant improvement over the earlier presented RNAi purification.[2] By eliminating the need for separate purification steps for each complementary strand, and annealing the strands on-column, our method allows the researcher to substantially reduce the time needed for sample preparation.

Following purification, collected fractions were analyzed with UPLC analysis (Figure 4), which was done in the highly-resolving mobile phase HAA at 20 °C to preserve the duplex. We also analyzed the duplex at 60 °C, which fully denatured the duplex and generated two single-stranded counterparts. Both analysis techniques indicated that the fraction collected contains the desired duplex and that the purity is greater than 98%.

Fraction analysis by UPLC

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	20 °C
Flow rate:	0.2 mL/min
Mobile phase A:	25 mM HAA, pH 7.0
Mobile phase B:	100% Acetonitrile
Gradient:	30% to 40% B in 10.0 min (1% ACN/min)
Detection:	PDA, 260 nm

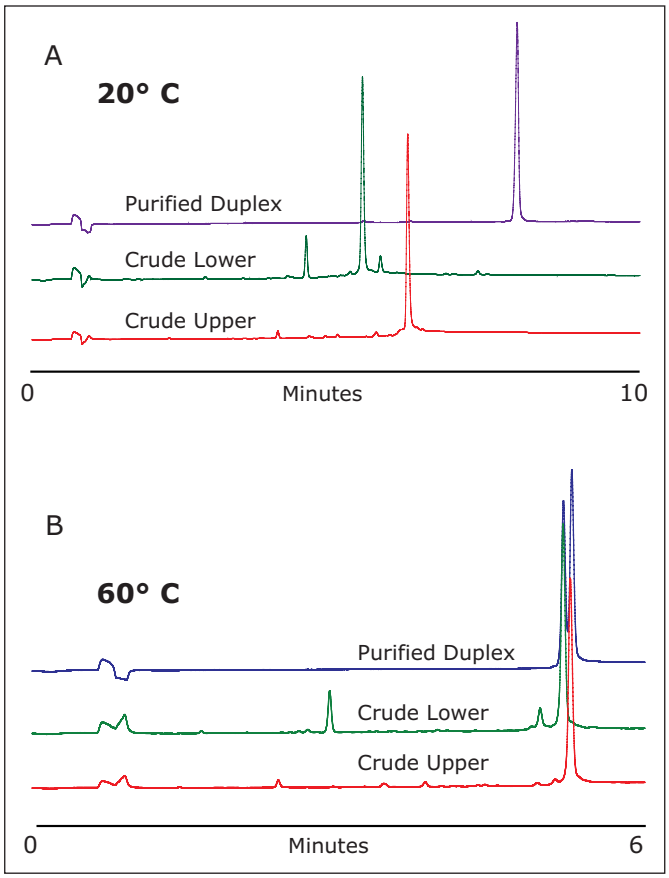


Figure 4. UPLC verification of RNAi duplex purity and composition.

Panel A: Analysis of single stranded and duplex RNAi at 20 °C.
Panel B: Analysis of single stranded and duplex RNA at 60 °C, which denatures the duplex.

CONCLUSION

In this application note, we have described a novel method for analysis and purification of double stranded siRNA. Using the Alliance HPLC Bioseparations and ACQUITY UPLC systems with ACQUITY UPLC and XBridge OST columns, we were able to efficiently resolve full-length siRNA duplexes from shorter truncated duplexes. The method can easily be scaled from analytical to preparative, allowing for fast purification of siRNA prior to gene silencing experiments.

The method utilized non-denaturing, mass spectrometry-compatible mobile phase comprising hexylammonium acetate and acetonitrile. The separation of single-stranded RNAi impurities and siRNA truncated duplexes was monitored by UV and MS. The retention order of impurities was confirmed by MS data. Non-denaturing mobile phases and low separation temperatures are necessary to maintain the stability of non-covalent complexes (duplex RNA) throughout the analysis.

This application note proposes a novel approach for purification of duplex siRNA using on-column annealing of RNA strands rather than purification of RNAi in single-stranded form (followed by off-line annealing). The presented method allows for high yields and purity of the desired duplex in a dramatically shorter time period. Volatile mobile phases allow for easy removal of mobile phase without the need for additional desalting.

The proposed purification strategy has potential to significantly improve the productivity of siRNA manufacturing. It allows manufacturers to ship the custom made siRNA product within a single day, which is often not attainable with the traditional purification strategies. Faster manufacturing of high quality siRNA probes will help to facilitate the adoption of silencing RNA technology.

References

1. Real-Time Analysis of RNAi Duplexes. Waters Application Note. 2008; 720002573en.
2. Semi-Preparative-Scale Single-Stranded RNA Purification. Waters Application Note. 2007; 720002602en.

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UPLC/MS ANALYSIS OF INTERFERING RNA OLIGONUCLEOTIDES

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INTRODUCTION

The discovery of RNA interference mechanisms, now broadly used for silencing the expression of target genes, has created a new category of synthetic RNA oligonucleotides containing a matrix of modifications. The primary intent of these modifications to native RNA molecules is to increase binding constants, increase nuclease resistance, or help preserve unique secondary structure.

Because these molecules are synthetically produced step-wise in a solid phase process, the final product may contain multiple truncated oligonucleotides and a mixture of process-related impurities. It is critical to not only be able to detect modifications, process impurities, or contaminants, but to quantitate them as well since they may directly affect compound efficacy and safety.

Similar to any pharmaceutical product, these molecules must also be assayed for identity and purity.

In order to accomplish full characterization of these complex molecules, multiple analysis techniques are typically used, requiring a number of different types of instruments and highly-specialized laboratory technicians. To satisfy the need for a single, sensitive, quantitative, and high-throughput method for RNAi analysis, a method has been developed utilizing an ACQUITY UltraPerformance LC® (UPLC®) System, Oligonucleotide Separation Technology (OST) Columns and the Q-Tof Premier™ Mass Spectrometer for detection.

The Waters® ACQUITY UPLC® System, combined with ACQUITY UPLC OST Columns packed with 1.7 µm sorbent, offers superior analytical performance for oligonucleotide analysis compared to HPLC, fast LC separations, and other techniques.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	15 mM TEA. 400 mM HFIP
Mobile phase B:	50% A, 50% methanol
Gradient:	20 to 40% B in 10 min
Detection:	ACQUITY UPLC PDA, 260nm

MS conditions

MS system:	Waters Q-Tof Premier Mass Spectrometer
Capillary:	500 V
Sample cone:	35 V
Extraction cone:	3 V
Ion guide:	2.5 V
Desolvation temp.:	200 °C
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Desolvation gas flow:	600 L/hr

Sample

RNAi 21 nt (nucleotides), 5' -UUC UGU AAU CUC UUG UCU ATT -3', and 20 nt, 5' -UC UGU AAU CUC UUG UCU ATT -3', were purchased from Integrated DNA Technologies, Coralville, IA, U.S. The samples were reconstituted in 0.1 M triethylamine acetate (TEAA) to make 40 pmole/µL concentration for LC/MS analysis.

RESULTS AND DISCUSSION

Exceptional resolution of the UPLC separation was achieved for the RNAi sample within 10 minutes, as shown in Figure 1. The full-length synthetic RNAi product was successfully resolved from its failure sequences.

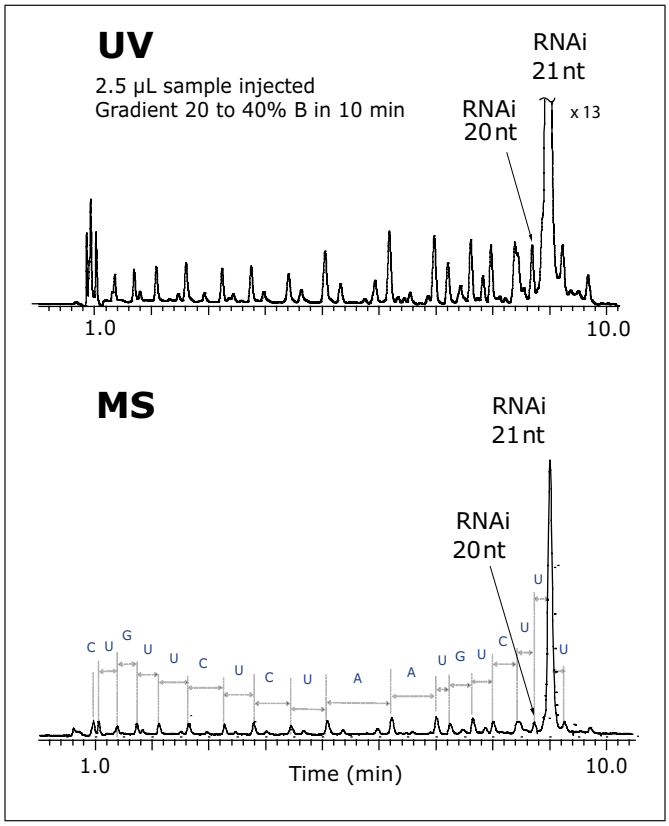

Figure 1. UV chromatogram (260 nm) and total ion chromatogram (TIC) of RNAi 21 nt.

This method is suitable for the oligonucleotide purity determination and monitoring of the chemical synthesis efficiency.

Mobile phases containing 15 mM triethylamine (TEA), 400 mM hexafluoroisopropanol (HFIP), pH 7.9, and methanol are compatible with MS electrospray ionization. Choosing the narrow (75 µm I.D.) silica capillary tubing between the PDA detector and the ESI source decreased a post-UV void volume and reduced the broadening of the chromatographic peaks prior to MS detection.

The acquisition of the accurate masses allowed for an assignment of the peaks of 5' -truncated oligomers (failed sequences generated during oligo synthesis), as well as some other impurities. The mass of each peak in the MS chromatogram was deconvoluted using MaxEnt1 software.

The tentative 5' -end failure products are assigned in Figure 2. Nearly the entire sequence of the parent oligonucleotide was elucidated. MS analysis also revealed a presence of an extra uridine mononucleotide added to the target 21-mer RNAi sequence.

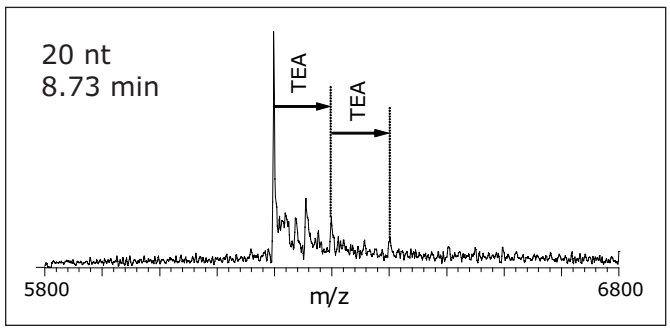

Figure 2. Deconvoluted mass spectrum of RNAi 20 nt using MaxEnt1.

In order to verify the origin of the peak adjacent to the target 21 nt oligomer, an original sample was spiked with its 20 nt (N-1) homolog. Both oligonucleotides were mixed 1:1 to obtain 40 pmole/µL of each oligomer in a vial; 5 µL of the sample was injected on column.

The high efficiency of the developed method allowed the separation to resolve 21 nt from 20 nt N-1 RNAi within an 8-minute analysis (Figure 3). This experiment confirmed the correct assignment of the 20 nt peak as a failed sequence peak in Figure 1. Faster separation, shown in Figure 3, was achieved by adjusting the initial gradient strength as noted. When maintaining the same gradient slope, resolution is not negatively affected.

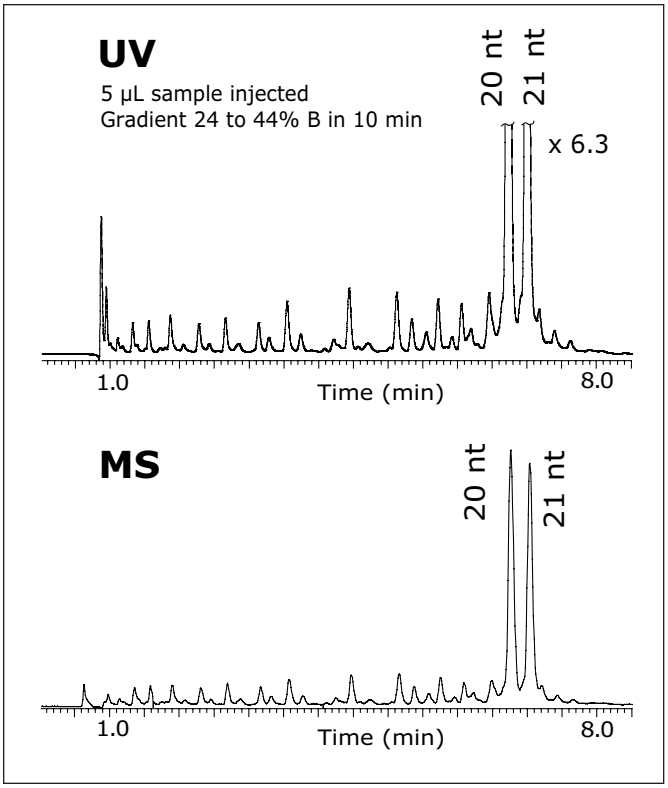

Figure 3. TIC and UV-chromatogram of the resolved oligomers of RNAi.

Formation of clusters and adducts from a buffer containing TEA was detected as 101 Da and 202 Da adducts in the deconvoluted mass spectra (Figure 2). Few sodium and potassium adducts were observed and did not obscure the mass spectral interpretation. MS parameters, including desolvation temperature, desolvation gas flow, cone gas flow, and cone voltage were chosen in order to achieve maximum declustering without compromising spectral intensity.

CONCLUSION

We have demonstrated the ability of a single method on the UPLC/MS system to perform routine analysis and quality control of small interfering RNA molecules. Superior UPLC resolution, together with the mass accuracy capability of the Q-Tof Premier Mass Spectrometer, allows for assignment of the low-intensity peaks and deciphering of the RNAi oligonucleotide sequence.

This method's exceptional dynamic range will enable the low-level quantitation of modifications, process impurities, or contaminants in the presence of the main RNAi molecule facilitating regulatory requirements compliance. Additionally, the comprehensive characterization and analysis results are generated by a single laboratory analyst on a single system, enabling greater overall laboratory throughput and efficiency.

References

1. UPLC Separation of Oligonucleotides: Method Development. Waters Application Note. 2007: 720002383EN.
2. UPLC/MS Separation of Oligonucleotides in Less than Five Minutes: Method Development. Waters Application Note. 2007: 720002387EN.
3. UPLC Analysis of Phosphorothioate Oligonucleotides: Method Development. Waters Application Note. 2007: 720002405EN.

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REAL-TIME ANALYSIS OF RNAi DUPLEXES

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INTRODUCTION

RNA interference (RNAi) is a rapidly emerging strategy for temporarily silencing genes and preventing protein translation. RNAi is a double-stranded non-coding RNA molecule designed to bind to a specific mRNA target, and via a cascade of biochemical reactions interfere with the protein production. This method of gene silencing is currently being utilized in a variety of animal studies and is receiving increased of attention as a potential therapeutic strategy for humans.

A major challenge for developing human therapeutics preventing protein translation remains the assurance of RNAi purity. The presence of certain related impurities may lead to the possibility of unwanted, and perhaps detrimental, non-targeted gene silencing.

Major sources of impurities in RNAi duplexes are often the result of degradation, intra-molecular hybridization mismatches, or more commonly incomplete syntheses of the complementary single-stranded RNA complementary strands. The presence of non-hybridized single stranded RNAi is also undesirable and often associated with a decrease in therapeutic potency.

In this application note, we describe a rapid and highly sensitive method for the routine analysis of RNAi duplexes preventing protein translation using the Waters® ACQUITY UPLC® System with Oligonucleotide Separation Technology (OST) Column chemistry.

METHODS

LC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	20 °C
Flow rate:	0.2 mL/min
Mobile phase A:	0.1 M TEAA, pH 7.5
Mobile phase B:	20% Acetonitrile in A
Gradient:	35 to 85% B in 10.0 min (1% ACN/min)
Detection:	ACQUITY UPLC PDA, 260 nm

Sample

RNAi complementary strands 5' -UCG UCA AGC GAU UAC AAG GTT -3' (upper) and 5' -CCU UGU AAU CGC UUG ACG ATT -3' (lower) were purchased from Integrated DNA Technologies and reconstituted in 110 µL of 0.1 M triethylammonium acetate (TEAA) to yield concentrations of approximately 2.5 nmol/µL. The samples were purified prior to use[1] and purity was verified prior to duplex formation experiments (Figure 1).

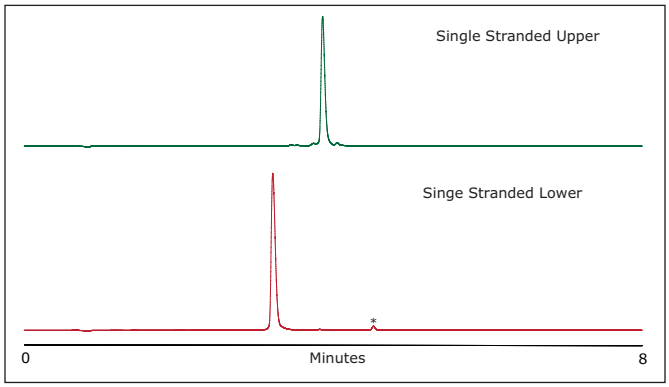

Figure 1. Determination of single-stranded RNA oligonucleotide purity. (= non-oligo impurity.)*

RESULTS AND DISCUSSION

RNAi duplex formation

RNAi duplexes were prepared by combining appropriate molar ratios of upper and lower (2:1 and 1:2) complementary strands in 0.1 M TEAA. Mixtures were heated at 90 °C for 5 minutes and gradually cooled to 20 °C. Samples were prepared immediately prior to use to minimize sample degradation.

UPLC conditions

RNAi duplex samples were separated on a Waters ACQUITY UPLC System using an ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm column using ion-pairing reversed phase chromatography.[2] Separated RNA species were detected with an ACQUITY UPLC PDA detector scanning from 19 to 350 nm.

As shown in Figure 2, this system offered exemplary component resolution with no evidence of on-column duplex degradation or melting. Additionally, this method offers impressive separation of the desired duplex from impurities present in the sample, primarily mismatched sequences.

In the presence of excess single-stranded RNA, retention times of both the excess single-stranded RNA and duplex remain constant, highlighting the utility of this method for purification of RNAi. The method also allows for the separation of failure sequence and other mismatch duplexes from the desired duplex product.

To fully investigate the utility of the chromatographic system and to confirm that there was no on-column melting of RNA duplexes with this method, we investigated whether our separation conditions would allow for the formation of RNA duplexes on an ACQUITY UPLC OST Column. To accomplish this, we separately injected each of the complementary RNAi strands under 100% binding conditions followed by gradient elution of the bound samples.

We found quantitative formation of an RNAi duplex following injection of upper and lower RNAi strands, with the resulting duplex peak exhibiting the identical retention time of authentic RNAi prepared via a separate annealing step (Figure 3). This data strongly indicates that under our UPLC separation conditions RNAi duplex does not melt during LC separation. In fact, it appears that spontaneous on-column annealing is favored as is indicated by the appearance of only one single-stranded peak present in a duplex/single-strand mixture.

Once quantitative duplex formation is accomplished, the duplex peak is predominant and well-separated from other impurities.

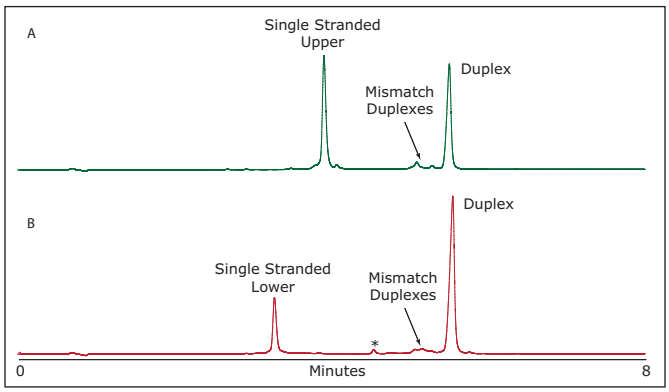

Figure 2. Separation of RNAi duplex from excess single-stranded upper (panel A) and lower (panel B) RNA. (= non-oligo impurity.)*

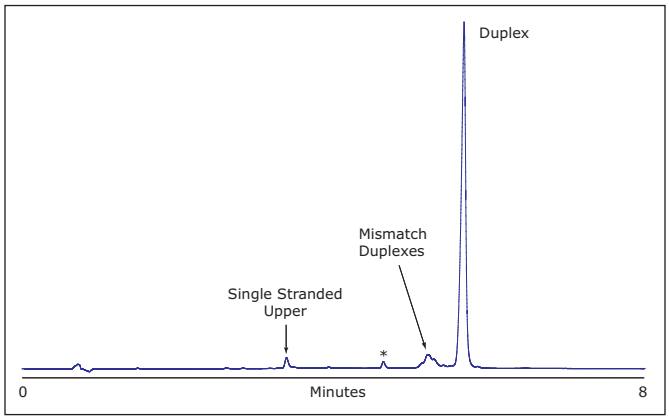

Figure 3. RNAi duplex formation on an ACQUITY UPLC OST Column. Single-stranded upper loaded on column at 35% B and single-stranded lower loaded immediately after. (= non-oligo impurity.)*

CONCLUSIONS

The data presented highlights the superior chromatographic resolution possible using the ACQUITY UPLC System and OST Column chemistry. This UPLC system solution offers superior performance for the efficient detection, quantification, and chromatographic resolution of RNAi duplexes from their single-stranded and mismatched counterparts, and also shows considerable utility in monitoring their formation.

The described UPLC method enables rapid and real-time quality control analysis of the reaction progress, eliminating the excessive RNAi characterization times associated with other analytical methods.

This method does not effect additional degradation of the duplex, enabling accurate, consistent, and reproducible analysis of RNAi duplexes. Finally, the UPLC method offers superior resolution, allowing for the detection and quantitation of excess single-stranded RNA, mismatched duplexes, and synthetic impurities.

Overall, this enables the direct analysis of reaction products and allows for purity determination in a high throughput manner.

References

1. Semi-Preparative Scale Single Stranded RNA Purification. Waters Application Note. 2008: 720002602en.
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SEMI-PREPARATIVE SCALE SINGLE-STRANDED RNA PURIFICATION

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INTRODUCTION

Oligonucleotide synthesis is a very efficient and high-yielding process. Typical yields of oligonucleotide reactions carried out on solid support range from 98 to 99.5% per coupling step. In a typical multi-step oligonucleotide synthesis, impurities accumulate and the overall yield of even a modest sized 21-mer oligonucleotide can range from 67 to 90%, with longer chain oligonucleotides giving correspondingly lower yields.

For researchers, it is often necessary to work with materials of higher purity than are available from crude synthetic mixtures. For this reason, oligonucleotides used for gene knockout, genotyping, and diagnostic purposes are typically purified following synthesis. Few economically viable solutions exist for lab-scale purification of oligonucleotides, and those that do exist – such as ion-exchange chromatography and polyacrylamide gel electrophoresis – are often cumbersome and/or time-consuming.

In this application note, we describe a cost-effective and rapid method for the purification of modest quantities of material, up to 140 nmoles in a single injection, with final purities of greater than 95% using the Waters® ACQUITY UPLC® System with Oligonucleotide Separation Technology (OST) Column chemistry. The purification scale presented matches well with typical oligonucleotide synthetic scales (50 to 250 nmol). The method described below allows for the purification of oligonucleotides with high purity products in 15 to 30 minutes.

RESULTS AND DISCUSSION

Sample

The RNA oligonucleotide 5' -CCU UGU AAU CGC UUG ACG ATT -3' was purchased from a vendor and reconstituted in 110 µL of 0.1 M triethylammonium acetate (TEAA) to yield a solution of approximately 2.8 nmol/µL. The sample was prepared immediately prior to use to prevent degradation.

HPLC conditions

The RNA oligonucleotide was purified using a Waters Alliance® HPLC Bioseparations System using a Waters XBridge™ BEH OST C_{18} 4.6 x 50 mm, 2.5 µm column using ion pair reversed-phase chromatography.[1]

LC system:	Waters Alliance HPLC Bioseparations System
Column:	XBridge OST BEH C_{18} 4.6 x 50 mm, 2.5 µm
Column temp.:	60 °C
Flow rate:	1.0 mL/min
Mobile phase A:	0.1M TEAA, pH 7.5
Mobile phase B:	20% Acetonitrile in A
Gradient:	30 to 52.5% B in 10.0 min (0.15% ACN/min)
Detection:	PDA, 260 nm

Separated products were detected with a Waters PDA detector at 290 nm. The mobile phase A consisted of 0.1 M triethylammonium acetate (TEAA); mobile phase B was 80:20 0.1 M TEAA/acetonitrile. The column temperature was maintained at 60 °C.

As shown in Figure 1, despite the high efficiency of oligonucleotide synthesis, there are many failed sequences present in a 21-mer.

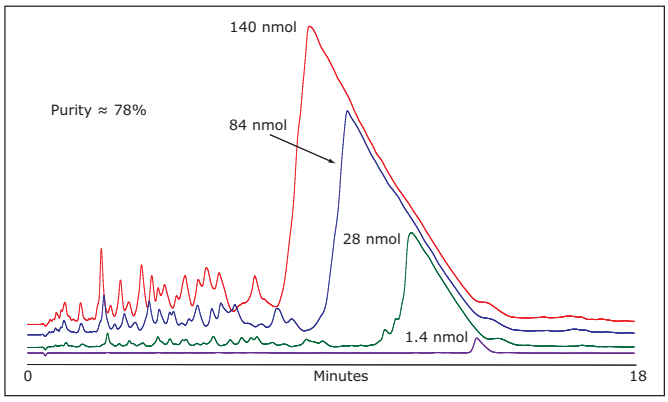

Figure 1. HPLC purification of a synthetic 21-mer oligonucleotide. Sample concentration was 2.8 nmol/µL, with on-column loading ranging from 1.4 to 140 nmol.

Although the column is overloaded with greater mass loads, the resolution is maintained with N-1, N-2... impurities eluting at the main peak front. Appropriate hearth-cutting of the main 21-mer oligonucleotide peak yields very high purity product.

The chosen fraction collection windows are indicated in Figure 2 for various mass loads. Following peak collection, samples can be aliquoted as needed and dried for long term storage. The volatility of TEAA allows for an easy removal of ion-pairing buffer components. The purified oligonucleotides after the solvent evaporation are practically salt free.

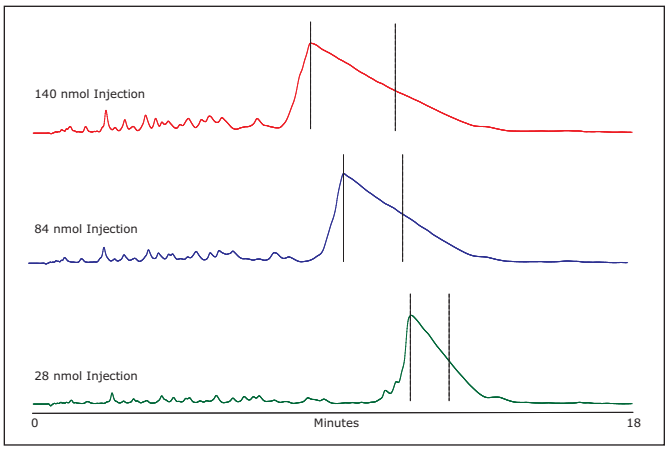

Figure 2. Fraction collection windows. Fractions were collected manually as indicated between dashed lines.

UPLC conditions

The purity of the purified RNA oligonucleotide was verified using the ACQUITY UPLC® System. As shown in Figure 3, our purification method efficiently reduces failed sequence impurities and generates an oligonucleotide of superior purity than is available from commercially available oligonucleotides without purification.

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	0.1M TEAA, pH 7.5
Mobile phase B:	20% Acetonitrile in A
Gradient:	35 to 85% B in 10.0 min (1% ACN/min)
Detection:	PDA, 260 nm

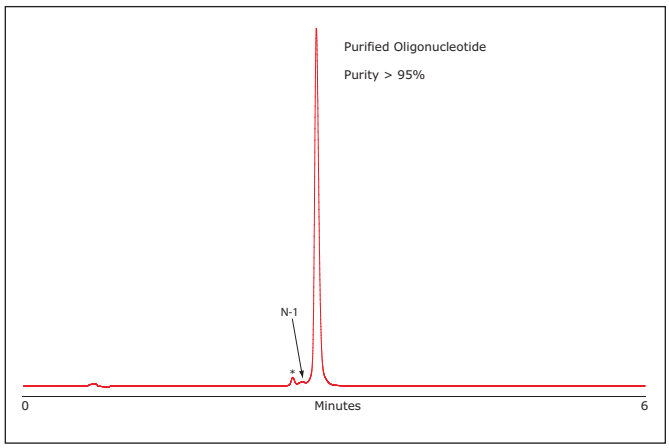

Figure 3. Verification of oligonucleotide purity via UPLC. (= non-oligo impurity.)*

CONCLUSION

The purification strategy for single-stranded RNA oligonucleotides presented here is rapid, cost effective, and yields high purity material. In a short time, using OST Column chemistry and the Alliance HPLC Bioseparations System, large quantities of crude single-stranded RNA can be successfully purified yielding material of high purity, ca. 95%, with an estimated yield of 55% based on collected peak area to the total peak area of the sample.

This method is particularly useful for the purification of single-stranded RNA for use in RNAi experiments where assurance of purity, and therefore specificity for the target, are of paramount importance.

Additionally, this strategy allows for storage of purified oligonucleotides in the absence of unwanted salts and other impurities often associated with other purification strategies due to the volatile nature of TEAA.

Taken together, this strategy offers a comprehensive purification strategy that is superior to those currently available. Furthermore, this purification method is very cost effective when considering the combined cost of time needed for sample purification, reagents, and the long lifetime of Waters XBridge OST columns.

References

1. UPLC Separation of Oligonucleotides: Method Development. Waters Application Note. 2007: 720002383EN.

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OLIGONUCLEOTIDE SEPARATION TECHNOLOGY: SYNTHESIS CHALLENGES AND HPLC ISOLATION OPTIONS

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INTRODUCTION

Origins of synthetic oligonucleotides impurities

Use of synthetic oligonucleotides is increasing in areas ranging from clinical diagnostics to novel biopharmaceutical therapeutics. While the automated synthesis of oligonucleotides is a highly efficient process, small amounts of impurities are created at each step throughout the synthesis cycle. Consequently, manufacturing organizations as well as individuals who depend on the quality of delivered products have a vested interest in cost effective and efficient ways to purify and analyze these important biological tools. Failure to achieve these goals can seriously impede the ability of an organization or individual to achieve desired results. An example might involve a delay in obtaining FDA approval for a new diagnostic reagent or drug.

A closer inspection of how synthesis coupling efficiency impacts the amount of manufactured full length product is shown in Figure 1. Regardless of average coupling efficiency, longer oligonucleotide sequences contain a greater concentration of shorter length contaminants. The failure products, typically labeled N-1, N-2..., N-x, are prematurely halted shorter oligonucleotides. Some are missing a nucleotide(s) in the middle of sequence, rather than at the end. These products are called mismatch failure sequences.

Some by-products of synthesis may have greater molecular weight (often labeled N+x) than the target oligonucleotide. This is a result of incomplete post-synthesis deprotection, or due to the branching of an oligo backbone during the synthesis. For labeled oligonucleotides, the failure products are also generated by failure to conjugate the label with the target sequence.

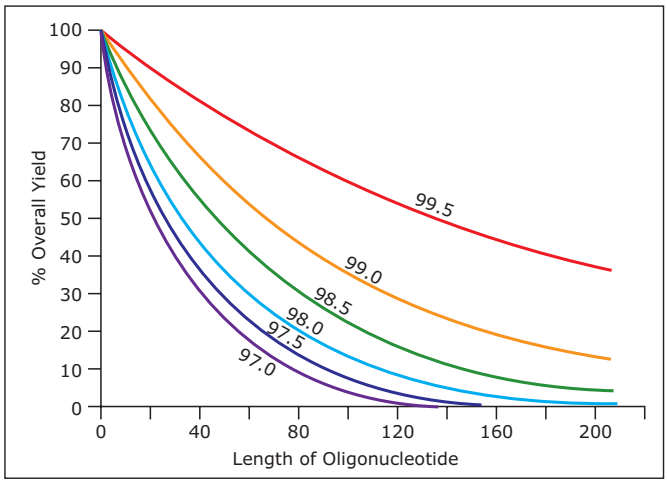

Figure 1. Synthetic oligonucleotide length compared to theoretical yield at various coupling efficiencies.

This application note addresses how Waters® Oligonucleotide Separation Technology Columns are the most viable option for handling the challenges of purification and isolation of synthetic oligonucleotides.

DISCUSSION

Lab-scale isolation options

Once a synthesis is complete, the synthetic oligonucleotide must be cleaved from the solid-phase support (e.g. controlled pore glass). The base and phosphate groups must then be fully deprotected prior to use of any subsequent purification technique. Table 1 highlights commonly used methods for the lab-scale purification (25 to 500 nmole) of synthetic oligonucleotides. The advantages as well as disadvantages of each technique are presented.

Technique	Advantages	Disadvantages
Polyacrylamide gel electrophoresis (PAGE)	Well-established and efficient method. It separates long oligonucleotides (>50 to 60 mer).	Low mass loading capacity. Gels are typically overloaded for purification and the resolution is compromised. PAGE does not separate N+x sequences. Manual band cutting. Excision is based on markers without detailed knowledge of target oligo retention. Samples need to be extracted from the gel and desalted; recovery of target oligonucleotides is low. Method is laborious; it is typically used only when no other technique is suitable for the task.
Ion exchange liquid chromatography (IEX-LC)	Trityl-off method. Separation of failure sequences is due to the backbone charge.	IEX-LC is efficient only for relatively short oligos (<20 to 25 mers); longer oligos are poorly resolved. Sample is contaminated with high concentration of salts; further desalting is required. IEX columns packed with non-porous sorbent offer improved resolution, but suffer with low mass load capacity. When loading exceeds 10 to 20 nmoles (for 4.6 mm I.D. columns), the resolution is compromised. IEX-LC does not separate N+x sequences.
Trityl-on liquid chromatography (Trytil-on LC; DMT-on LC)	Elegant, fast, and universal method for oligos of various length and sequence. RP columns used with this method have sufficient mass load capacity.	Does not adequately remove mismatch failure sequences (similarly as the target oligo, they carry DMT group). DMT group is labile; part of the product may be lost due to the spontaneous detritylation. DMT residue and remaining acid have to be removed after the detritylation.
Trityl-off liquid chromatography (Trityl-off LC; DMT-off LC)	Effectively removes practically all types of failure products. Uses volatile solvents; samples do not have to be further desalted. Collected fractions are simply lyophilized and ready for use. RP columns used with this method have sufficient mass load capacity. Labeled and dually-labeled oligonucleotide probes can be also purified. Method is suitable for LC/MS analysis (with MS compatible ion-pairing buffers).	Method requires efficient columns packed with small particle size sorbent. Oligo retention and resolution partially depends on the sequence. Method development for different oligo sequence and length probes is necessary.

Table 1. Advantages vs. disadvantages of synthetic oligonucleotide lab isolation techniques.

Oligonucleotide Separation Technology

Waters Oligonucleotide Separations Technology (OST) Columns are specifically designed for the HPLC purification and HPLC or UltraPerformance LC® (UPLC®) analysis of synthetic oligonucleotides. Its separation mechanism is based on highly efficient ion-pairing reversed-phase (IR-RP) chromatography of the "trityl-off" synthetic oligonucleotide species, where the oligonucleotide is detritylated at the last step of synthesis. IP-RP LC separates the trityl-off full length product from failure sequences.

Waters OST columns were developed following a series of comprehensive investigations that helped Waters scientists and engineers better understand limitations of existing technologies for this application area. Our flexible separation chemistry technology is designed to assist manufacturers deliver quality products that can help researchers make profound discoveries (e.g. via siRNA research) that lead to novel drug therapies or diagnostic reagents.

As shown in Figure 2, separation of N from N-1 species on OST Columns rivals separations obtained using capillary gel eletrophoresis techniques. OST Columns are useful for the purification and analysis of DNA or RNA-based oligonucleotide products. This method has significant advantages over current technologies used to purify oligonucleotides. For example, compared to purification with cartridges, gel electrophoresis, desalting, or ion-exchange chromatography, OST Columns offer the highest level of product purity without sacrificing product recovery (Table 2). As such, OST Columns represent a new standard in synthetic oligonucleotide purification.

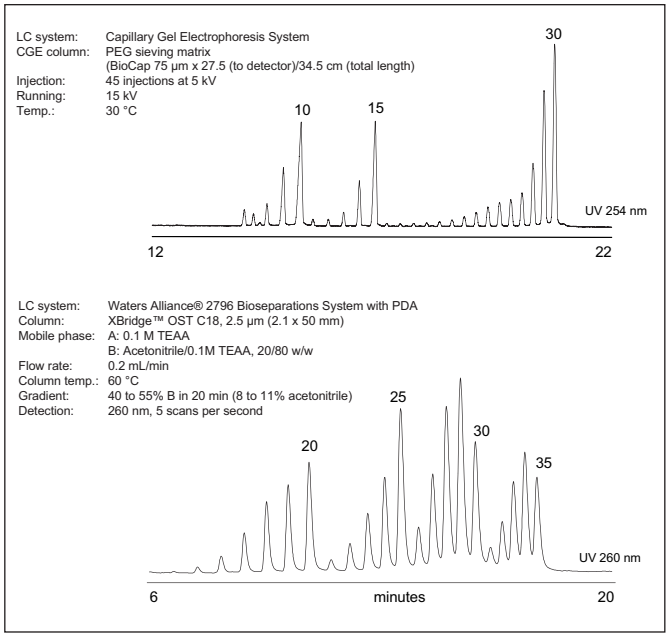

Figure 2. Separation of detritylated oligodeoxythymidine ladders by capillary gel electrophoresis (CGE) vs. ion-pair reversed-phase (IR-RP) chromatography.

Technique	Published expected purity	Actual purity* estimated by HPLC/CGE	Target recovery*
Desalted (gel filtration)	60 to 70%	70%	~80%
Anion exchange	85 to 95%	90%	~37%
PAGE	85 to 95%	91%	~8%
Waters OST Column	>95%	>95%	>90%

*At standard mass loads.

Table 2. Comparison of purity between available methods. Comparison of methods was performed with 100 nmole of 25 mer oligonucleotide. The IP-RP HPLC purification was accomplished in a single injection using a Waters OST C_{18} 2.5 µm, 4.6 x 50 mm column.

The XBridge™ OST C_{18} Column chemistry consists of Waters' patented Bridged Ethyl Hybrid (BEH) base particles (Figure 3) functionalized with C_{18} ligands. The small particles (e.g. XBridge OST 2.5 µm particles and ACQUITY UPLC® OST 1.7 µm particles) and large surface area of the BEH sorbent material yields high separation efficiency and large sample capacity. In particular, the small particle size of sorbent improves the mass transfer of the oligo macromolecules in the stationary phase and is key for successful separation efficiency.

Furthermore, compared to the use of traditional silica-based small particle C_{18} offerings, Waters BEH-based OST Columns demonstrate outstanding packed bed stability over repeated conditions of elevated temperature and pH conditions.[1]

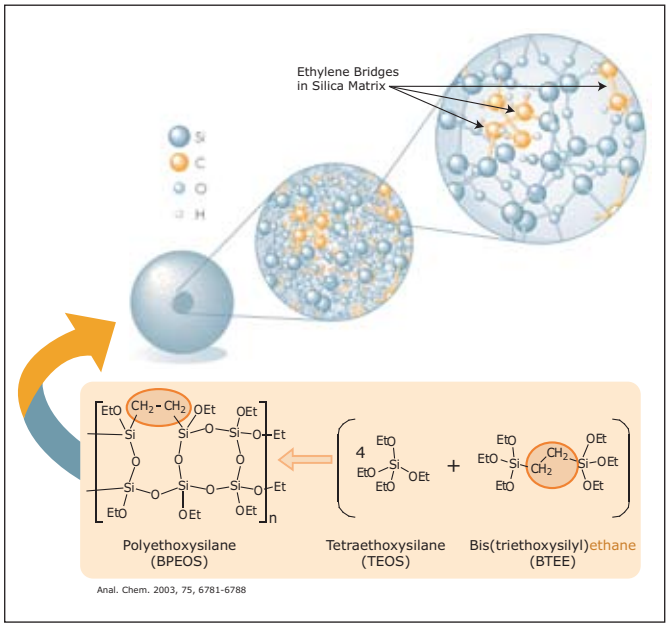

Figure 3. BEH Technology™ particles are prepared from two high purity monomers: tetraethoxysilane (TEOS) and bis (triethoxysilyl) ethzane (BTEE, which incorporates the pre-formed ethylene bridge). This structure results in greater temperature and pH stability compared to that seen with traditional silica-based, reversed-phase materials.

CONCLUSION

Scalable separations with OST Columns

XBridge OST C_{18} Columns are the preferred offering for detritylated oligonucleotide purifications due to their resolving ability (Figure 4) and availability of column sizes designed to meet laboratory-scale isolation requirements in a cost effective yet efficient manner.

LC conditions

LC system:	Waters Alliance® HPLC 2695 System
Column:	Waters XBridge OST C_{18}, 2.5 µm 4.6 x 50 mm
Column temp.:	80 °C
Flow rate:	1.26 mL/min.
Mobile phase A:	0.1M TEAA, pH 7.5
Mobile phase B:	Acetonitrile
Gradient:	5 to 50% B in 9.6 min
Detection:	UV 260 nm

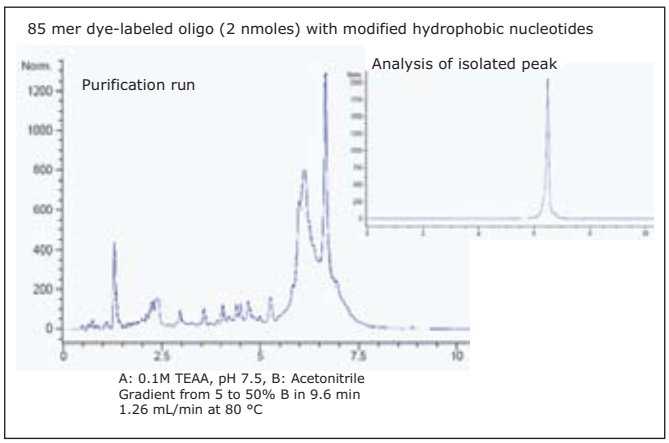

Figure 4. XBridge OST C_{18} isolation (2 nmoles injected) and analysis of isolated 85 mer dye-labeled oligo with modified hydrophobic nucleotides.

As indicated in Table 3, the choice of XBridge OST C_{18} column dimension and operating flow rate depends primarily on the scale of the synthesis reaction mixture. Typically, 2.5 µm particle sorbents are used for HPLC analytical- or lab-scale purification applications using 4.6 x 50 mm columns. Additional column dimensions are offered for larger-scale applications. Up to 0.5 µmole of synthetic oligonucleotide material can be successfully purified on a 10 x 50 mm column without compromising isolation product purity or recovery.

Higher mass loads, up to 2.5 µmole, can be purified with the same high purity and only moderate reduction in recovery. Selection of the appropriate column size for the amount of oligonucleotide sample loaded is recommended to maximize component resolution and recovery of the target product from non-desired failure sequences.

Column (mm)	Approx. mass load (µmoles)**	Flow rate (mL/min)
2.1 x 50	0.04	0.2
4.6 x 50	0.20	1.0
10.0 x 50	1.00	4.5
19.0 x 50*	4.00	16.0
30.0 x 50*	9.00	40.0
50.0 x 50*	25.00	110.0

**Custom OST Column*
***Values are only approximates and vary depending on oligonucleotide length, base composition, and "heart-cutting" fraction collection method used.*

Table 3. XBridge OST C_{18} Column selection guide for detritylated oligonucleotide purification.

Figure 5. Oligonucleotide Separation Technology (OST) Columns.

For the latest listing of Waters XBridge OST and ACQUITY UPLC Column offerings for the high-resolution HPLC isolation and UPLC or HPLC analysis of synthetic oligonucleotides, go to www.waters.com/ost.

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1. Wyndham K, et al. Characterization and evaluation of C18 HPLC stationary phases based on ethyl-bridged hybrid organic/inorganic particles. Anal. Chem. 2003; 75: 6781. Waters Application Note WA32741.

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UPLC/UV-MS ANALYSIS OF OLIGONUCLEOTIDES

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INTRODUCTION

Synthetic oligonucleotides are used extensively in the field of molecular biology, clinical diagnosis, and the development of new therapeutic agents. Quantitative and qualitative methods are required for the analysis of these oligonucleotides.

With a growing number of antisense- and RNAi-based drugs in development and clinical trials, a reliable and sensitive liquid chromatography method with mass spectrometry detection (LC/MS) is highly desirable.

The inherently unique characteristics of therapeutic oligonucleotides combined with the multiple-step manufacturing process make analysis of these oligonucleotides challenging. Post-purification analysis is a difficult and time-consuming process, typically requiring multiple orthogonal methods (CGE and SAX HPLC), adding significant costs and burden to an analytical laboratory. Furthermore, CGE and SAX HPLC are unable to resolve and quantitate many of the process-related impurities and degradent products that may exist after primary purification. Additionally, neither technique can provide significant structural data about the oligonucleotide, requiring the use of additional techniques.

The Waters® ACQUITY UltraPerformance LC® (UPLC®) System combines with Oligonucleotide Separation Technology (OST) Columns, packed with 1.7 µm sorbent, to provide superior analytical performance for oligonucleotide separations compared to HPLC and fast LC separations.

This application note describes the use of the ACQUITY UPLC® System, the OST Column, and the Q-Tof Premier™ Mass Spectrometer for the study of oligonucleotides. This methodology demonstrates outstanding separation efficiency and sensitivity together with high mass accuracy, resulting in an improved quality and high throughput analysis.

RESULTS AND DISCUSSION

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	15 mM TEA. 400 mM HFIP
Mobile phase B:	50% A, 50% methanol
Gradient:	38 to 48% B in 10 min
Detection:	ACQUITY UPLC PDA, 260nm

MS conditions

MS system:	Waters Q-Tof Premier Mass Spectrometer
Capillary:	2500 V
Sample cone:	35 V
Extraction cone:	3 V
Ion guide:	2.5 V
Desolvation temp.:	200 °C
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Desolvation gas flow:	600 L/hr
Lock mass:	10 mg/mL, CsI, 5 µL/min
Scan time:	1 sec
Frequency:	30 sec

Sample

A MassPREP™ OST standard (PN 186004135) consisting of 15, 20, 25, 30, and 35 nt (nucleotides) long oligodeoxythymidines was used as a sample to demonstrate the performance of ACQUITY UPLC System, OST Columns, and the Q-Tof Premier Mass Spectrometer. The approximate quantity of each oligomer in the vial is listed in Table 1. The oligomers were reconstituted in 500 µL of 0.1 M triethylamine acetate (TEAA) before LC injection.

oligomer	15 nt	20 nt	25 nt	30 nt	35 nt
nmole	1.9	1.0	0.7	1.0	0.8

Table 1. Oligomer quantities.

UPLC/MS conditions

UPLC separation of oligonucleotides was performed with MS-compatible mobile phases comprised of aqueous solution of 15 mM triethylamine (TEA) and 400 mM hexafluoroisopropanol (HFIP), pH 7.9, and methanol. The resulting chromatogram shows an efficient separation of 15, 20, 25, 30, and 35 nt oligonucleotides from the by-products of synthesis, customarily termed failed sequences (Figure 1).

Both UV and MS detection was used in series. The ACQUITY UPLC PDA detector was connected to the Q-Tof Premier using 75 µm x 70 cm silica capillary tubing. The MS scan time was 0.45 sec to collect at least 20 data points across the chromatographic peak.

UV and MS limits of quantitation

The UV limit of quantitation (LOQ) given chromatographic system (S/N=10) at UV 260 nm was estimated from Figure 1. The LOQ for 26 nt was ~70 fmoles.

Mass spectrometry data were acquired with the Q-Tof Premier operating in negative ion mode. Results are shown in Figure 2. The LOQ estimate for MS was ~700 fmoles (25 nt). This is more than sufficient for MS analysis of minor peaks corresponding to the failed sequences of oligonucleotides.

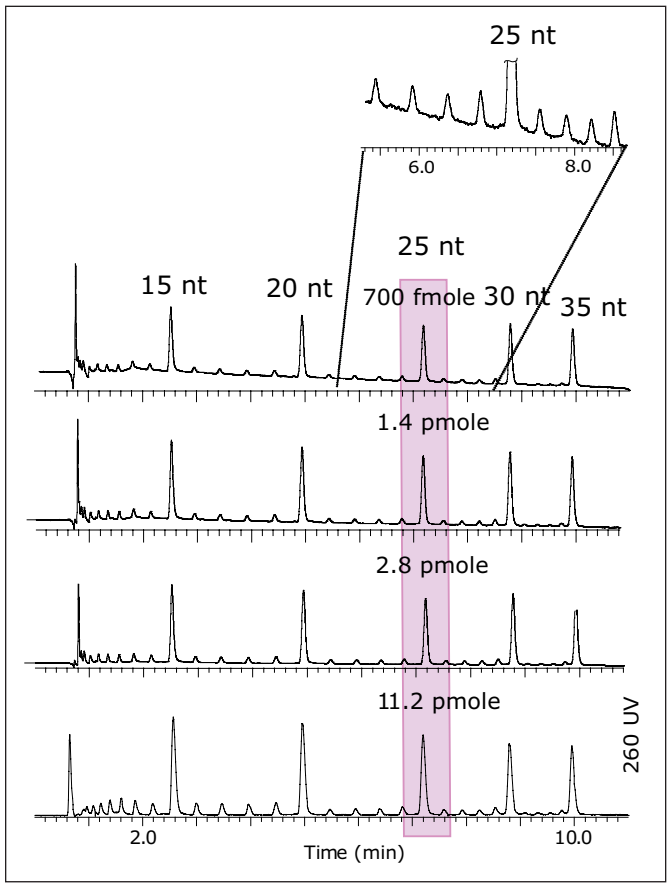

Figure 1. UV chromatograms (260 nm) of 15 to 35-mer oligonucleotide mixture. Injected quantities are highlighted based on 25 nt, which has the lowest concentration in the mixture.

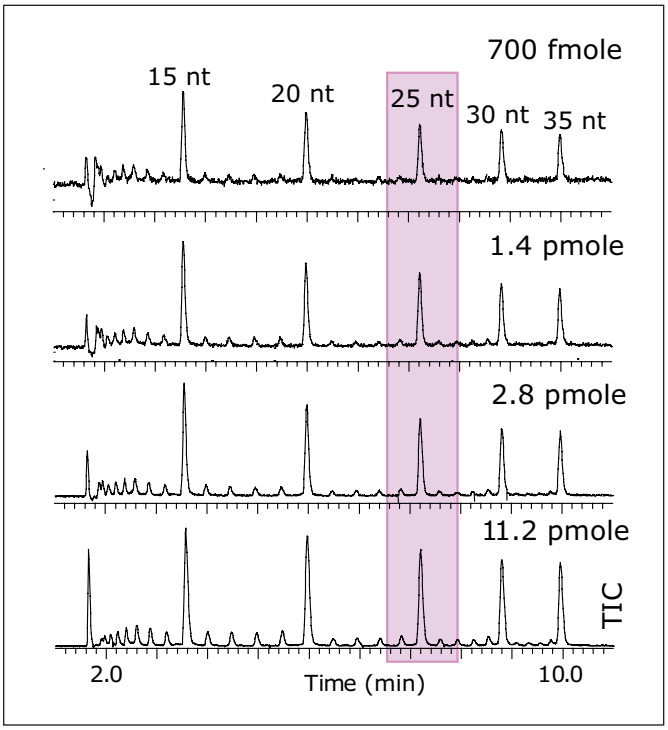

Figure 2. MS chromatograms of oligonucleotide sample (negative ion mode). The indicated injected amounts relate to 25 T oligonucleotide (700 fmole to 11.2 pmole).

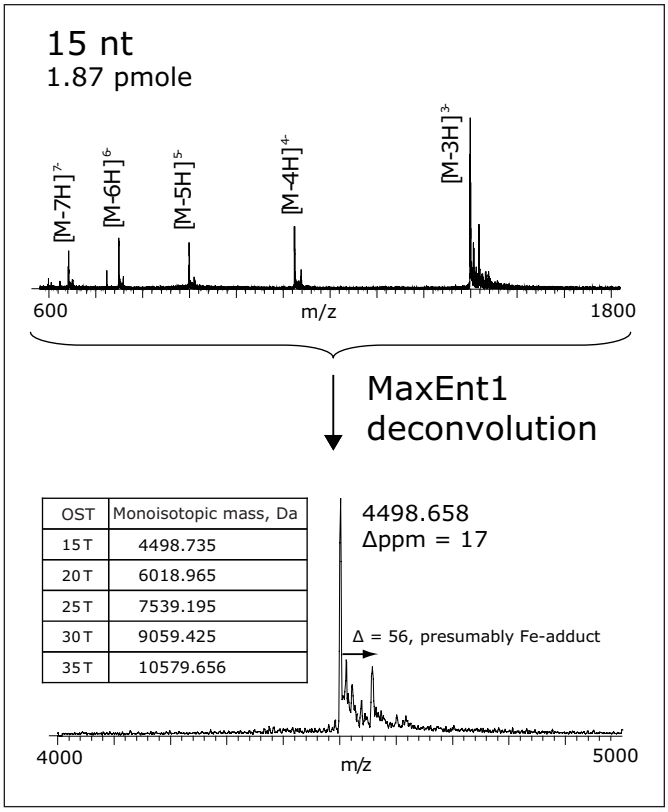

Figure 3. Mass deconvolution of 15 T oligonucleotide.

Average mass accuracy of 17 ppm was obtained for the OST Column (15 nt), internally calibrated by "lock-mass" method, as shown in Figure 3. CsI (10 mg/mL in isopropanol/water, 1:1) was used as the lockmass reference.

CONCLUSION

The Q-Tof Premier Mass Spectrometer has been applied for sensitive analysis of synthetic oligonucleotides following high resolution UPLC separation. The ACQUITY UPLC OST Column, gradient conditions, and MS parameters were designed to perform high-throughput and reproducible separations, with sensitive and accurate mass detection. Sub-picomole LOQ's were achieved using the proposed method.

This UPLC/MS methodology demonstrates the ability to use Waters technology to quantitate oligonucleotides while providing significant structural information, resulting in both improved quality as well as productivity for biopharmaceutical laboratories, making UPLC/MS an enabling technology for analysis of DNA/RNA based therapeutics.

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1. UPLC analysis of phosphorothioate oligonucleotides: method development. Waters Application Note. 2007: 720002405EN.
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HPLC AND UPLC COLUMNS FOR THE ANALYSIS OF OLIGONUCLEOTIDES

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INTRODUCTION

Typical oligonucleotides used as therapeutic or potential therapeutic compounds are 15 to 30 nucleotides (nt) long, with the exception of aptamers, which are often 40 to 60 nucleotides in length. While shorter oligonucleotides (<15 nt) can be readily resolved by liquid chromatography (LC), the separation of longer sequences becomes progressively more challenging. Ion-pair reversed-phase (IP-RP) LC has been traditionally used for oligonucleotide analysis.

Recently, the Waters® ACQUITY UltraPerformance LC® (UPLC®) System has been introduced offering separation benefits for a variety of compounds including oligonucleotides.

The traditional IP-RP LC eluent system typically employs 100 mM triethylammonium acetate (TEAA) at pH~7. An alternative IP-RP eluent consists of 8.6-15 mM triethylamine (TEA) and 100 to 400 mM hexafluoroisopropanol (HFIP). The ion-pairing agent in both IP-RP LC eluent systems is the triethylammonium ion.[1, 2, 3]

The separation is most often carried out using C_{18} columns at 60 °C. Using an elevated temperature is important to prevent the potential contribution of oligonucleotide secondary structure from impacting retention. Under such conditions, the column's hydrolytic stability becomes crucial.

Waters Oligonucleotide Separation Technology (OST) Columns have been developed to ensure excellent oligonucleotide resolution and column life time.

RESULTS AND DISCUSSION

Column lifetime

Figure 1 illustrates Waters XBridge™ OST Column lifetime for ~1000 injections, demonstrating no loss of retention or resolution. Traditional silica-based columns operated at similar separation conditions frequently fail after only tens of injections.

The hydrolytic stability of OST Columns is achieved by a patented second-generation bridged ethyl hybrid (BEH) organic-inorganic sorbent. This BEH Technology™ is used in Waters ACQUITY UPLC Columns and XBridge HPLC Columns (Figure 2).

BEH Technology columns demonstrate outstanding performance over a wide pH range (pH 1 to 12) typical of polymer packings, yet attain peak shape, efficiency, retention properties, and high temperature stability equal to or better than silica-based reversed-phase columns.

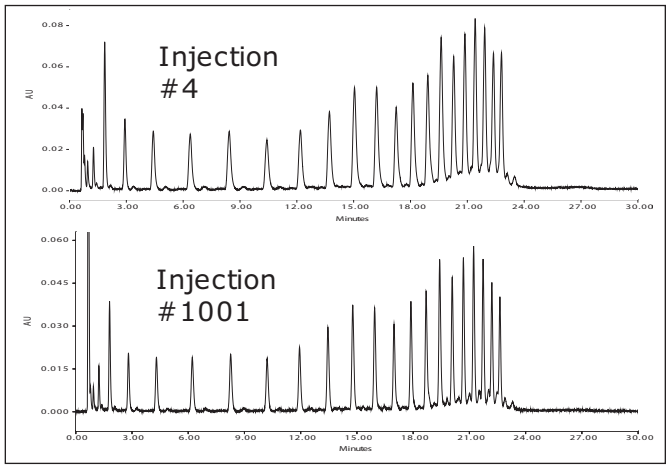

Figure 1. BEH OST Column longevity exceeds 1000 injections. Separation of 5 to 25 nt oligodeoxythymidine ladder.

LC conditions (Figure 1)

LC system:	Waters® Alliance® HPLC 2695 System with 2996 PDA Detector
Column:	Waters XBridge BEH OST C_{18} 4.6 x 50 mm, 2.5 µm
Column temp.:	60 °C
Flow rate:	1 mL/min
Mobile phase A:	10% methanol, 90% aqueous, 14.3 mM Triethylamine (TEA), 385 mM Hexafluoroisopropanol (HFIP), pH 7.9

Mobile phase B:	25% methanol, 75% aqueous, 14.3 mM Triethylamine (TEA), 385 mM Hexafluoroisopropanol (HFIP), pH 7.9
Gradient:	0 to 100% B in 30 min
Detection:	UV 260 nm

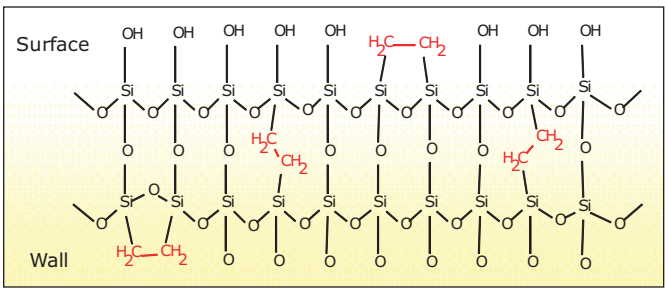

Figure 2. Schematic structure of BEH sorbent. Hydrolytic stability is achieved by bridging ethyl groups. For oligonucleotide analysis, the surface of sorbent is alkylated by C_{18} functional groups.

BEH OST Column separation performance

Column separation performance in gradient elution mode is frequently measured as peak capacity. The peak capacity represents the maximum theoretical number of peaks that can be resolved within the gradient time. For oligonucleotides, where the target compound (N) elutes in close proximity to shorter species (N-1, N-2, etc.), and the separation selectivity cannot be easily altered, column peak capacity is critical.

In order to maximize peak capacity, OST Columns are packed with small sorbent particles. XBridge HPLC OST Columns are packed with 2.5 µm C_{18} sorbent, while ACQUITY UPLC OST Columns are packed with 1.7 µm C_{18} sorbent. The impact of sorbent particle size on the resolution of oligonucleotides is illustrated in Figure 3. An oligodeoxythymidine ladder (15 to 60 nt) is analyzed using 2.1 x 50 mm XBridge OST C_{18} Columns packed with 2.5, 3.5, and 5 µm sorbent. All separation conditions are identical for each tested column. Since the selectivity of separation does not change, the improvements in resolution are achieved by the higher peak capacity of columns packed with increasingly smaller sorbent.

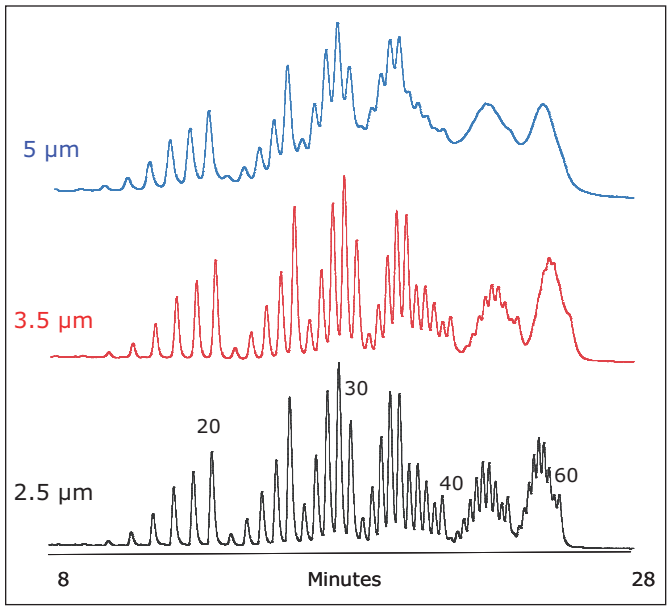

Figure 3. Impact of sorbent particle size on oligonucleotide ladder separation. Improved resolution of 15 to 60 nt oligodeoxythymidine ladder is observed for columns packed with smaller particles.

LC conditions (Figure 3)

LC system:	Waters Alliance HPLC 2796 Bioseparations System with 2996 PDA detector
Column:	Waters XBridge OST C_{18} 2.1 x 50 mm (sorbent size is indicated in labeled chromatograms)
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	100 mM TEAA, pH 7
Mobile phase B:	80% A, 20% acetonitrile
Gradient:	40 to 62.5% B in 30 min
Detection:	UV 260 nm

Understandably, using 2.5 µm sorbent for XBridge OST Columns leads to elevated backpressure. For relatively short OST Columns operated at elevated temperatures, the pressure is well within the range of conventional HPLC pumps.

UPLC separation of oligonucleotides

The ACQUITY UPLC System enables the use of columns packed with sub-2 µm sorbent. Enhanced diffusion of macromolecules in such columns leads to greater peak capacity and faster analyses than those attained with HPLC technology. Figure 4 illustrates the improved resolution of 30 to 60 nt oligodeoxythymidines; rapid, high resolution separation conditions were optimized for UPLC.

ACQUITY UPLC BEH OST column dimensions are listed in Table 1. The sorbent is identical in HPLC and UPLC OST columns, enabling simple method transfer from HPLC to the ACQUITY UPLC System.

Description	Particle size	Pore size	Dimension	Part number
XBridge OST C_{18}	2.5 µm	135Å	2.1 x 50 mm	186003952
XBridge OST C_{18}	2.5 µm	135Å	4.6 x 50 mm	186003953
XBridge OST C_{18}	2.5 µm	135Å	10 x 50 mm	186003954
Custom XBridge OST C_{18}	--	--	--	On request
ACQUITY UPLC OST C_{18}*	1.7 µm	135Å	2.1 x 50 mm	186003949
ACQUITY UPLC OST C_{18}*	1.7 µm	135Å	2.1 x 100 mm	186003950
Custom ACQUITY UPLC OST C_{18}*	--	--	--	On request

**For use on Waters ACQUITY UPLC Systems.*

Table 1. BEH OST Columns information.

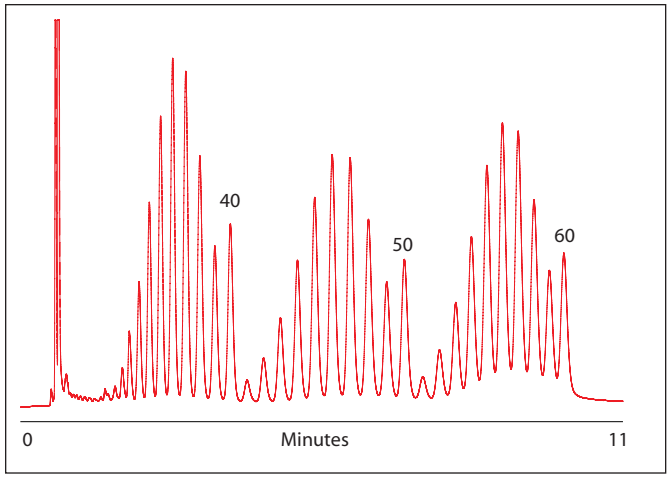

Figure 4. Resolution of 30 to 60 nt oligodeoxythymidine ladder in UPLC mode.

LC conditions (Figure 4)

LC system:	Waters ACQUITY UPLC System for oligonucleotide analysis, with ACQUITY UPLC PDA detector
Column:	Waters ACQUITY UPLC OST C_{18} 1.7 µm, 2.1 x 50 mm
Column temp.:	60 °C
Flow rate:	0.2 mL/min.
Mobile phase A:	15 mM TEA, 400 mM HFIP in water, pH 7.9
Mobile phase B:	50% m.p. A, 50% methanol
Gradient:	45 to 49.5% B in 15 min
Detection:	UV 260 nm

CONCLUSION

The second generation of hybrid sorbents, utilizing bridged ethyl hybrid (BEH) chemistry, are extremely stable, providing for a robust platform for separation of oligonucleotides at neutral-basic pH and at elevated temperature. Waters OST Columns packed with 2.5 µm particle size sorbent exhibit a superior performance for oligonucleotide separations. Routinely, 15 to 25 nt oligonucleotides can be baseline-resolved within 10 minutes with UPLC, compared to traditional LC and capillary electrophosresis analyses that usually take 30 to 60 minutes to accomplish. Longer oligonucleotides can be separated at moderately longer retention times.

OST Columns for UPLC analyses are suitable for efficient and fast separations of oligonucleotides in lengths up to 60 nt or longer. Smaller oligonucleotides can be analyzed within minutes using the ACQUITY UPLC System and columns. The faster analysis will result in savings in both time and resources, allowing for faster sample throughput and higher laboratory productivity.

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Figure 5. ACQUITY UPLC System and Oligonucleotide Separation Technology (OST) Columns.

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UPLC SEPARATION OF OLIGONUCLEOTIDES: METHOD DEVELOPMENT

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INTRODUCTION

The Waters® ACQUITY UltraPerformance LC® (UPLC®) System, combined with Oligonucleotide Separation Technology (OST) Columns packed with 1.7 µm sorbent, offer superior analytical performance for oligonucleotide separations compared to HPLC and fast LC separations.

The Waters UPLC-based OST solution for the high-resolution, high-throughput analysis of synthetic oligonucleotides was developed following a series of comprehensive investigations that helped Waters scientists and engineers better understand limitations of existing analytical techniques for compounds. This research led to innovations designed to assist manufacturers deliver quality products that can help researchers make impactful discoveries that can lead to novel diagnostics or drug therapies. Failure to achieve these goals can seriously impede the ability of an organization to achieve desired results, such as obtaining the necessary FDA approval for product commercialization.

This application note illustrates the impact of chromatographic parameters on UPLC oligonucleotide separations and general guidelines for developing high resolution, fast analytical methods. For further method development guidelines for separation of oligonucleotides, please refer to other available application notes.

EXPERIMENTAL

LC conditions

LC system:	Waters ACQUITY UPLC System with ACQUITY UPLC PDA Detector
Column:	ACQUITY UPLC OST C_{18} 4.6 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min, unless indicated otherwise in figures
Mobile phase A:	100 mM TEAA, pH 7
Mobile phase B:	80% A, 20% acetonitrile
Gradient figure 1:	40 to 62.5% B, for gradient time see figure
Gradient figure 2:	45 to 64.5% B, for gradient time see figure
Gradient figure 3:	Gradient started at 50, 45, 40, and 35% B, respectively.
0.75%	B/min (0.15% acetonitrile/min)
Detection:	UV 260 nm
Sample:	15 to 60 nt oligodeoxythymidines

RESULTS AND DISCUSSION

Oligonucleotide analysis in ion-pairing reversed-phase liquid chromatography (IP-RP LC) is typically performed with shallow gradients. The impact of gradient slope on oligonucleotide resolution is illustrated in Figure 1.

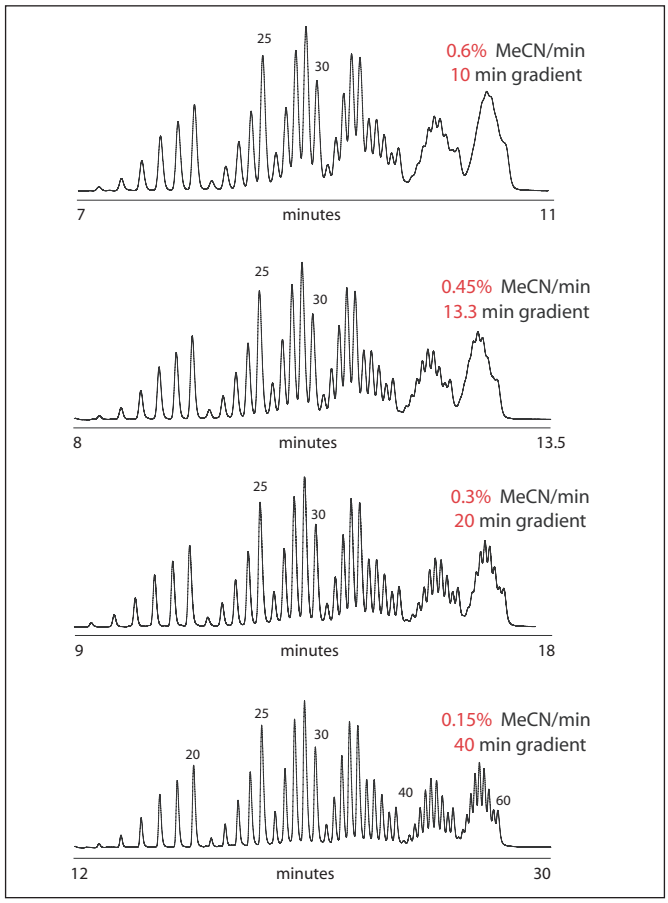

Figure 1. Impact of the gradient slope on separation of 15 to 60 nt oligodeoxythymidines and analysis time.

As expected, decreasing gradient slope increases resolution, but negatively impacts analysis throughput by increasing the run time. Another approach to maintaining resolution while decreasing analysis time is to increase mobile phase flow rate while proportionally reducing the gradient time (Figure 2). In such a scenario, the number of column volumes remains constant. Therefore, the separation selectivity remains unchanged with only the potential for some loss of resolution (Figure 2a). The constant gradient volume method is preferable as it enables faster analysis times with minimal deterioration in resolution. The increased operational pressures generated by 1.7 µm sorbent and higher flow rates require the capabilities of the ACQUITY UPLC System.

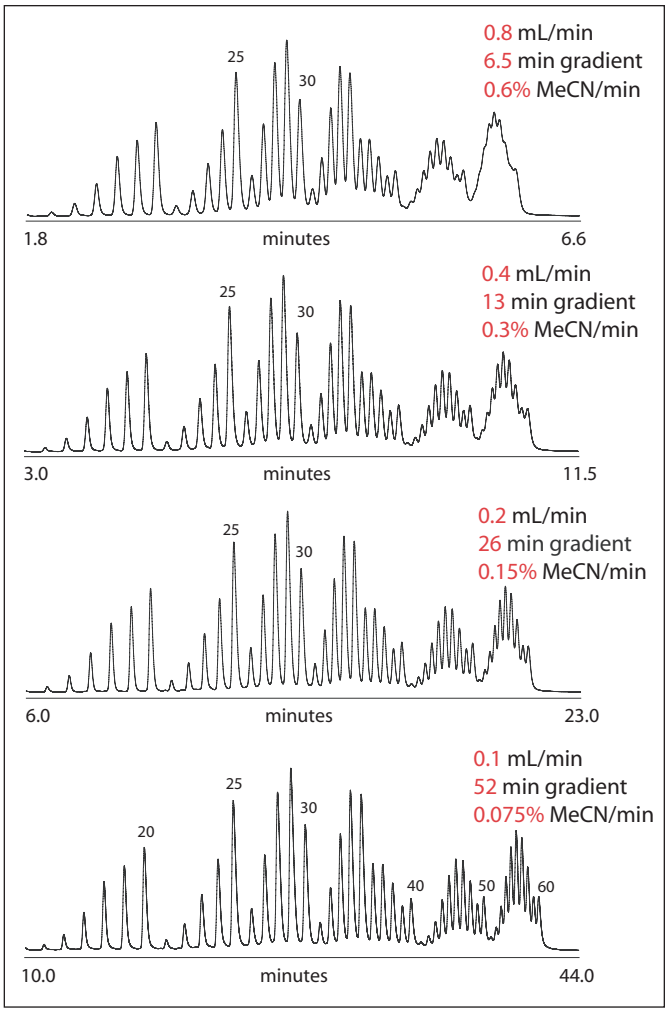

Figure 2. Separation of 15 to 60 nt oligodeoxythymidines at constant gradient volume in various mobile phase flow rates.

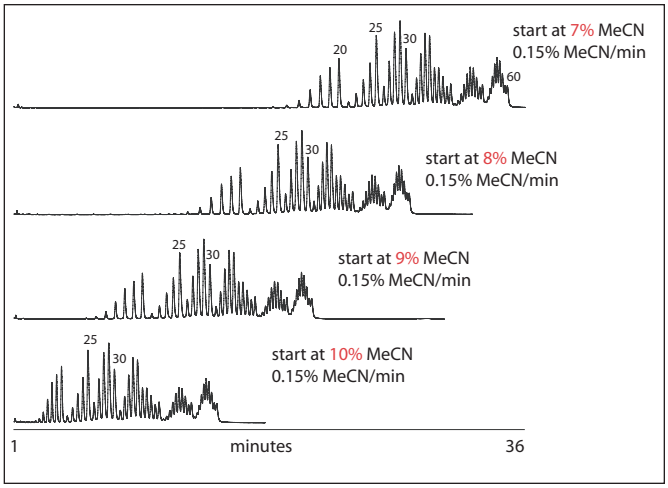

Figure 3. Reducing the analysis time by adjustment of initial gradient strength. Gradient slope remains constant.

Oligonucleotides tend to elute in very narrow gradient ranges (mobile phase elution strength). If initial and final gradient conditions are not optimized properly, the resulting analysis time can be considerably longer than necessary, as the majority of the separation space in the typical HPLC chromatogram is unused for the separation. The preferable UPLC approach is to select a gradient slope providing high resolution and adjust the gradient initial conditions while keeping the gradient slope constant. In this way it is possible to significantly reduce analysis time without sacrificing resolution, as shown in Figure 3.

CONCLUSION

The Waters ACQUITY UPLC System with OST Columns solution offers significant advantages to manufacturers or researchers who require improved technology for the analysis of oligonucleotides. The impact of optimized gradient slope, flow rate, and initial gradient strength on the separation of oligonucleotides has been demonstrated. UPLC enables improved resolution, resulting in improved separations with very fast run times.

High-resolution, high-throughput methods offer easier quantitative analysis with increased throughput, generating better data in shorter time with cost savings. The ACQUITY UPLC System will increase the productivity of any laboratory developing methods and analyzing oligonucleotides.

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UPLC/MS SEPARATION OF OLIGONUCLEOTIDES IN LESS THAN FIVE MINUTES: METHOD DEVELOPMENT

Martin Gilar
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INTRODUCTION

The ACQUITY UltraPerformance LC® (UPLC®) System, when combined with Oligonucleotide Separation Technology (OST) Columns packed with 1.7 µm sorbent, offers superior analytical performance for oligonucleotide separations compared to HPLC. UPLC also enables users to significantly reduce analysis time.

In this work, we illustrate the UPLC analytical method development process and extend the topic discussed in a previous note, "UPLC Separation of Oligonucleotides: Method Development"[1] providing guidelines for producing high resolution, fast, LC/MS compatible oligonucleotide separations.

RESULTS AND DISCUSSION

Oligonucleotide separation in ion-pairing reversed-phase (IR-RP) liquid chromatography is typically performed with shallow gradients. Recently, a novel ion-pairing buffer compatible both with UV and electrospray MS detection has been described.[2, 3] The buffer is comprised of triethylamine (TEA, an ion-pairing agent) and aqueous hexafluoroisopropanol (HFIP, a volatile weak acid used as buffering component to bring the pH to ~ 8).

While triethylammonium acetate (TEAA) is useful for oligonucleotide analysis, it is not compatible with MS detection. The LC retention behavior of oligonucleotides strongly depends on their nucleotide composition, requiring careful optimization of gradient elution conditions for each specific oligonucleotide.[4] TEA-HFIP ion-pairing buffer yields more consistent and predictable oligonucleotide retention behavior over a broad range of compositional differences as compared to TEAA.

Oligonucleotide retention also varies depending on its length (charge). Differences are also observed for different classes of oligonucleotides and chimeric oligonucleotides (DNA, RNA, LNA, phosphorothioates, morpholino backbone, 2'O-methylated species, and combination of all above).

Waters UPLC method development guidelines for oligonucleotide analysis can be summarized in three steps:

1) Identify a suitable initial gradient strength. If elution behavior of the oligonucleotide is unknown, start with a scouting gradient. Recommended flow rate for a 2.1 x 50 mm OST Column is 0.2 mL/min and separation temperature is 60 °C.

2) Adjust the gradient slope to achieve a desirable separation. In general, shallower gradients provide increased resolution.

3) Faster analyses can be achieved by increasing the flow rate while maintaining gradient column volumes. If the gradient range and the gradient slope in column volumes remain constant, the separation selectivity is not negatively affected.

LC conditions

LC system:	Waters ACQUITY UPLC System with ACQUITY UPLC PDA Detector
Column:	ACQUITY UPLC OST C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min unless indicated otherwise
Mobile phase A:	15 mM TEA, 400 mM HFIP, pH 7.9
Mobile phase B:	50% A, 50% MeOH
Detection:	PDA TIC or UV 260 nm
Sample:	oligodeoxythymidines

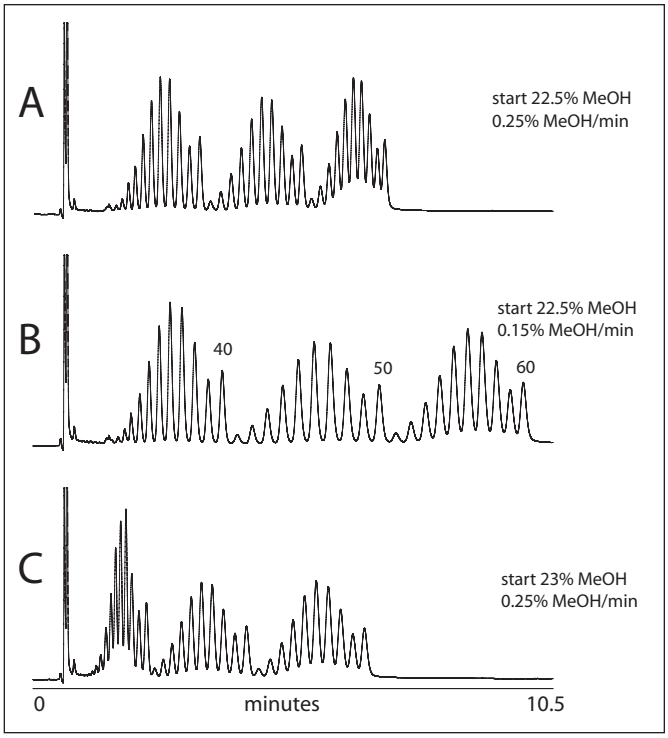

Figure 1. Separation of 30 to 60 nt oligodeoxythymidines using 2.1 x 50 mm, 1.7 µm ACQUITY UPLC OST C_{18} Column.

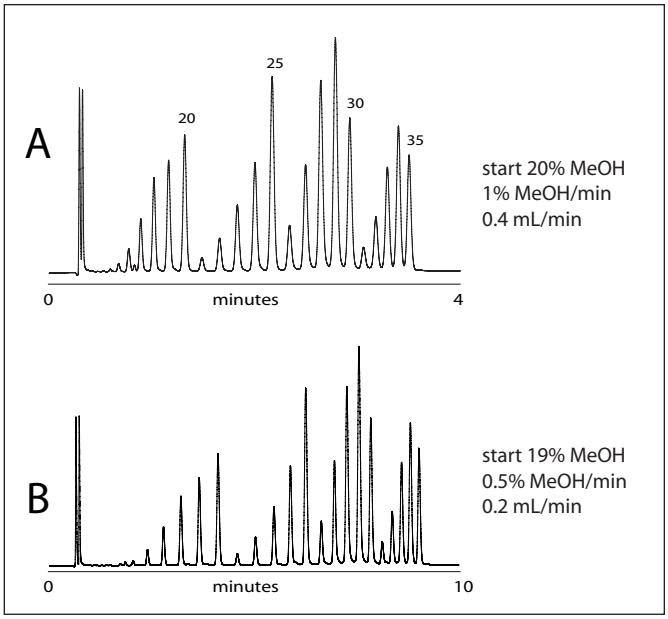

Figure 2. Separation of 15 to 35 nt oligodeoxythymidines.

Figure 1 illustrates method development for a 30 to 60 nt oligonucleotide using TEA-HFIP. The high resolution separation in chromatogram Figure 1A has an initial mobile strength of 22.5% MeOH and slope 0.25% MeOH/min. The resolution can be further improved by using a shallower gradient (0.15% MeOH/min, Figure 1B), but at the expense of analysis time – which will negatively impact throughput.

The analysis time can be shortened by adjusting the initial gradient conditions (Figure 1C). Since the gradient slope was unchanged, the high resolution separation was preserved, with the possible exception of the early eluting peaks.

Figure 2 highlights a strategy for reducing analysis time without compromising high resolution by using faster flow rates. The chromatogram in Figure 2B shows baseline resolution of 15 to 35 nt peaks in less than 10 minutes. To optimize the separation for throughput while maintaining high resolution, the flow rate was doubled and the initial gradient conditions were adjusted while holding constant the gradient slope in column volumes. The resulting chromatogram in Figure 2A shows a final separation accomplished in less than four minutes.

CONCLUSION

UPLC has significant advantages for the LC and LC/MS analysis of different classes of oligonucleotides. The impact of optimized gradient slope, flow rate, and initial gradient strength on the separation of oligonucleotides has been demonstrated.

UPLC enables improved resolution, resulting in improved separations with very fast run times. High resolution, high throughput, LC/MS compatible methods offer easier quantitative analysis with increased throughput generating better data in shorter time. ACQUITY UPLC systems and columns thus will increase the productivity of any laboratory developing LC and LC/MS methods and analyzing oligonucleotides.

References

1. UPLC Separation of Oligonucleotides: Method Development, Part 1. Waters Technical Note. 2007: 720002383EN.
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3. Gilar M, Fountain KJ, Budman Y, Holyoke JL, Davoudi H, Gebler JC. Oligonucleotides. 2003; 13: 229.
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UPLC SEPARATION OF DNA DUPLEXES

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INTRODUCTION

Over the past 20 years there has been a considerable amount of effort focused on the determination of detailed maps of genomes from various species and individual genome analysis. This work is leading to an increased understanding of susceptibility to disease and provides putative sequence targets for oligonucleotide-based therapeutic strategies.

In general, molecular biology methods for manipulation of DNA rely on restriction enzymes, polymerase chain reaction (PCR), and sequencing techniques. Using these methods, genomic DNA is typically converted into shorter double-stranded (ds) DNA sequences, typically 100 to 1000 base pairs (bp) in length. The shorter dsDNA molecules are often analyzed or isolated by methods such as slab gel or capillary electrophoresis and anion-exchange LC. The method outlined in this application note uses a volatile ion-paring system, reducing post-purification processing.

Besides gel electrophoresis, Waters UltraPerformance Liquid Chromatography (UPLC®) Technology can also be used for rapid and cost-effective separation and purification of wide array of dsDNAs.

This application outlines a UPLC method for the separation/purification of dsDNA sequences ranging from 50 to 600 base pairs in length, in under 20 minutes. The separation is based on DNA length, rather than on the sequence. Because of the substantial column mass load capacity, large amounts of dsDNA fragments can be isolated.

This presented method dramatically reduces analytical time and effort compared to gel electrophoresis, and can be utilized for separation of dsDNA fragments produced by hydrodynamic shearing. Additionally, isolated samples may be used for next-generation sequencing. With current research efforts focused on reducing total genome processing times and sequencing costs, the Waters ACQUITY UPLC® System solution provides for a superior tool to those currently used for dsDNA isolation, purification and analysis.

EXPERIMENTAL

The HaeIII digest of pBR322 plasmid was purchased from Sigma-Aldrich. MspI digest of pBR322 was obtained from New England Bio Labs. These digests were chosen because they contain few dsDNA fragments of the similar or same length, but cover a broad range of dsDNA lengths. The stock solutions were diluted 1:10 in 100 mM triethylammonium acetate (TEAA). Typical injection volumes were 10 µL, giving on column loads of 0.6 µg.

dsDNA mixtures were separated using the ACQUITY UPLC System with an ACQUITY UPLC Peptide Separation Technology BEH300 C_{18} 2.1 x 50 mm, 1.7 µm column using ion-pair, reversed-phase chromatography.[1] The pore size of the sorbent was 300 Å. Separated products were detected with a Waters ACQUITY UPLC PDA detector at 260 nm. Mobile phase A consisted of 0.1 M triethylammonium acetate (TEAA); mobile phase B was composed of 20 % acetonitrile in mobile phase A. The column temperature was maintained at 50 °C. Gradient was 57.5 to 84.5% B (11.5 to 16.9% ACN) in 20 minutes.

RESULTS AND DISCUSSION

The BEH300 column material was selected for the dsDNA separation for several reasons. First, BEH Technology™ offers exceptional stability, allowing for use of a single column over the long term. Secondly, the 300 Å pore size allows for an efficient separation of longer dsDNA fragments. This is primarily due to the increased accessibility of pores to large molecular weight analytes.

As shown in Figure 1, the UPLC separation provides good resolution of dsDNA, especially in the 50 to 300 base-pairs (bp) region. Eluting peaks can be easily collected, unlike using gel electrophoresis where the band needs to be excised and isolated from the gel and desalted.

To determine whether the UPLC separation strategy provided predictable dsDNA retention, we plotted retention time vs. oligonucleotide length for the HaeIII digest. The data were fit to an inverse third order polynomial, Equation 1, yielding an excellent correlation coefficient of 0.9999.

$$RT = \frac{a}{bp^3} + \frac{b}{bp^2} + \frac{c}{bp} + d$$

Equation 1. Equation used for fit of dsDNA data for MspI and HaeIII digests of pBR322. RT is the predicted retention time, and bp is the desired oligonucleotide duplex length.

Additionally, we plotted and fit the MspI data in the same manner yielding a correlation coefficient of 0.9996 (curve not shown). Excellent correlation between the expected and observed retention times was found regardless of which digest was used for curve fitting.

This correlation strongly indicates that our separation system provides exemplary resolution of dsDNA sequences, up to 600 bp, and provides a predictable elution order regardless of oligonucleotide sequence.

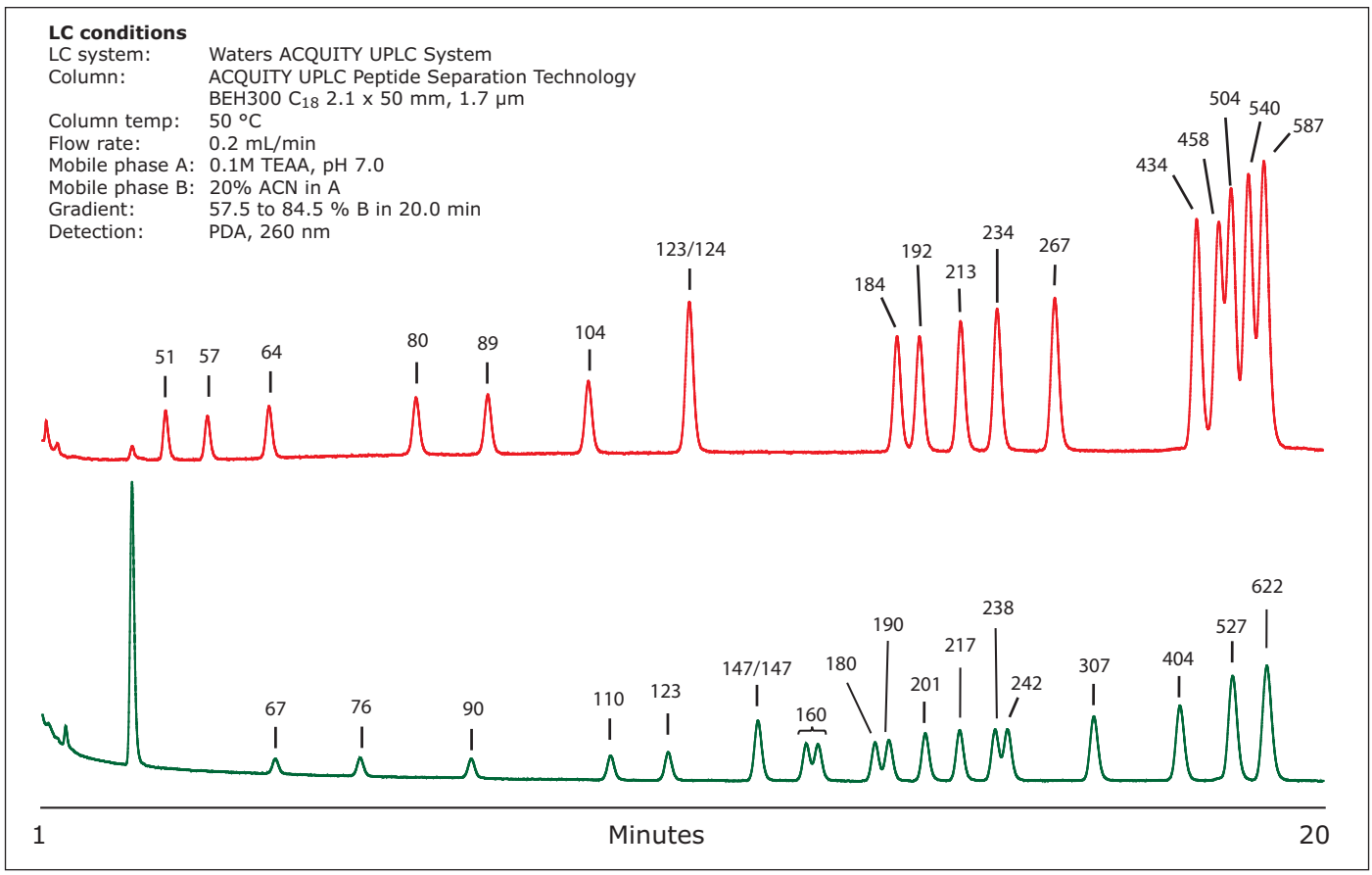

Figure 1. Separation of HaeIII (top trace) and MspI (bottom trace) digests of the plasmid pBR322.

Accuracy in retention time prediction allows for the collection of fractions of specific lengths if desired. In order to investigate the sizing accuracy of the UPLC method, we calculated the expected bp length from the retention times for the MspI digest using the curve for the HaeIII digest (Figure 2). These values were compared to the actual bp lengths and expressed as absolute and percent bp error.

As shown in Figure 3, there is very good correlation between the expected and observed bp lengths. The red line indicates that there is less than 5% variation in the predicted oligonucleotide length compared to the fitted curve (fitted for HaeIII data). This error translates to less than a 10 bp error for oligonucleotide lengths up to 400 bp.

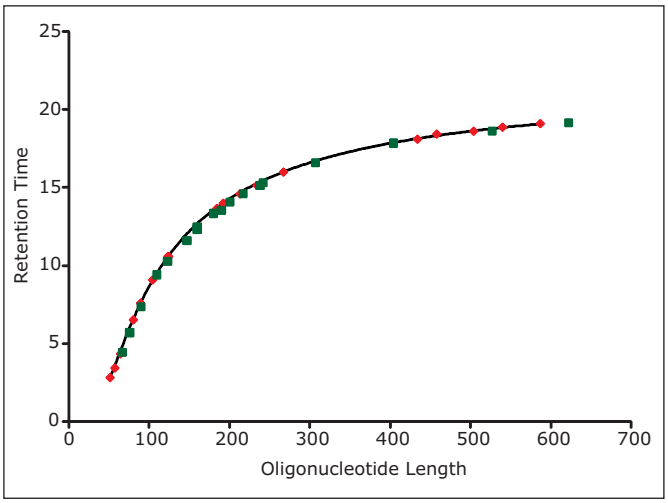

Figure 2. Fit of data between dsDNA fragment length (bp) and observed retention time. The fit was constructed for HaeIII pBR322 restriction digest. Red dots represent data for HaeIII pBR322 digest used for fitting. Green squares represent MspI pBR322 digest data points (not used for curve fitting).

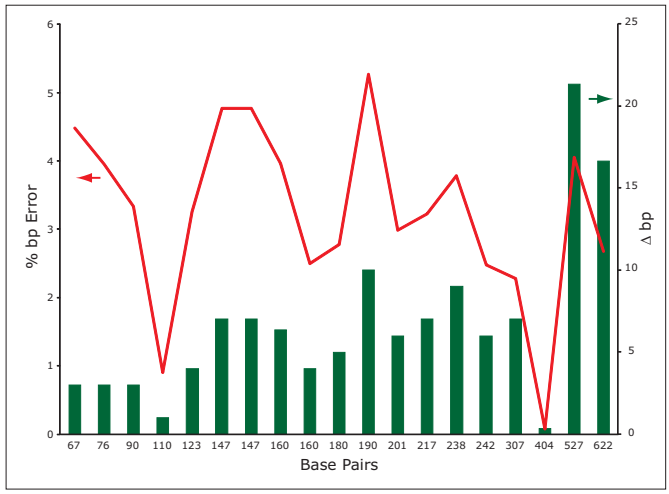

Figure 3. Absolute (green bars) and relative (red line) errors between predicted and measured retention of dsDNA fragments in ion-pair UPLC method.

One can note that there is more significant absolute deviation for 527 and 622 bp fragments (15 to 22 bp), but the relative difference remains below 5%. This sizing accuracy is acceptable for many molecular biology applications.

Some deviation in retention behavior was detected for shorter dsDNA, generally below 40 bp in length (Figure 2). This is due to gradient delay effect at the beginning of analysis. It is advisable to start the gradient with lower elution strength solvent when working with shorter dsDNA fragments.

Additionally, there appears to be a limited contribution of oligonucleotide dsDNA sequence to the observed retention time. Although this effect is limited, it can be observed as imperfect co-elution of dsDNA fragment of the same length, but different sequence. While minor sequence contribution was observed, it is clear from our data that elution in UPLC correlates very closely to dsDNA length.

We also investigated the utility of our method using more rapid chromatographic separations. To accomplish this we increased the gradient slope by a factor of two at constant flow rate. As shown in Figure 4, there is a slight loss in resolution, primarily for oligonucleotide lengths above 300 bp, however very good resolution for moderate length oligonucleotides is achieved.

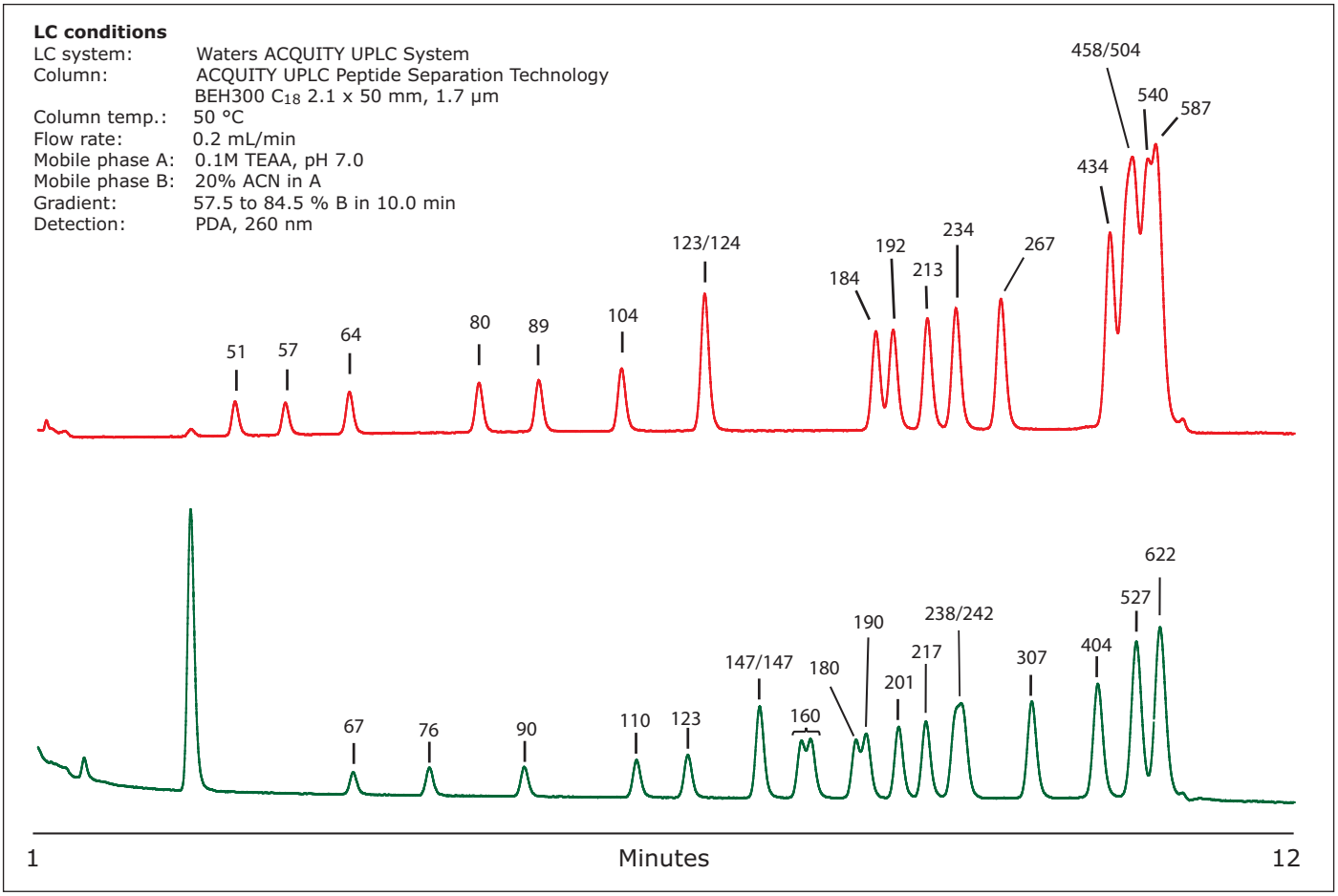

Figure 4. Separation of HaeIII (top trace) and MspI (bottom trace) digests of the plasmid pBR322.

CONCLUSION

The dsDNA separation strategy utilizing the ACQUITY UPLC System presented here provides an accurate, high throughput, and reproducible method that allows for the prediction of retention time for a desired oligonucleotide length, and collection of oligonucleotide lengths of interest.

The method can be scaled by using larger column configurations available from Waters, which is useful for the researcher seeking to do large-scale separations of dsDNA.

When combined, the accuracy, high throughput, and ease of this method offer a significant advantage other methods currently in use.

As illustrated, this UPLC method allows separations to be performed at a variety of gradient slopes, allowing for analysis and sample collection at a variety of timescales, depending on the needs of the researcher, with minimal loss of resolution for moderate length oligonucleotides.

This powerful separation solution relies on the outstanding stability and reproducibility offered by Waters BEH column chemistry. Following peak collection, samples can be aliquoted and dried for long-term storage. The volatility of TEAA allows for an easy removal of ion-pairing buffer components, yielding oligonucleotides that are practically salt-free and suitable for storage as necessary, offering another advantage over currently used methods.

References

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OPTIMIZATION OF LCT PREMIER XE MS SETTINGS FOR OLIGONUCLEOTIDE ANALYSIS

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INTRODUCTION

The analysis of oligonucleotides via liquid chromatography and mass spectrometry is becoming a common practice. Many applications require the identification of oligonucleotides at low concentrations. For this reason, it is advantageous to utilize highly sensitive mass spectrometers such as Waters® LCT Premier™ XE System.

The exceptional sensitivity of the LCT Premier XE System is in part achieved by greater efficiency of ion transition into the electrospray source. For oligonucleotides that are often analyzed with mobile phase containing triethylammonium and hexafluoroisopropanol aqueous solutions, some degree of TEA and/or HFIP adducts are also often present in the MS spectra. The majority of adducts are observed at low charge states of oligonucleotides, while the high charge states typically have comparatively less adduction.

In this work, we outline the critical parameters that were adjusted to yield significantly lower adduct formation for oligonucleotide analysis at moderate LC flow rates.

EXPERIMENTAL

Sample

For our study, we utilized the Waters Oligonucleotide Separation Technology (OST) standard, which contains oligo deoxythymidine sequences up to 35-mer reconstituted in 500 µL of 0.1 M triethylammonium acetate (TEAA) to yield a solution of 2 pmol/µL per oligonucleotide, and a 25-mer phosphorothioate (5' -CTC TCG CAC CCA TCT CTC TCC TTC T -3') at 1 µg/µL in TEAA.

LC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	Waters ACQUITY UPLC OST 2.1 x 50 mm, 1.7 µm (P/N 186003949)
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Mobile phase A:	15 mM TEA/400 mM HFIP
Mobile phase B:	50 % MeOH in Mobile A (v/v)

Gradient (OST standard)		
Time	%A	Curve
0 min	31%	
10 min	47%	6

Gradient (Phosphorothioate 25mer)		
Time	%A	Curve
0 min	31%	
5 min	50%	6

RESULTS AND DISCUSSION

Figure 1 shows a typical LC/MS chromatogram of Waters OST standard under the specified conditions using the LCT Premier XE System. The OST sample is a mixture of 15-, 20-, 25-, 30-, and 35-mer oligonucleotide standards, and their synthetic N-x impurities. Under normal operating conditions, outlined in the instrument parameters in Table 1, we found evidence for significant adduct formation. The adduct formation is more significant for longer oligonucleotides (ca. 25- and 35-mer, as shown in Figure 2).

Due to the different chemical nature of phosphorothioate oligonucleotides, the adduct formation is more pronounced. Figure 3 shows abundant multiple TEA adducts in both raw and deconvoluted 25-mer phosphorothioate spectra. It can be seen that adduct formation is more pronounced at lower charge states, with -2 and -3 charge states exhibiting a greater extent of adductation than the other charge states.

In an attempt to decrease adduct formation, we adjusted various LCT Premier XE System parameters including aperture 1, aperture 2, aperture 3, cone voltage, desolvation temperature, desolvation gas flow, and cone gas flow. Of these parameters, we found the largest benefit from the adjustment of desolvation temperature and desolvation gas flow.

While we did find evidence that adjustment of aperture 1, ca. from 15 to 30, yielded modest improvement in adduct formation, the benefits were not sufficient to justify a change from normal conditions.

The parameters providing the best LC/MS results and efficient desolvation for oligonucleotides are listed in Table 2. As shown in Figures 4 and 5 for OST and phosphorothioate analysis respectively, the optimal parameter settings yield significantly less adduct formation as compared to Figures 2 and 3. This benefit was particularly evident for the phosphorothioate, with virtually all TEA adducts eliminated, as shown in Figure 5.

Additionally, increasing the desolvation temperature and gas flow yielded a significant improvement in signal-to-noise (S/N), which is particularly evident when compared to 25- and 35-mer OST oligonucleotides under normal and optimal conditions. This was likely due to increased population of the parent ions from a decrease in adduct formation, further highlighting the benefits of this change.

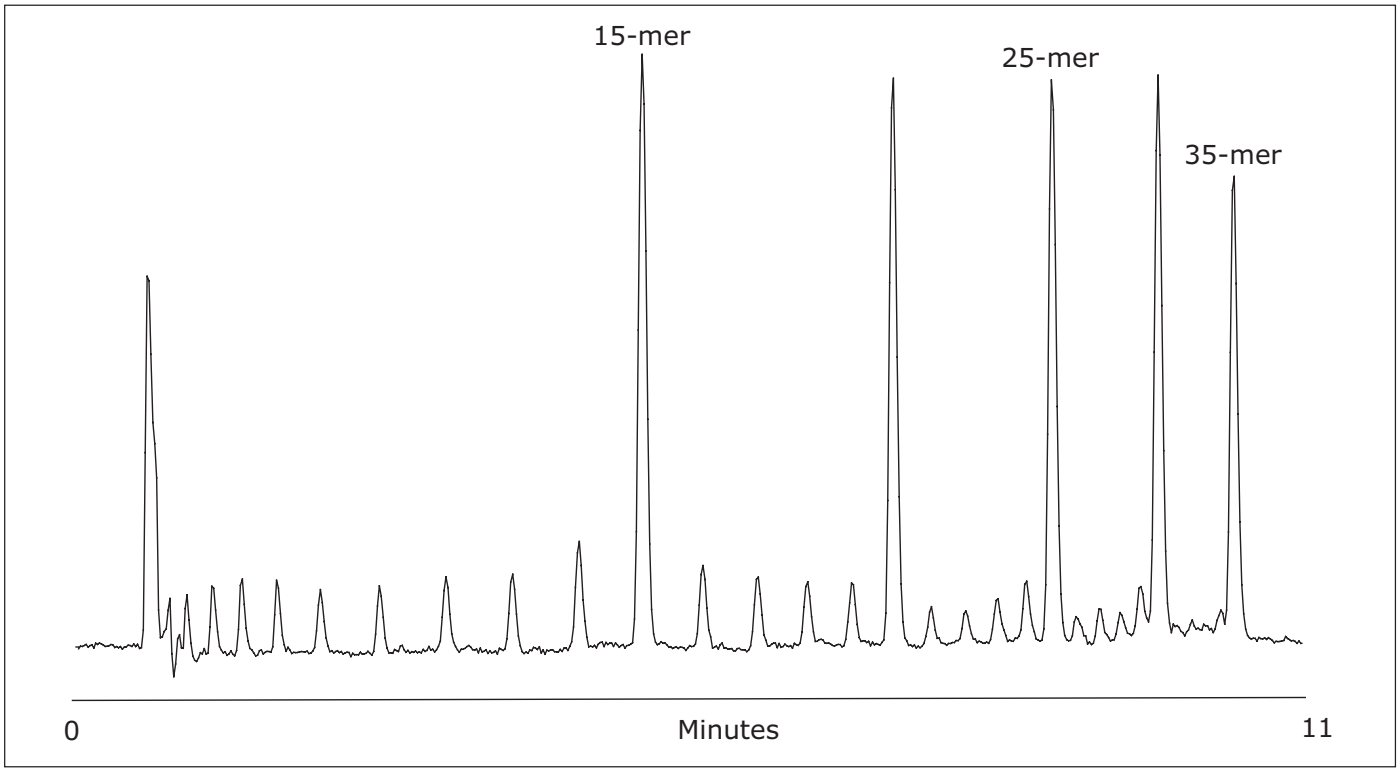

Figure 1. Total Ionic Current (TIC) for the separation of Waters OST standard detected with the LCT Premier XE System.

Polarity	ES-	Puller Offset Voltage	0.00
Analyser	V Mode	MCP Detector (V)	2000.0
Capillary (V)	2600.0	Pusher Cycle Time	Auto (68.0)
Sample Cone (V)	*37.0*	Pusher Frequency	14705.88
Desolvation Temp (C)	**250.0**	Pusher Width	4.00
Source Temp (C)	150.0	Centroid Threshold	1.0
Cone Gas Flow	*50.0*	Min Points	4.0
Desolvation Gas Flow	**500.0**	Np Multiplier	0.70
Syringe Type	Hamilton 250uL	Resolution	6000.0
Ion Guide One	5.0	Lteff	1081.0000
Aperture 1 Voltage	*15.0*	Veff	5681.4629
Ion Energy (V)	105.0	Trigger Threshold (mV)	600.0000
Aperture 2 Voltage	*6.0*	Signal Threshold (mV)	40.0000
Hexapole DC Voltage	6.0	Data Threshold	0.0000
Aperture 3 Voltage	*5.0*	DXC Temperature	25.0
Acceleration (V)	200.0	IonGuide1InitialRF	150.0
Y Focus (V)	0.0	IonGuide1FinalRF	150.0
Steering (V)	0.0	IonGuide2InitialRF	200.0
Tube Lens (V)	192.0	IonGuide2FinalRF	200.0
Attenuated Z Focus (V)	500.0	Fixed Hexapole RF	True
Normal Z Focus (V)	65.0	HexapoleRF	180.0
TOF Flight Tube (V)	5630.0	DRE Mass 0.0000 Setting	5.0000
Reflectron (V)	1780.0	DRE Mass 280.0000 Setting	50.0000
Pusher Voltage	839.0	DRE Mass 1000.0000 Setting	50.0000
Pusher Offset Voltage	-1.47	DRE Mass 2000.0000 Setting	50.0000
Puller Voltage	769.0	DRE Mass 3000.0000 Setting	50.0000

Table 1. Normal LCT Premier XE System operating parameters.

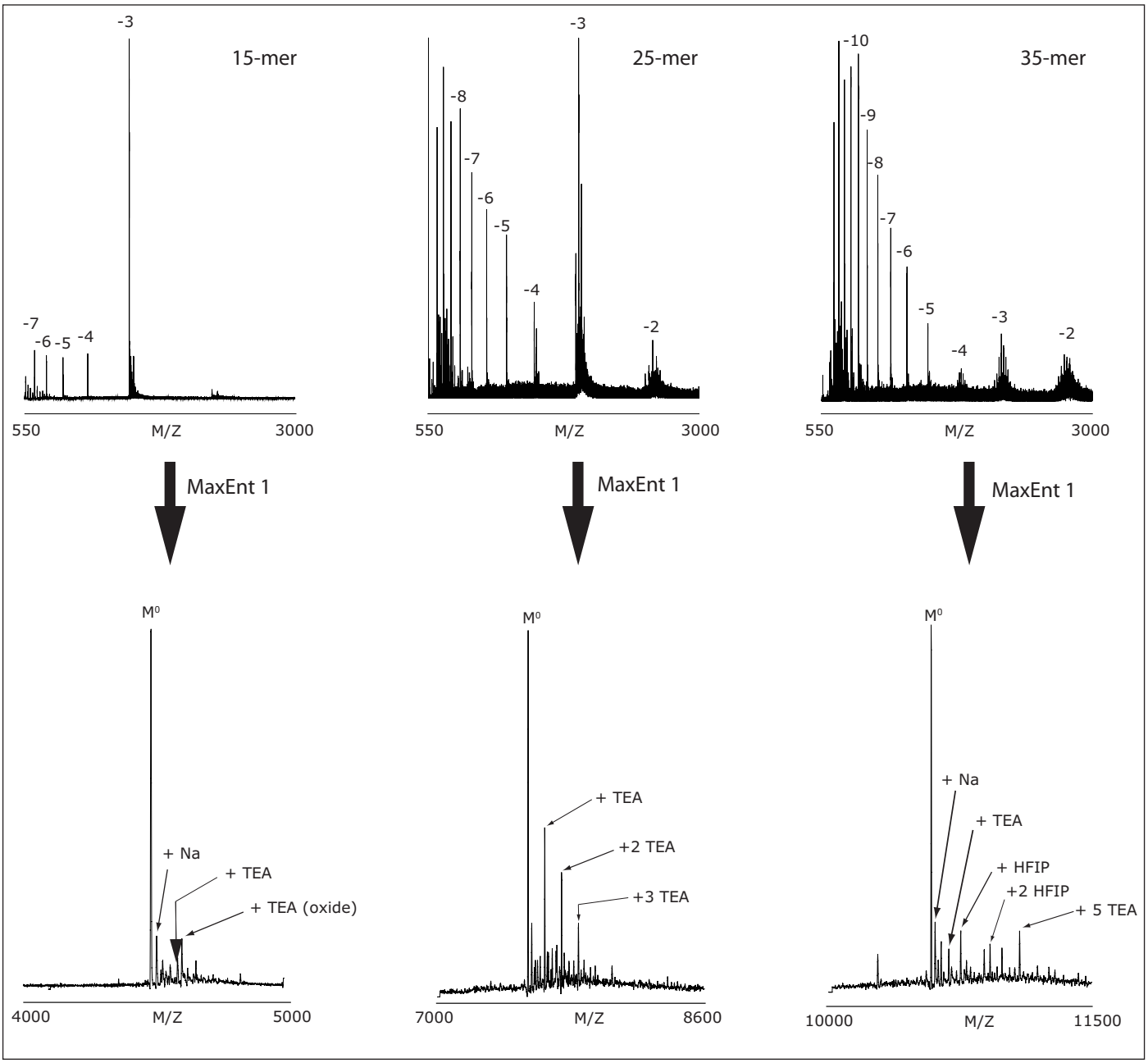

Figure 2. Raw and MaxEnt1 deconvoluted data for 15-, 25-, and 35-mer oligonucleotides of Waters OST standard under normal operating conditions given in Table 1.

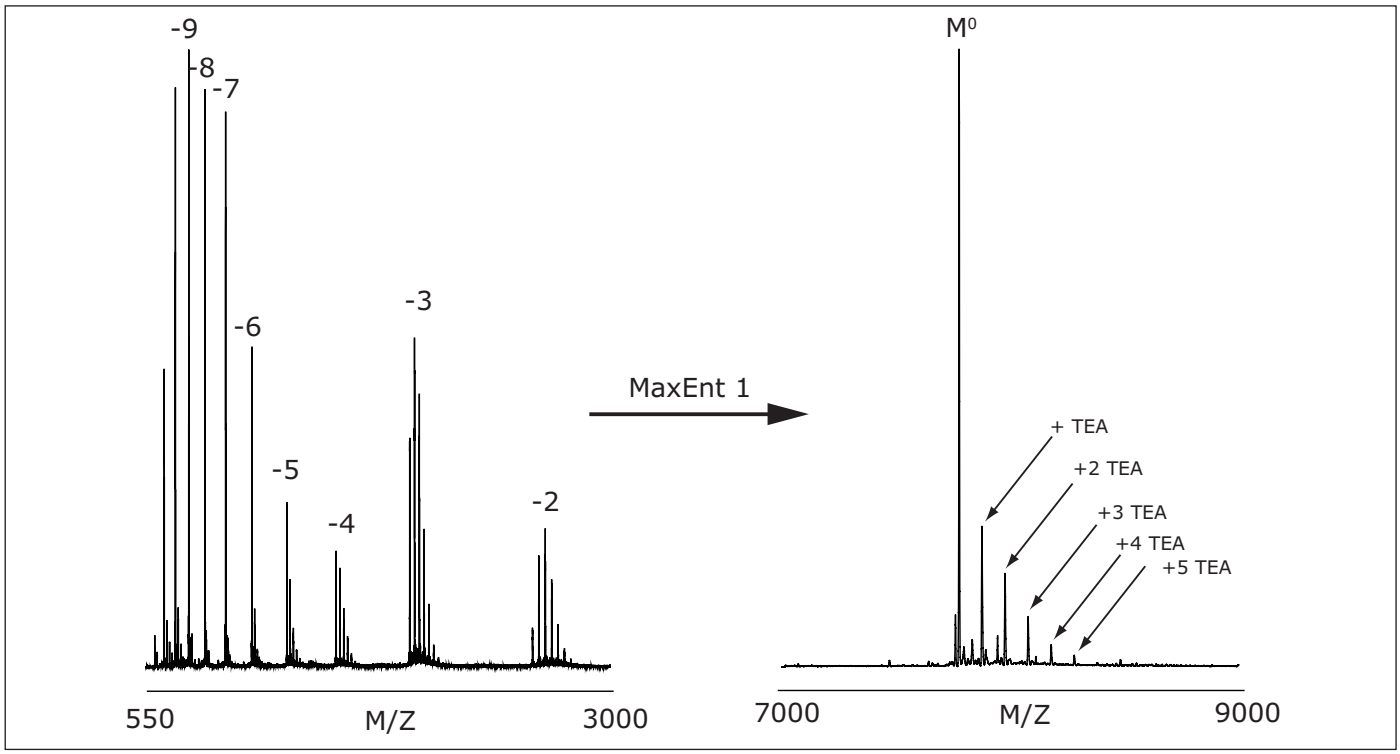

Figure 3. Raw and MaxEnt1 deconvoluted data for 25-mer phosphorothioate under normal operating conditions given in Table 1.

Polarity	ES-	Puller Offset Voltage	0.00
Analyser	V Mode	MCP Detector (V)	2000.0
Capillary (V)	2600.0	Pusher Cycle Time	Auto (68.0)
Sample Cone (V)	*37.0*	Pusher Frequency	14705.88
Desolvation Temp (C)	**500.0**	Pusher Width	4.00
Source Temp (C)	150.0	Centroid Threshold	1.0
Cone Gas Flow	*50.0*	Min Points	4.0
Desolvation Gas Flow	**800.0**	Np Multiplier	0.70
Syringe Type	Hamilton 250uL	Resolution	6000.0
Ion Guide One	5.0	Lteff	1081.0000
Aperture 1 Voltage	*15.0*	Veff	5681.4629
Ion Energy (V)	105.0	Trigger Threshold (mV)	600.0000
Aperture 2 Voltage	*6.0*	Signal Threshold (mV)	40.0000
Hexapole DC Voltage	6.0	Data Threshold	0.0000
Aperture 3 Voltage	*5.0*	DXC Temperature	25.0
Acceleration (V)	200.0	IonGuide1InitialRF	150.0
Y Focus (V)	0.0	IonGuide1FinalRF	150.0
Steering (V)	0.0	IonGuide2InitialRF	200.0
Tube Lens (V)	192.0	IonGuide2FinalRF	200.0
Attenuated Z Focus (V)	500.0	Fixed Hexapole RF	True
Normal Z Focus (V)	65.0	HexapoleRF	180.0
TOF Flight Tube (V)	5630.0	DRE Mass 0.0000 Setting	5.0000
Reflectron (V)	1780.0	DRE Mass 280.0000 Setting	50.0000
Pusher Voltage	839.0	DRE Mass 1000.0000 Setting	50.0000
Pusher Offset Voltage	-1.47	DRE Mass 2000.0000 Setting	50.0000
Puller Voltage	769.0	DRE Mass 3000.0000 Setting	50.0000

Table 2. Optimal LCT Premier XE System operating parameters for oligonucleotide analysis.

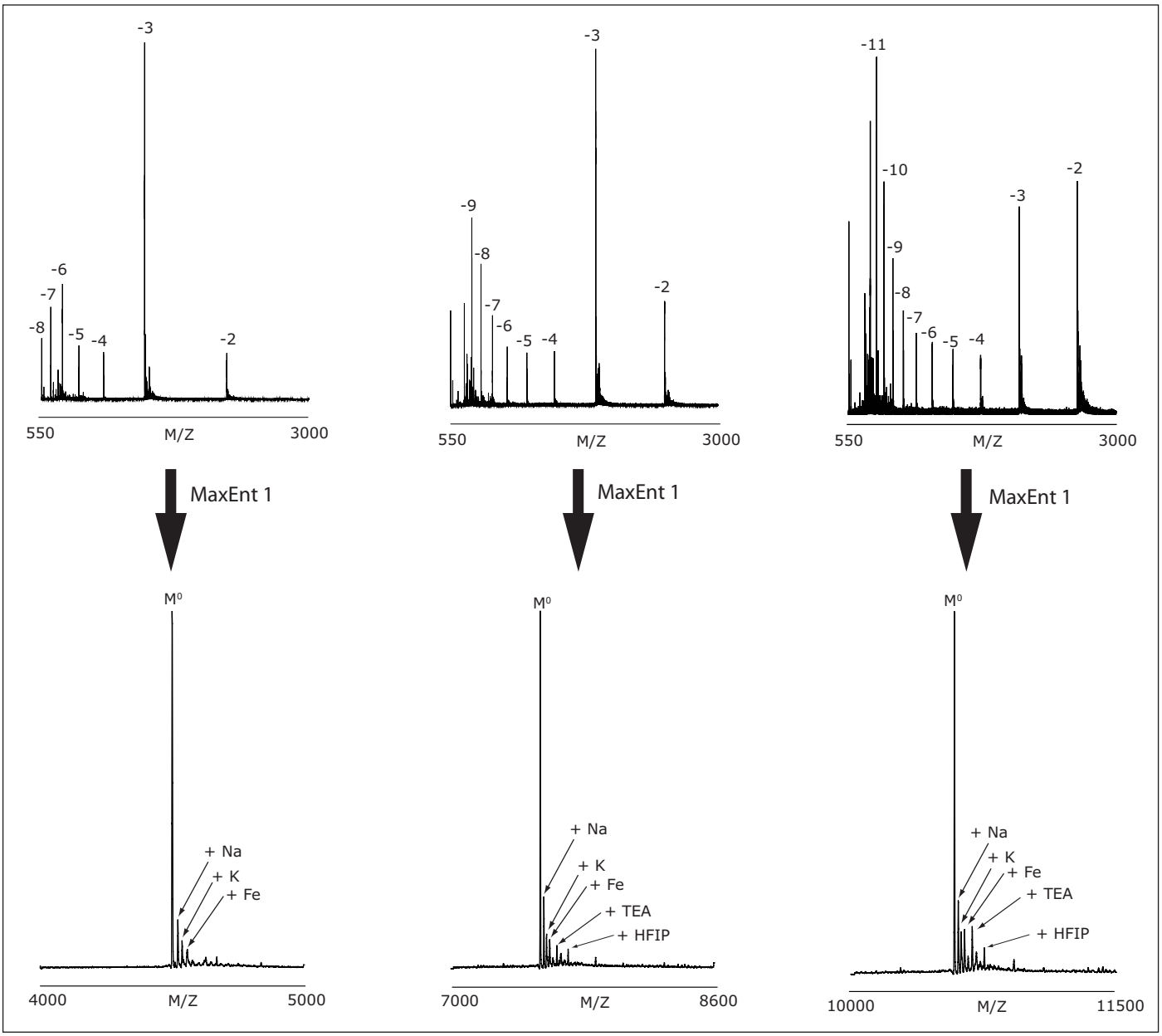

Figure 4. Raw and MaxEnt1 deconvoluted data for 15e, 25e, and 35-mer oligonucleotides of Waters OST standard under optimal operating conditions given in Table 2.

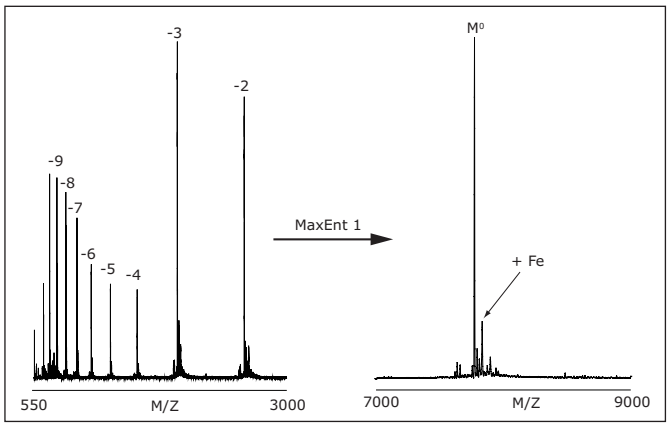

Figure 5. Raw and MaxEnt1 deconvoluted data for 25-mer phosphorothioate under normal operating conditions given in Table 2.

CONCLUSION

This technical note illustrates that the LCT Premier XE System is a good choice for sensitive LC/MS analysis of synthetic oligonucleotides and oligonucleotide-based biotherapeutic compounds.

By adjusting the desolvation parameters, one can achieve efficient desolvation with the LCT Premier XE System at typical LC flow rates of ~0.2 mL/min. The desolvation at lower flow rates is more efficient.

The data presented here illustrates that by modifying the normal operating conditions to our recommended setup, one can significantly reduce adduct formation, resulting in improvements in S/N.

Sensitive and adduct free LC/MS analysis of oligonucleotides are very important for the identification of structurally related components and degradation products in synthetic and therapeutic oligonucleotide compounds.

The LCT Premier XE System, coupled with the separation efficiency offered by the ACQUITY UPLC System and OST Column Chemistry, offers biopharmaceutical laboratories a complete system solution for achieving their research goals. With a fast development of oligonucleotide therapies, such tools become more desired by the biopharmaceutical industry.

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UPLC ANALYSIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES: METHOD DEVELOPMENT

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INTRODUCTION

The ACQUITY UltraPerformance LC® (UPLC®) System combines with Oligonucleotide Separation Technology (OST) Columns, packed with 1.7 µm sorbent, to offer superior analytical performance for oligonucleotide separations compared to HPLC and fast LC separations. As a result, method development and analysis are accomplished in dramatically shorter time, saving valuable time and analytical resources.

In this work, we illustrate UPLC method development for the analysis of phosphorothioate oligonucleotides. For further information on UPLC method development for oligonucleotides, refer to Waters Application Notes 720002383EN and 720002387EN.

Here, we provide guidelines for fast and efficient analysis of therapeutic phosphorothioate oligonucleotides.

RESULTS AND DISCUSSION

The ACQUITY UPLC System and OST Columns are used for fast and efficient separation of oligonucleotides using ion-pairing reversed-phase liquid chromatography (IP-RP LC) mode. Figure 1 illustrates the oligonucleotide separation on the mix of 15, 20, 25, 30, and 35 nt oligodeoxythymidines. The minor peaks are by-products of failed synthesis. Baseline n-1 resolution of all species is achieved in less than 10 minutes.

Phosphorothioate oligonucleotides are more difficult to analyze than phosphorodiester ones. When replacing an oxygen atom in the oligo backbone for sulfur, multiple diastereomers are created. Partial separation of isomers broadens the peaks in both capillary electrophoresis (CE) and liquid chromatography (LC) and complicates the analysis.

While the traditional triethylammonium acetate (TEAA) ion-pairing system is useful for phosphorodiester oligonucleotides, it fails when applied for separation of phosphorothioate oligonucleotides. Recently Fountain[1] and Gilar[2] described a novel ion-pairing buffer suitable for and efficient analysis of therapeutic phosphorothioate oligonucleotides. The buffer is comprised of triethylamine (TEA, an ion-pairing agent) and aqueous hexafluoroisopropanol (HFIP, a volatile weak acid used as buffering component to bring the pH to ~ 8). In addition, this ion-pairing system is compatible with both UV and electrospray MS detection.

The method development for oligonucleotide separation includes an optimization of gradient slope and initial mobile phase elution strength. The method development for analysis of modified oligonucleotides should reflect the fact that these are often more retained in IP-RP LC. An adjustment of initial mobile phase strength may be necessary, especially for 2'O-methylated oligos.

Figure 2 shows the separation of 25 nt phosphorothioate oligonucleotide that was partially hydrolyzed with snake venom phosphodiesterase (3'-exonuclease). The main 25 nt peak was clearly resolved from the N-x 3' truncated species. The identity of the peaks was confirmed by their mass (data are not shown).

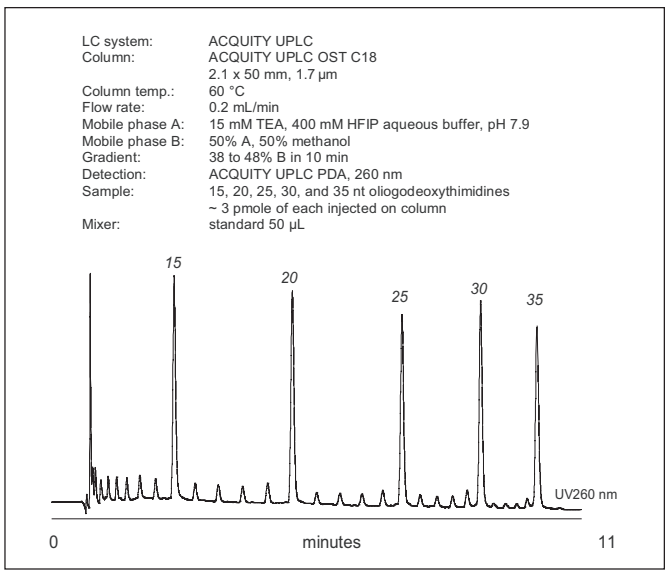

Figure 1. Separation of synthetic oligodeoxythymidines phosphorodiester oligos on a 2.1 x 50 mm, 1.7 µm UPLC OST C_{18} Column.

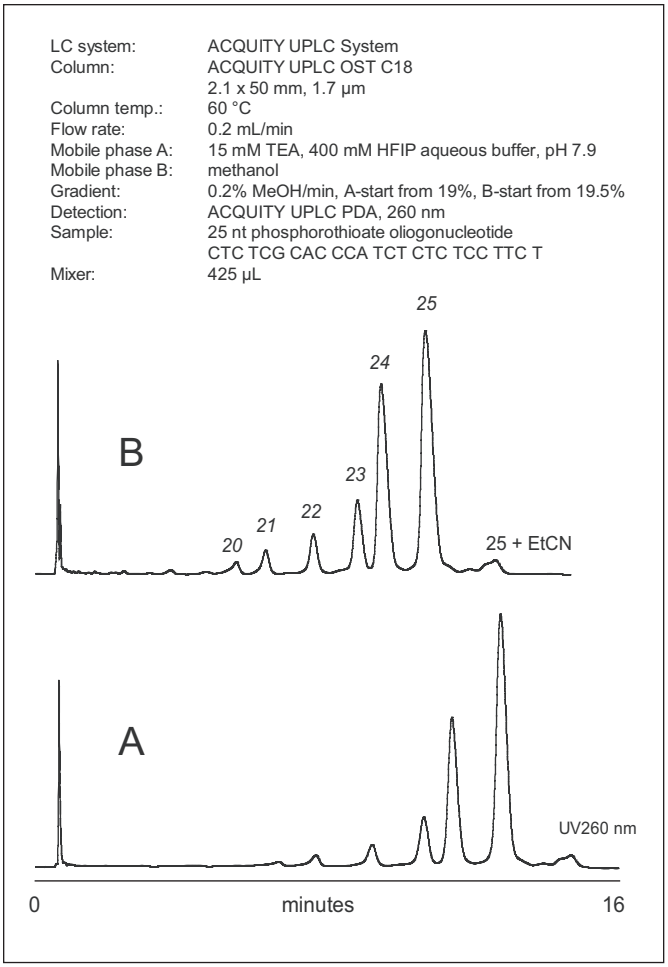

Figure 2. Separation of 25 nt phosphorothioate oligonucleotide from 3' truncated metabolites (3'-exonuclease digested sample). Gradient slope was 0.2% MeOH/min. Gradient started at 19% (A) or at 19.5% MeOH (B).

The gradient slope used for phosphorothioate separation was 0.2% MeOH per minute. In order to maintain a smooth gradient profile when generating the gradient from 100% aqueous and 100% organic mobile phases, the larger mixer (425 µL) is recommended. Figure 2 illustrates that the analysis time can be reduced without sacrificing a resolution. This is achieved by appropriately adjusting the initial gradient strength while keeping the gradient slope constant.

Figure 3 shows the analysis of a purified synthetic 25 nt phosphorothioate oligonucleotide. Interestingly, the failed synthesis by-products correspond to 3'-truncated parent oligonucleotide fragments. N+x peak (cyanoethyl protection group adduct; EtCN) was resolved from the target compound.

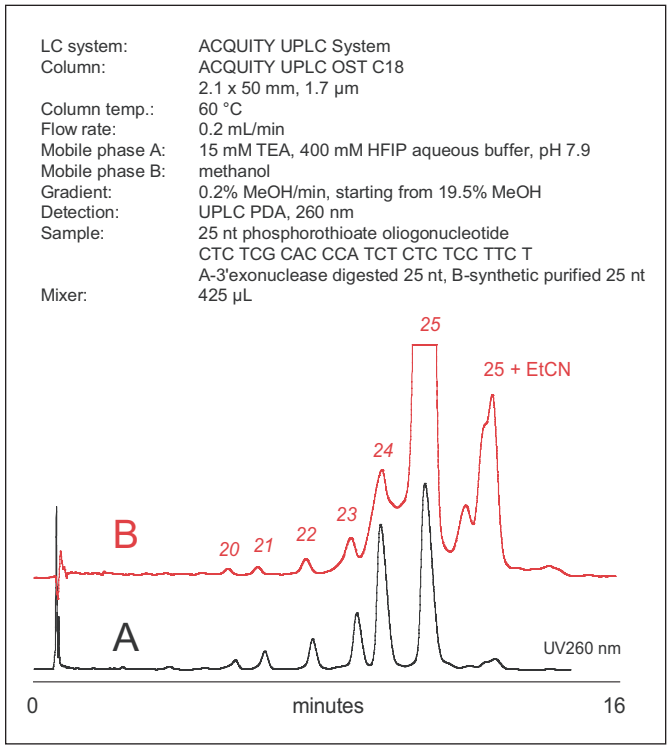

Figure 3. Separation of 25 nt phosphorothioate oligonucleotide from shorter species. (A) 3'exonuclease digested 25 nt, (B) synthetic 25 nt oligo contaminated with a trace amounts of N-x peaks and EtCN 25 nt synthetic by-products.

CONCLUSION

The ACQUITY UPLC System with OST Columns enables improved resolution of oligonucleotides. Fast analyses of native and modified oligonucleotides can be achieved with little method development time.

UPLC technology will increase the productivity of any laboratory developing LC and LC/MS methods and performing analysis of oligonucleotides.

References

1. Fountain KJ, Gilar M, Gebler JC, Rapid Commun. Mass Spectrom. 17 (2003) 646.
2. Gilar M, Fountain KJ, Budman Y, Holyoke JL, Davoudi H, Gebler JC, Oligonucleotides 13 (2003) 229.

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UPLC/UV MS ANALYSIS OF PHOSPHOROTHIOATE OLIGONUCLEOTIDES

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INTRODUCTION

Phosphorothioate (PS) oligonucleotides belong to a class of therapeutic agents intended for various indications including cancer and HIV treatment. As therapeutic candidates, PS oligonucleotides must be purified and the remaining minor impurities characterized. The most common contaminants are shorter failure products of synthesis.

Chromatographic resolution of PS oligonucleotides can be difficult or impossible to resolve by conventional ion-pairing reversed-phase methods. These nucleic acid-based therapeutics represent a new class of biopharmaceutical compounds. Analysis of PS oligonucleotides is becoming more important with the revival of antisense and RNAi-based drugs.

The unique LC/MS method presented in this application note resolves the undesirable failed sequences from the target oligonucleotide peak, and characterizes their respective masses.

Previously-described methods employing Waters® UltraPerformance LC® (UPLC®) Technology for the analysis of phosphorothioate oligonucleotides were applied,[1] pairing the ACQUITY UPLC® System and Oligonucleotide Separation Technology (OST) Columns with MS detection using the Q-Tof Premier™ Mass Spectrometer.

The exceptional resolution and sensitivity provided by UPLC analysis, used in combination with the high mass accuracy of the Q-Tof Premier, provided an identification of failed phosphorothioate impurities within a 15-minute analysis. Sample throughput has been significantly improved in comparison to HPLC's 60-minute analysis time.

EXPERIMENTAL

Method

Mixtures of the phosphorothioate oligonucleotides were analyzed to demonstrate the performance of ACQUITY UPLC System, OST Columns (PN 186003949), and Q-Tof Premier Mass Spectrometer.

Phosphorothioate samples consisting of 25 nucleotides (nt), 5' -CTC TCG CAC CCA TCT CTC TCC TTC T -3', and its 24-, 23-, and 22-mer metabolites truncated from the 3' end were purchased from Integrated DNA Technologies (Coralville, IA).

The samples were reconstituted in mobile phase A to a final concentration of 1 mg/mL. Solvent A consisted of an aqueous solution of 15 mM triethylamine (TEA) containing 400 mM hexafluoroisopropanol (HFIP), pH 7.9. Solvent B contained 50% methanol and 50% solvent A (v/v). Water was used as weak and strong wash solvent. Oligonucleotides of different sizes were premixed in a vial at approximately equimolar ratios.

UPLC/MS conditions

Introduction of phosphorothioate moieties in the oligonucleotide phosphate backbone creates multiple diastereomers. The number of isomers can be calculated as 2^n, where "n" represents the number of nucleotide linkages. The isomers are often partially resolved chromatographically, which results in wider peaks than expected.

Some mobile phases tend to suppress the diastereomeric resolution, and are therefore more suitable for analysis of PS oligonucleotides. Ion-pairing aqueous buffers composed of TEA and HFIP are recommended.[1] In addition, the TEA/HFIP based mobile phases are more appropriate for LC/MS and do not cause ion suppression.

RESULTS AND DISCUSSION

To demonstrate a separation of target PS oligonucleotide from its shorter length fragments, we prepared a mixture of the 25 nt and (N-x) homologs, mimicking 3' exonuclease digestion, because 3' digestion is the primary *in vivo* degradation mechanism. Baseline UPLC separation of the target phosphorothioate product from its (N-x) failure sequences was achieved within 15 minutes (Figure 1). To obtain the best elution profile, the oligonucleotide sample should be prepared in the solvent similar to the initial gradient of mobile phase.

Detection was performed using a photodiode array detector, which was connected to the MS using 75 µm I.D. x 80 cm silica capillary tubing. The narrow I.D. capillary was chosen to minimize the post-column peak broadening prior to MS detection.[2]

MS analysis was performed using parameters optimized for the most efficient electrospray ionization of the oligonucleotides in the negative ion mode.[3] The MS chromatogram demonstrated efficient separation of the peaks corresponding to phosphorothioate failed sequences (Figure 1). Another type of impurity, (N+x), that was also resolved from the targeted sample, was a 25 nt carrying an unremoved cyanoethyl protection group used during oligonucleotide synthesis.

Deconvolution of MS peaks was performed by Waters® MassLynx™ Software with automated MaxEnt™1 data processing. The 3'-truncated oligomers and their depurinated fragments were assigned by their molecular mass (Figure 1).

The high-quality spectra that were generated demonstrates this method's ability to adequately desalt the phosphorothioates for mass analysis with minimal interference due to mobile phase components. Undesired cyanoethylated oligonucleotide contaminants were assigned based on mass difference comparisons.

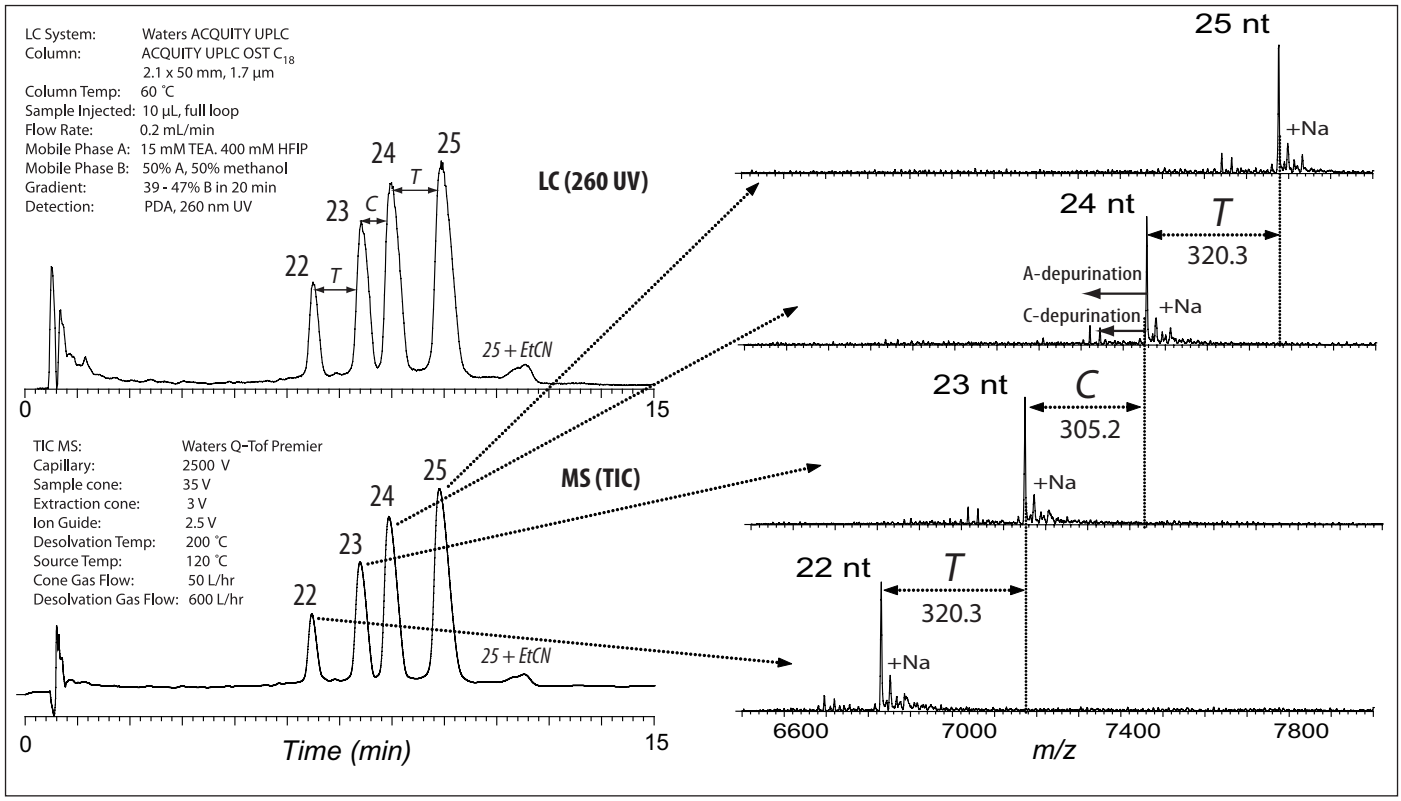

Figure 1. Left: UV and MS chromatograms of 25-mer phosphorothioate oligonucleotide and 3' truncated 24-, 23-, and 22-mer samples. Right: Corresponding MS spectra of the failed sequences (negative ion mode).

CONCLUSIONS

A fast, robust, and sensitive UPLC/MS method was developed for the analysis of synthetic phosphorothioate (PS) oligonucleotides. Failed sequences were resolved from the target 25 nt within 15 minutes. The chosen mobile phase allows for an efficient and robust LC/MS analysis of therapeutic PS oligonucleotides.

To the best of our knowledge, no other method offers the required resolution for the analysis of PS class of oligonucleotides. Because of the revival of nucleic acid-based drug research, the biopharmaceutical industry is in critical need for methods for oligonucleotide analysis. The above-described LC/MS method has been successfully adopted in many industrial laboratories around the world.

Among the benefits of the UPLC methodology is its reduced sample analysis time, which improves sample throughput by approximately four-fold compared to traditional HPLC methods for PS oligonucleotide characterization.[4]

The adoption of LC/UV and LC/MS methods based on Waters UPLC Technology will increase productivity and reduce analysis costs for laboratories involved in the discovery, design, and commercialization of this class of biologically-significant compounds.

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3. UPLC/UV-MS Analysis of Oligonucleotides. Waters Application Note. 2008; 720002413en.
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CHARACTERIZING POLYETHYLENE GLYCOL (PEG) BY SYNAPT HIGH DEFINITION MASS SPECTROMETRY (HDMS)

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INTRODUCTION

Polyethylene glycol (PEG) is a polymer composed of repeating subunits of ethylene oxide. PEG and its functionalized derivatives can be formed in linear or branched shapes with different molecular masses, resulting in significant material complexity and diversity.

Due to the many unique properties of PEG materials – highly water soluble, non-toxic – PEG is often attached to biopharmaceuticals (i.e. PEGylation) to improve pharmacological properties. It is critically important to determine the quality of a batch of PEG prior to attaching it to a biopharmaceutical. Attaching a low-quality batch of PEG to a biopharmaceutical leads to poor end product performance, and increases costs because the final product does not meet specifications.

Because of the complexity associated with PEG materials, PEG characterization by conventional methods has been extremely challenging. In this application note, we present a method to characterize PEG using the Waters SYNAPT™ High Definition MS™ (HDMS™) System, a novel instrument that combines high-efficiency ion mobility (IMS) based measurements and separations with high performance tandem mass spectrometry. The additional ion mobility based gas-phase separation of the system provides a unique method to examine – in great detail – the composition of PEG materials. This better enables analysts to identify potential contaminants contained in the material and thus assess the quality of the material, providing for more confidence in the release of a PEGylated biopharmaceutical product.

EXPERIMENTAL

PEG 4450 was obtained from a Waters GPC molecular weight standard kit (part number WAT035711). PEG 20000 was purchased from Sigma [20% (w/v)]. The polymers were prepared at a concentration of 0.5% (w/v) in 50:50 acetonitrile/H_2O for mass spectrometric analysis. Samples were introduced to MS directly by infusion, using a syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 5 µL/min.

MS conditions

MS system:	Waters SYNAPT HDMS System
IMS gas:	N_2 gas
IMS gas pressure:	0.8 mbar
Pulse height:	Variable, 7 to 15 V
Ionization mode:	ESI positive
Capillary voltage:	3200 V
Cone voltage:	40 V
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Source temp.:	150 °C
Acquisition range:	100 to 4000 m/z
Trap collision energies:	8 V

RESULTS

An electrospray ionization time-of-flight (ESI-TOF) mass spectrum of PEG 4450 [0.5% (w/v) in 50:50 H_2O/ACN solution] results in a distribution of several charge envelopes (Figure 1, left panel). Each charge envelope contains multiple peaks representing a molecular weight distribution of the material. The overlap between each of the charge states, the polydisperse nature of the material, and the presences of low molecular weight PEGs/contaminants all make the complete characterizations of the material via conventional ESI-TOF a formidable task to undertake, even for a medium-size PEG.

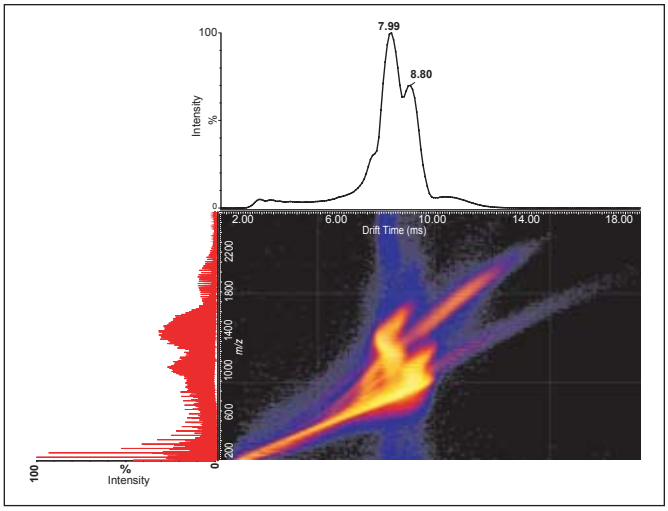

Figure 1. Data for analysis of PEG 4450 using the SYNAPT HDMS System, displayed in DriftScope™ Software. Each pixel represents an ion, with color representing its intensity (blue-low, to orange-high). To the left of the plot is the ESI-TOF spectrum without IMS separation. On the top of the DriftScope plot is the composite IMS spectrum from the projection of DriftScope on the drift time axis.

By analyzing the sample in HDMS mode (IMS-MS), Triwave™ Technology can be used to rapidly separate components in complex mixtures in tens of milliseconds. Here, we have taken advantage of this capability to separate complex PEG ions formed during the ionization process. In these experiments, the time required for IMS separations is <20 ms. Ions with different charge states, or different conformers of the same m/z ions, were readily resolved by IMS (Figure 2). The separation greatly simplifies the complexity of the spectrum such that some of the minor components in the samples that cannot be observed otherwise can be easily identified from the sample (Figure 3).

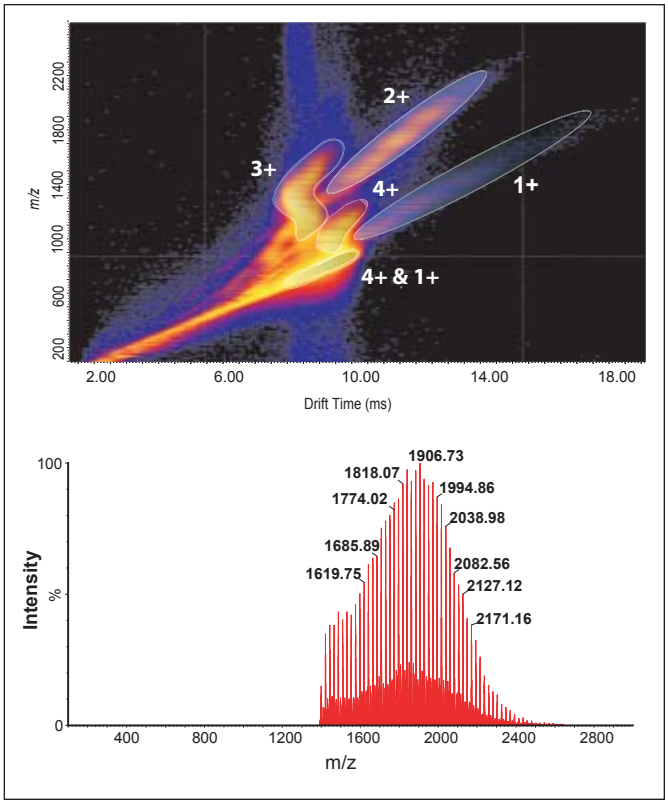

Figure 2. Analysis of PEG 4450 using HDMS. Top panel: HDMS data show the gas-phase separation power of the SYNAPT HDMS System in the analysis of PEG 4450. Components with different charge states are separated via ion mobility, thus enabling the examinations of different (minor) components in the PEG materials. Bottom panel: Mass spectrum showing the ions with +2 charge state.

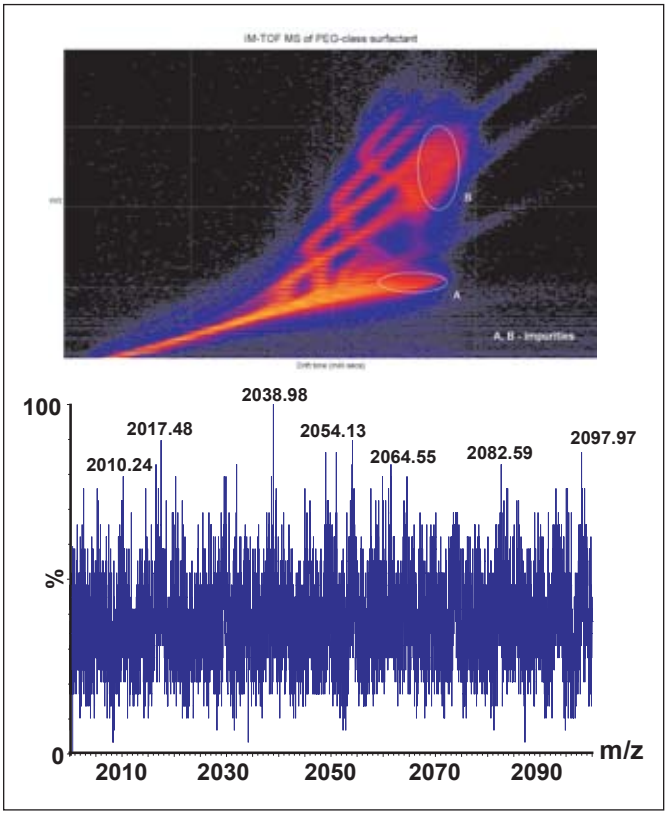

Figure 3. Analysis of PEG 20,000 using HDMS. Top panel: Using DriftScope Software, the HDMS data exhibit the separation of potential impurities (labeled as circle A and B) contained in the PEG materials from the rest of PEG components. These impurities would not be readily discovered without the gas-phase separations. Bottom panel: Zoomed mass spectrum showing the ions in circled region B. The mass difference between neighboring peaks indicated that they are not pure PEG material.

CONCLUSION

By employing IMS separations in HDMS mode with the SYNAPT HDMS System, the general molecular weight distribution of PEG material used in biopharmaceuticals can be rapidly assessed and potential contaminants in the materials can be quickly identified. Fast, more detailed characterizations of PEG are readily achieved. With the level of analytical detail provided by the SYNAPT HDMS System, analysts can be more confident that their PEGylated biopharmaceutical product will pass quality control tests towards product release.

The consequence of attaching a low-quality batch of PEG to a therapeutic protein is failure of the bioactivity test and the need to scrap a batch of very expensive product.

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The travelling wave device described here is similar to that described by Kirchner in U.S. Patent 5,206,506 (1993).

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