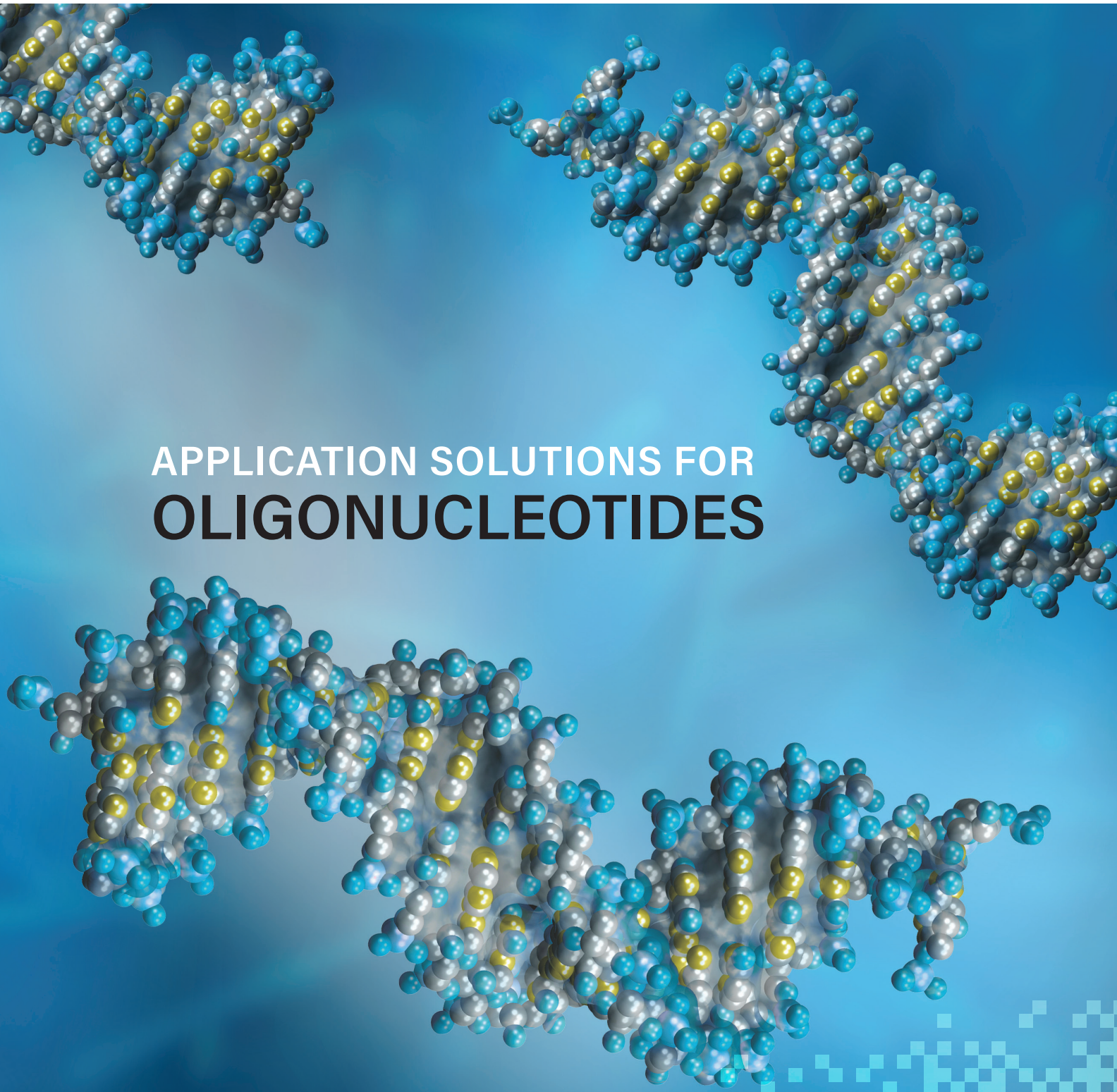

APPLICATION SOLUTIONS FOR OLIGONUCLEOTIDES

Waters

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Table of Contents

[HPLC & UPLC Separations]

[UPLC-MS]

[UPLC-HRMS]

[UPLC-HDMS]

Introduction – UPLC Analysis of Synthetic Oligonucleotides

Oligonucleotides are synthesized, polymeric sequences of nucleotides (RNA, DNA, and their analogs) that are utilized extensively as PCR and microarray-based reagents in life science research, as primer and probe reagents in DNA-based diagnostic test kits, and increasingly they are being developed as direct therapeutic agents against a wide range of disease conditions. Only a few FDA-approved oligonucleotide therapeutic drugs are on the market today, but well over 100 are in the clinical pipeline and many more are in pre-clinical development.

Oligonucleotides as therapeutic drugs come in a variety of forms from antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), and anti-micro RNAs (anti-miRs)—which all affect "gene silencing;" to aptamers, which are short nucleic acid sequences that fold into unique, three-dimensional shapes and bind to proteins/disease targets like small molecule drugs; to messenger RNAs (mRNAs), which are long gene transcript sequences that can augment gene expression; to CRISPR/Cas9 constructs that enable gene editing and hold tremendous promise.

In their natural state, oligonucleotides tend to be unstable and break down quickly in the body. To enhance their stability as therapeutic drugs, chemically-modified nucleotides are often incorporated. And to improve cellular uptake and delivery, the molecules are sometimes PEGylated, or conjugated to other chemical moieties (e.g. GalNAc for liver uptake). Like all biologic drugs, it is critically important for drug developers to fully characterize these molecules, and to separate and purify them from a host of synthesis-related byproducts and impurities.

Waters® offers fit-for-purpose oligonucleotide analysis solutions that stem from our many years of applications development experience. These solutions deliver industry-leading chromatographic performance, resolution, sensitivity, and throughput—enabling laboratories to be more productive.

As referenced in this application notebook, Waters oligonucleotide analysis solutions bring together a full complement of leading-edge technologies that deliver exceptional performance, including:

- ACQUITY UPLC® systems
- ACQUITY UPLC Oligonucleotide BEH C_{18} Columns, method validation kits, and standard reagents
- Oasis® HLB Solid-Phase Extraction (SPE) Cartridges and Plates
- Flexible detection capabilities, with high-sensitivity ACQUITY UPLC Tunable UV (TUV), photodiode array (PDA), fluorescence (FLR), and mass spectrometry (MS)
- Robust software for processing and managing data

With Waters as your biopharmaceutical laboratory technology partner, you can meet your analytical characterization requirements and be more productive at the same time.

DESIGNED FOR OPTIMUM PERFORMANCE

Waters oligonucleotide analysis solutions start with our award-winning ACQUITY UPLC systems and industry-leading oligonucleotide columns, which together deliver the ultimate in high-resolution chromatographic performance. Add to this our many detection options, including tunable UV, photodiode array, and fluorescence detectors, as well as nominal mass and high-resolution mass spectrometry equipment—all optimized for UPLC®—and you have a comprehensive set of tools to meet all of your oligonucleotide analysis needs.

In creating complete system solutions, Waters considered every aspect of oligonucleotide analysis, 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 enabled by the separation efficiencies of sub 2 micron UPLC chemistries, with surfaces optimized for oligonucleotide separation selectivity. Just as significant, the proprietary surface chemistry of our second-generation Bridged Ethylene Hybrid (BEH Technology™) ACQUITY UPLC Oligonucleotide Columns enable exceptional reproducibility and long column life, even at high temperature and pH settings.

Optimal performance from these columns is obtained using our ACQUITY UPLC systems, which feature 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 precise flow rate and gradient control of our ACQUITY UPLC systems combined with our robust ACQUITY UPLC Oligonucleotide BEH C_{18} column chemistry delivers highly reproducible chromatography. Long-term assay stability is ensured with columns that are quality control-tested specifically for oligonucleotide analysis, using the very same MassPREP™ Oligonucleotide Standard we make available to customers.

Qualitative and quantitative analysis of impurities and synthesis artifacts

All the elements of our UPLC-based oligonucleotide analysis solutions are designed to work together to enable the successful qualitative and quantitative analysis of oligonucleotides, their variants (insertions/truncations) and other trace-level impurities. With our ACQUITY UPLC Oligonucleotide BEH C_{18} Columns and ACQUITY UPLC systems, oligonucleotides can be separated, 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
- ACQUITY UPLC Oligonucleotide BEH C_{18} Columns deliver exceptional chromatographic resolution and undistorted peak shape, even at extreme molar ratios

The advanced optical and mass detection technologies included with our oligonucleotide analysis solutions cover a wide dynamic range, ensuring oligonucleotides and impurities at extreme molar ratios are detected, even at the lowest limits of quantitation.

FIT-FOR-PURPOSE ANALYTICAL SOLUTIONS

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 exceedingly low concentrations. With versatile fit-for-purpose configurations that can match your application requirements, Waters' portfolio of MS technologies can provide the most appropriate solution for your laboratory's needs.

UPLC-MS FOR OLIGONUCLEOTIDES

The ACQUITY QDa® Mass Detector combines the resolution, sensitivity, and speed of UPLC technology with single quadrupole MS detection. Designed specifically for chromatographers with little to no mass spectrometry experience, it offers a simple yet powerful solution for rapid oligonucleotide ID confirmation and impurity analysis in routine laboratory environments—delivering robust and reliable performance, and walk-up operation for any expertise level.

Rapid oligonucleotide ID confirmation

- Peak purity and impurity profiling
- Process monitoring

UPLC-HRMS FOR OLIGONUCLEOTIDES

When exact mass measurements are needed, or sequence confirmation via MS/MS analysis is required, the Waters Xevo® G2-XS QTof Mass Spectrometer is an excellent choice that delivers high-sensitivity and high-resolution performance.

- High MS resolution provides the selectivity needed to discern analyte spectra from isobaric interferences and background chemical noise
- High sensitivity achieves very low detection limits
- High linear dynamic range allows experiments to be run across a range of concentration levels

The Xevo G2-XS QTof 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 sequence confirmation.

UPLC-HDMS FOR OLIGONUCLEOTIDES

The Waters SYNAPT® G2-S*i* High Definition Mass Spectrometry® (HDMS®) System offers unique functionality designed for researchers working at the limits of conventional mass spectrometry capabilities who need to further characterize and define their oligonucleotides, or associated impurities.

Combining high-efficiency ion mobility-based measurements and separations with quadrupole time-of-flight (Tof) mass spectrometry, the SYNAPT G2-S*i* 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.

Offering both Tof and HDMS operation modes, the SYNAPT G2-S*i* HDMS expands your capabilities beyond conventional MS:

- Triwave® Technology provides access to the unique benefits of ion mobility spectrometry (IMS)
- 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

ACQUITY UPLC Oligonucleotide BEH C_{18} 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.

ACQUITY UPLC Oligonucleotide BEH C_{18} Columns contain second-generation, hybrid-silica BEH Technology particles functionalized with C_{18}. The separation and analysis of synthetic oligonucleotide samples is based on the well-established method of ion-pair, reversed-phase chromatography.

ACQUITY UPLC Oligonucleotide BEH C_{18} Column 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.

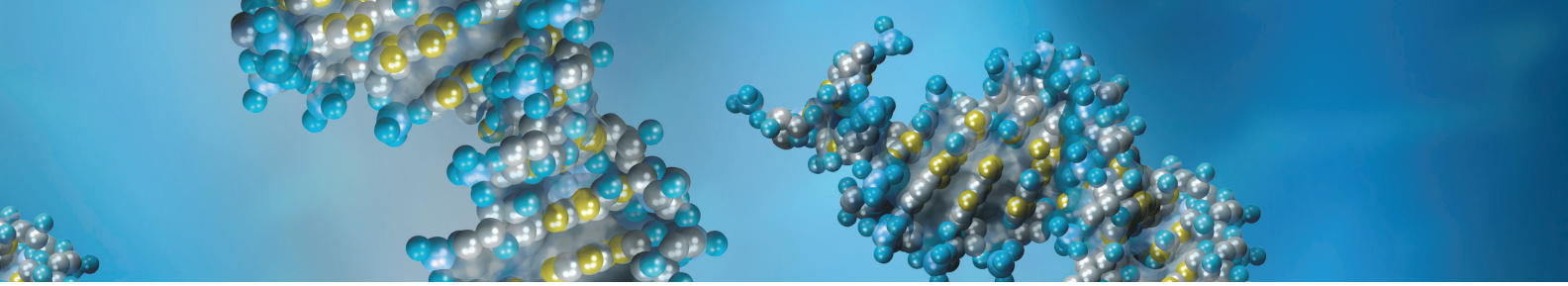
The availability of 1.7 µm UPLC technology and 2.5 µm HPLC/UHPLC particles in a portfolio of optimized column dimensions gives you the flexibility to meet oligonucleotide analysis and laboratory-scale isolation needs.

- Scalable reversed-phase columns for lab-scale purifications
- Increased sample throughput via 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

To learn more about our oligonucleotide LC-MS workflows, please visit **www.waters.com/oligos**.

For details on our various column chemistries and reagents, please visit **www.waters.com/biosep**.

Note: This application notebook includes application notes spanning 10+ years of applications work here at Waters. During this time, there have been some modifications in our product nomenclature. In particular, ACQUITY UPLC Oligonucleotide BEH C_{18} Columns were formerly referred to as Oligonucleotide Separation Technology (OST) columns. In the following application notes, you will see these columns referenced by both names.



[HPLC & UPLC Separations]

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Real-Time Analysis of RNAI Duplexes

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

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.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC
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).

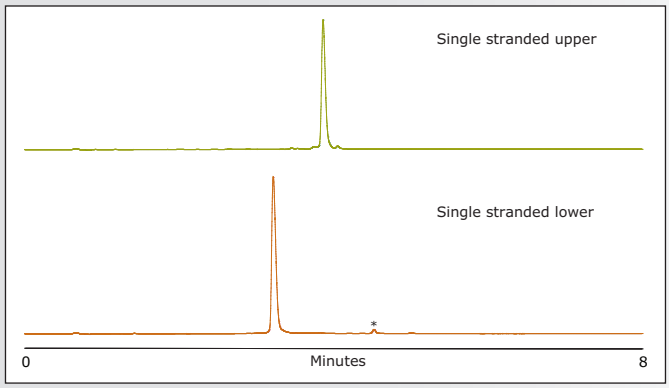

Figure 1. Determination of single-stranded RNA oligonucleotide purity. (= non-oligo impurity)*

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 an 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.

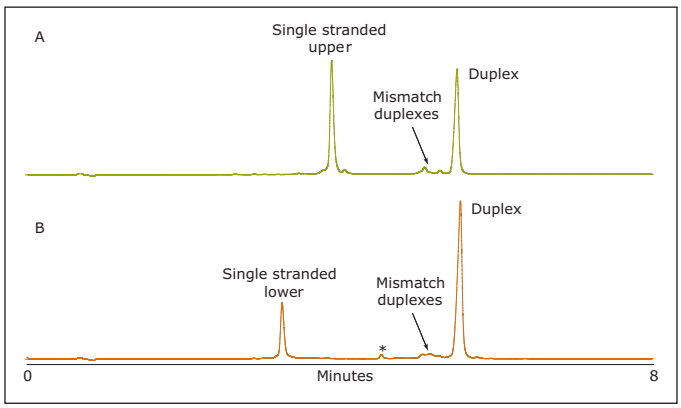

Figure 2. Separation of RNAi duplex from excess single-stranded upper (panel A) and lower (panel B) RNA. (= non-oligo impurity)*

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.

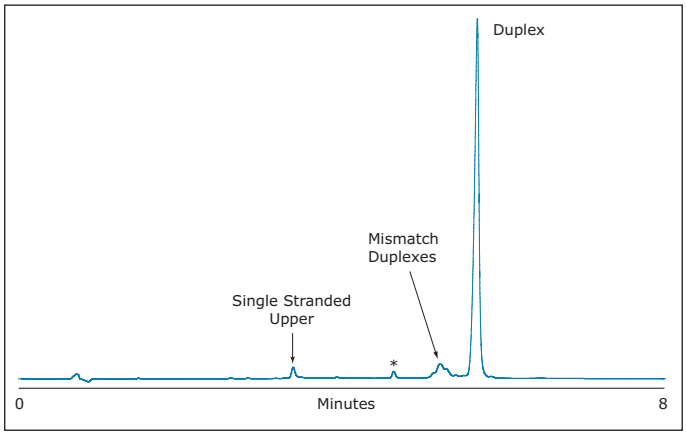

Figure 3. RNAi duplex formation on an ACQUITY UPLC OST Column. Single stranded upper loaded on column at 35% B and single-stranded lower loaded immediately after. (= non-oligo impurity)*

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 (P/N 720002602EN).
2. UPLC/MS Analysis of Interfering RNA Oligonucleotides. Waters Application Note. 2008 (P/N 720002412EN).

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Semi-Preparative Scale Single-Stranded RNA Purification

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

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.

EXPERIMENTAL

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.

Separated products were detected with an ACQUITY UPLC 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.

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:	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:	0.1 M 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

UPLC conditions

LC system:	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:	0.1 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:	PDA, 260 nm

RESULTS AND DISCUSSION

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

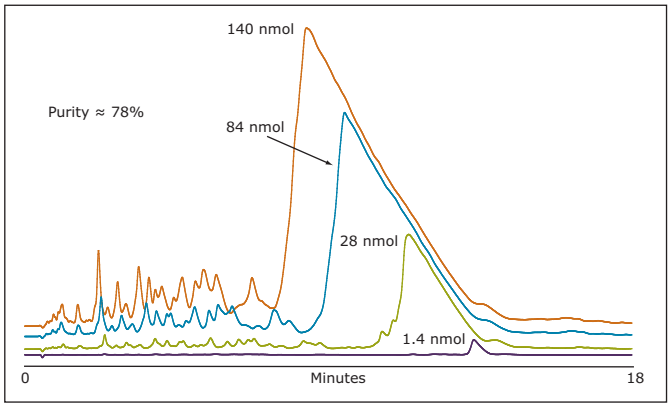

Figure 1. HPLC purification of a synthetic 21-mer oligonucleotide. Sample concentration was 2.8 nmol/µL, with on-column loading ranging from 1.4 to 140 nmol.

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.

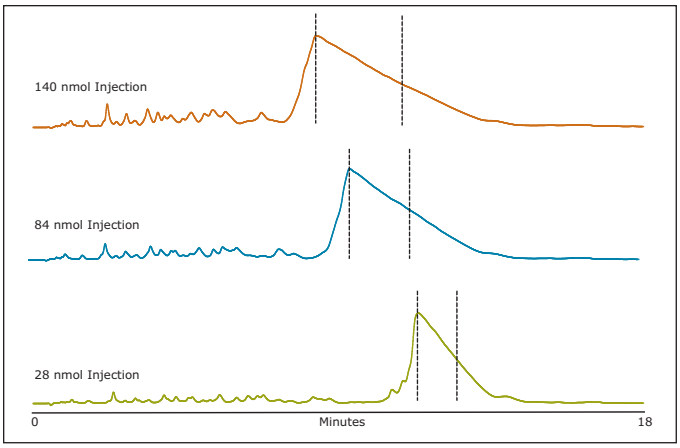

Figure 2. Fraction collection windows. Fractions were collected manually as indicated between dashed lines.

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.

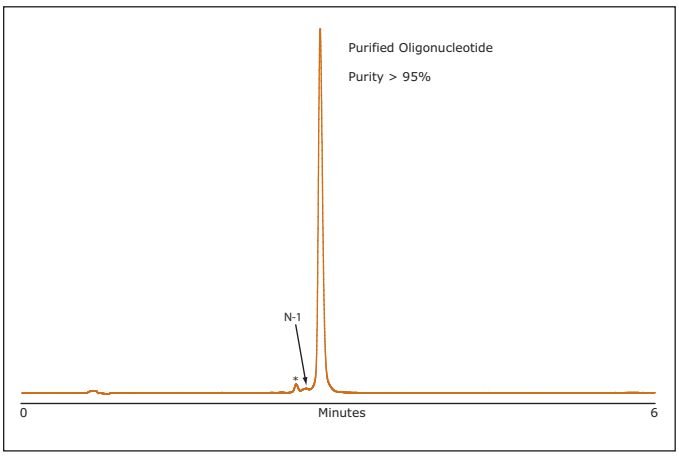

Figure 3. Verification of oligonucleotide purity via UPLC. (= non-oligo impurity.)*

CONCLUSIONS

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 singlestranded 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 singlestranded 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 (P/N 720002383EN).

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Oligonucleotide Separation Technology: Synthesis Challenges and HPLC Isolation Options

Martin Gilar
Waters Corporation, Milford, MA, USA

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.

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.

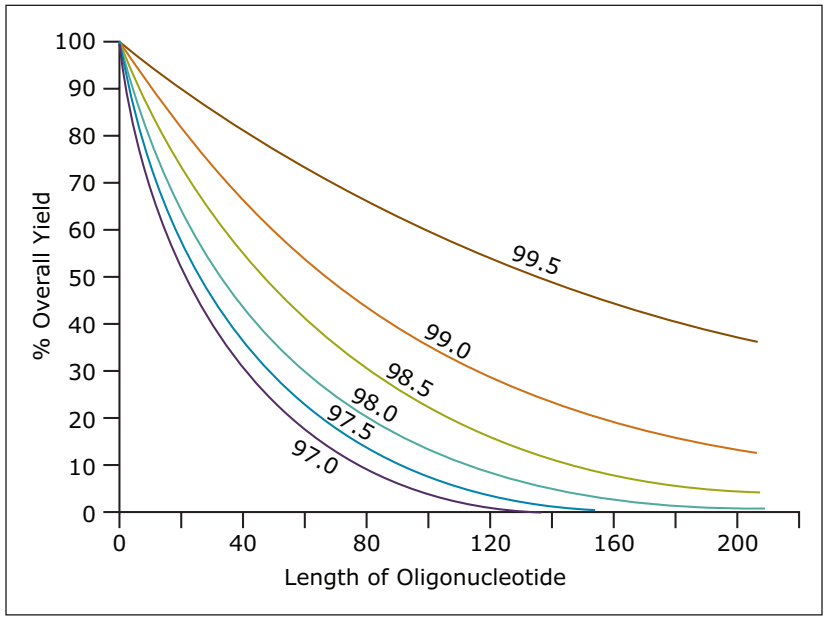

Figure 1. Synthetic oligonucleotide length compared to theoretical yield at various coupling efficiencies.

EXPERIMENTAL

LC conditions

LC system:	Alliance® HPLC 2695
Column:	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

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 "trityloff" 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.

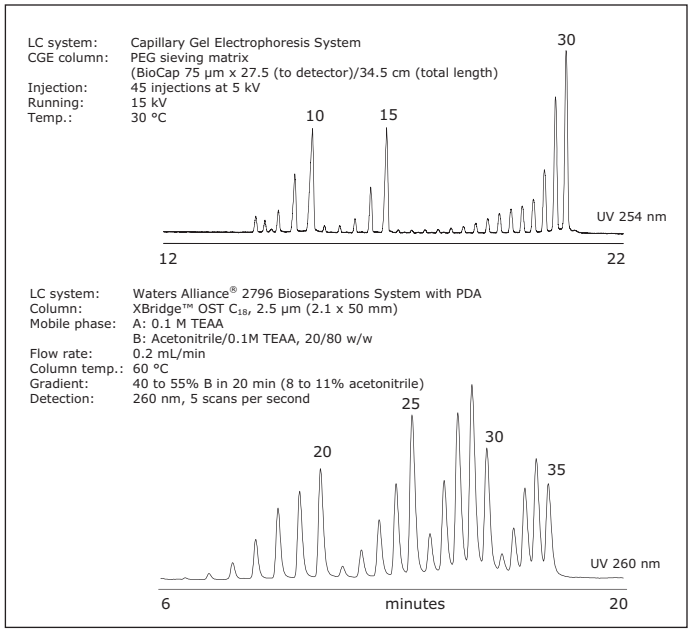

Figure 2. Separation of detritylated oligodeoxythymidine ladders by capillary gel electrophoresis (CGE) vs. ion-pair reversed-phase (IR-RP) chromatography.

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]

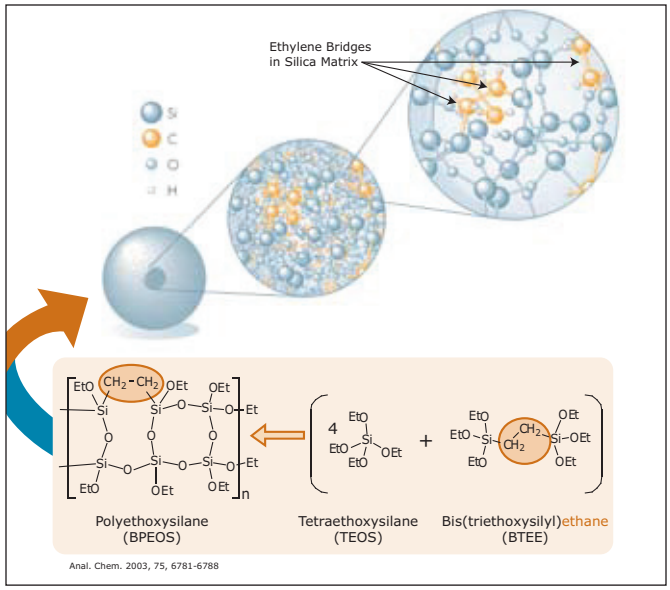

Figure 3. BEH Technology™ particles are prepared from two high purity monomers: tetraethoxysilane (TEOS) and bis (triethoxysilyl) ethzane (BTEE, which incorporates the pre-formed ethylene bridge). This structure results in greater temperature and pH stability compared to that seen with traditional silica-based,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

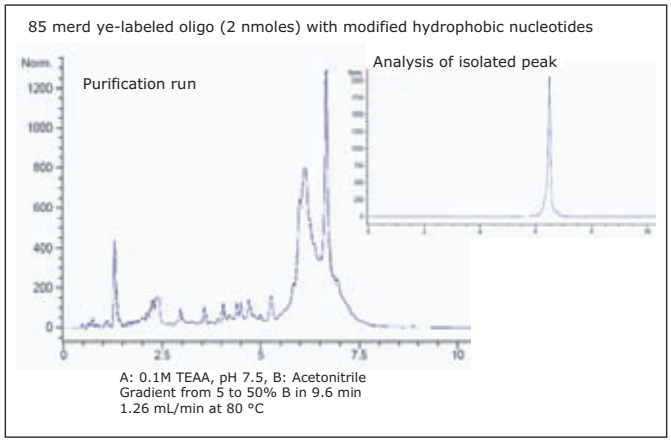

Figure 4. XBridge OST C_{18} isolation (2 nmoles injected) and analysis of isolated 85 mer dye-labeled oligo with modified hydrophobic nucleotides.

Figure 5. Oligonucleotide Separation Technology (OST) Columns.

CONCLUSIONS

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.

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.

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.

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.

References

1. Wyndham K, et al. Characterization and evaluation of C_{18} HPLC stationary phases based on ethyl-bridged hybrid organic/inorganic particles. *Anal. Chem.* 2003; 75: 6781. Waters Application Note WA32741.

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HPLC and UPLC Columns for the Analysis of Oligonucleotides

Martin Gilar, Hua Yang, Edouard S. P. Bouvier, and Bill Warren
Waters Corporation, Milford, MA, USA

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.

The ACQUITY UltraPerformance LC® (UPLC®) System offers 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.

EXPERIMENTAL

LC conditions (Figure 1)

LC system:	Alliance® HPLC 2695 HPLC System with 2996 PDA Detector
Column:	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

LC conditions (Figure 3)

LC system:	Alliance HPLC 2796 Bioseparations System with 2996 PDA Detector
Column:	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

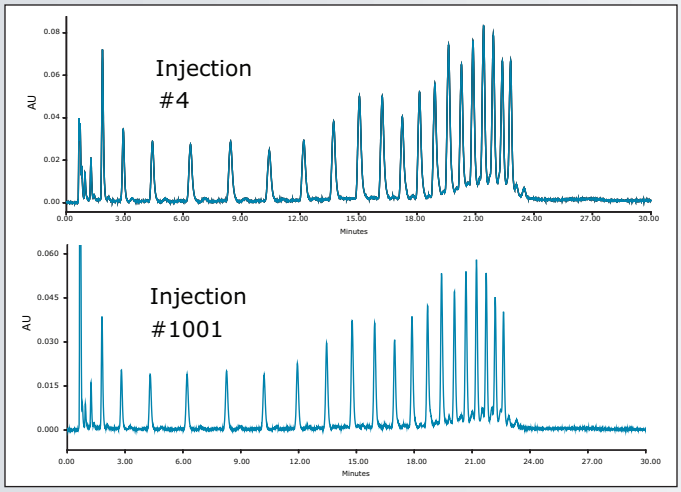

Figure 1. BEH OST Column longevity exceeds 1000 injections. Separation of 5 to 25 nt oligodeoxythymidine ladder.

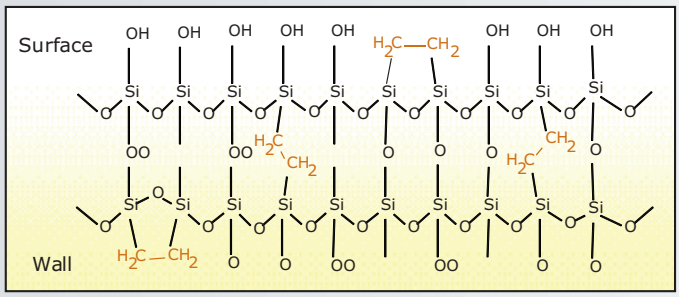

Figure 2. Schematic structure of BEH sorbent. Hydrolytic stability is achieved by bridging ethyl groups. For oligonucleotide analysis, the surface of sorbent is alkylated by C_{18} functional groups.

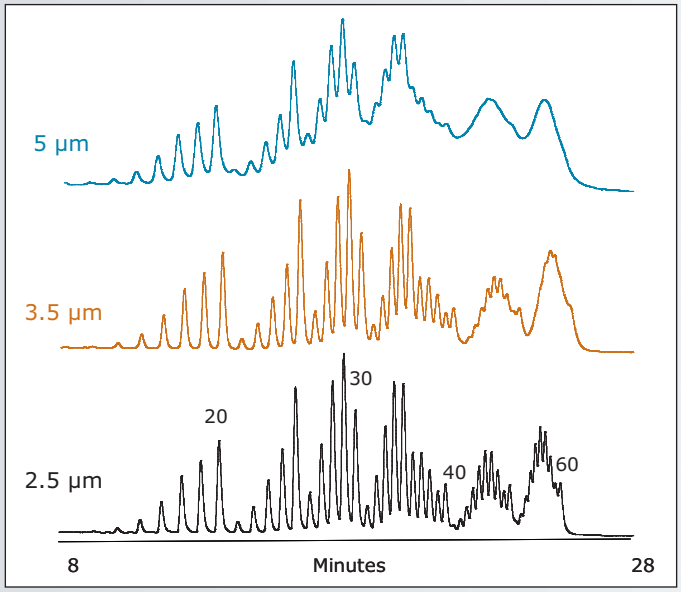

Figure 3. Impact of sorbent particle size on oligonucleotide ladder separation. Improved resolution of 15 to 60 nt oligodeoxythymidine ladder is observed for columns packed with smaller particles.

RESULTS AND DISCUSSION

Column lifetime

Figure 1 illustrates 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.

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.

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.

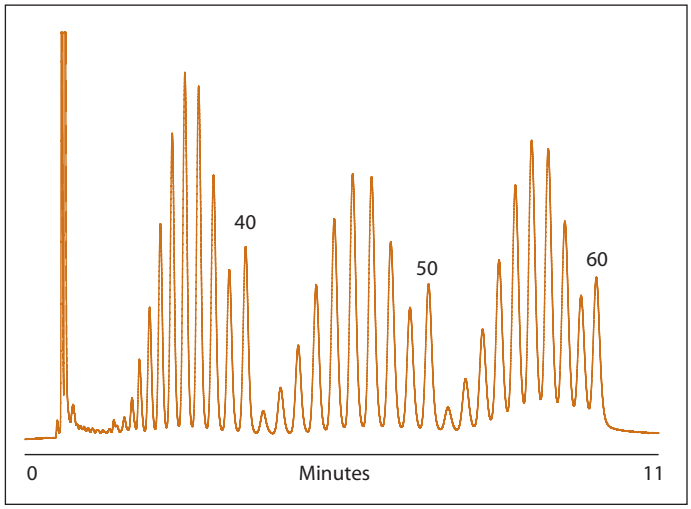

Figure 4. Resolution of 30 to 60 nt oligodeoxythymidine ladder in UPLC mode.

CONCLUSIONS

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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UPLC Separation of Oligonucleotides: Method Development

Martin Gilar
Waters Corporation, Milford, MA, USA

INTRODUCTION

The Waters® ACQUITY UltraPerformance® (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, highthroughput 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.

Waters ACQUITY UPLC System.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC 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 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.

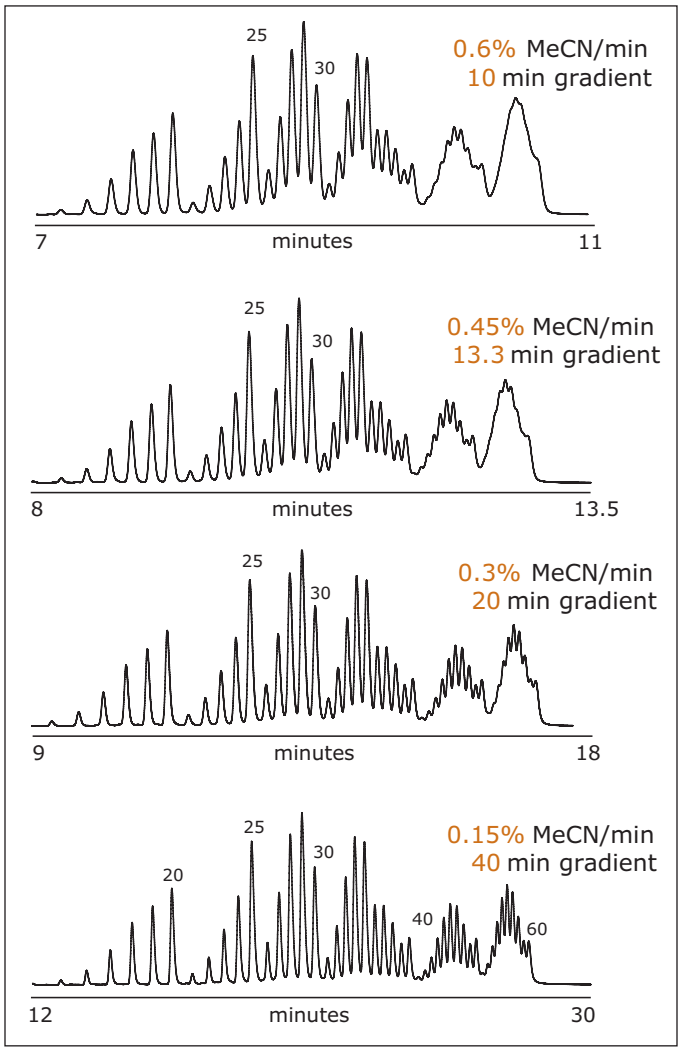

Figure 1. Impact of the gradient slope on separation of 15 to 60 nt oligodeoxythymidines and analysis time.

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.

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.

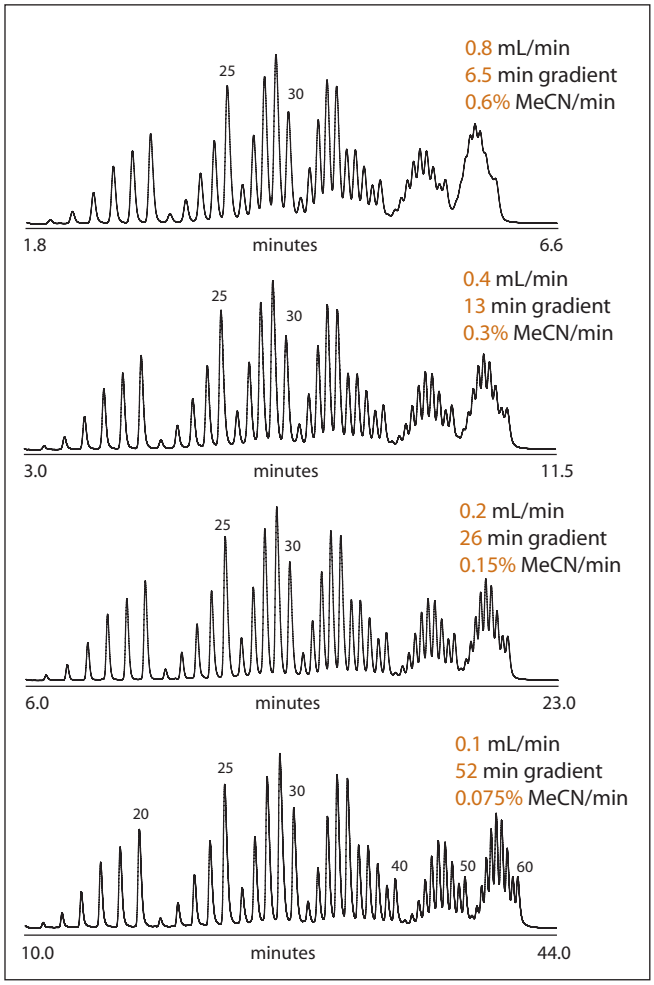

Figure 2. Separation of 15 to 60 nt oligodeoxythymidines at constant gradient volume in various mobile phase flow rates.

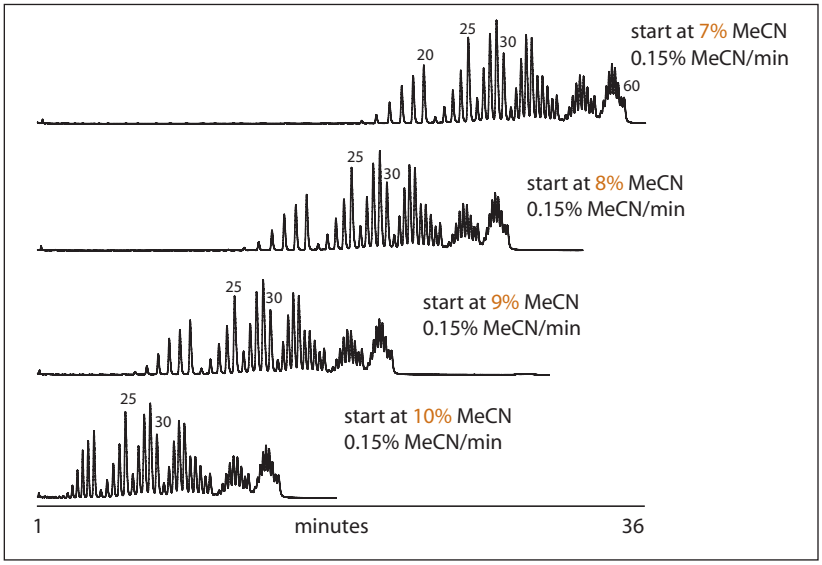

Figure 3. Reducing the analysis time by adjustment of initial gradient strength. Gradient slope remains constant.

CONCLUSIONS

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
Waters Corporation, Milford, MA, USA

INTRODUCTION

The ACQUITY UltraPerformance LC® (UPLC®) System, when combined with Oligonucleotide Separation Technology (OST) Columns packed with 1.7 µm sorbent, offer 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 ACQUITY UPLC 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.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC® 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 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.

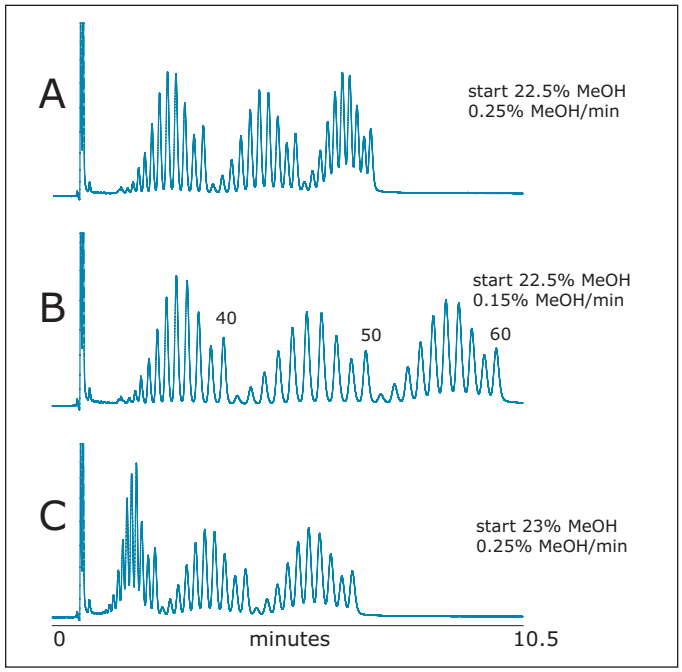

Figure 1. Separation of 30 to 60 nt oligodeoxythymidines using 2.1 x 50 mm, 1.7 µm ACQUITY UPLC OST C_{18} Column.

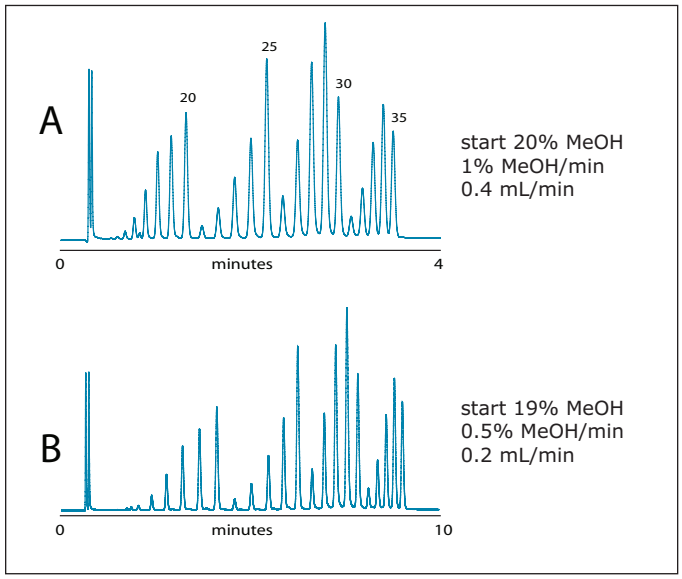

Figure 2. Separation of 15 to 35 nt oligodeoxythymidines.

CONCLUSIONS

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.

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UPLC Separation of DNA Duplexes

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

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 HspIII 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.

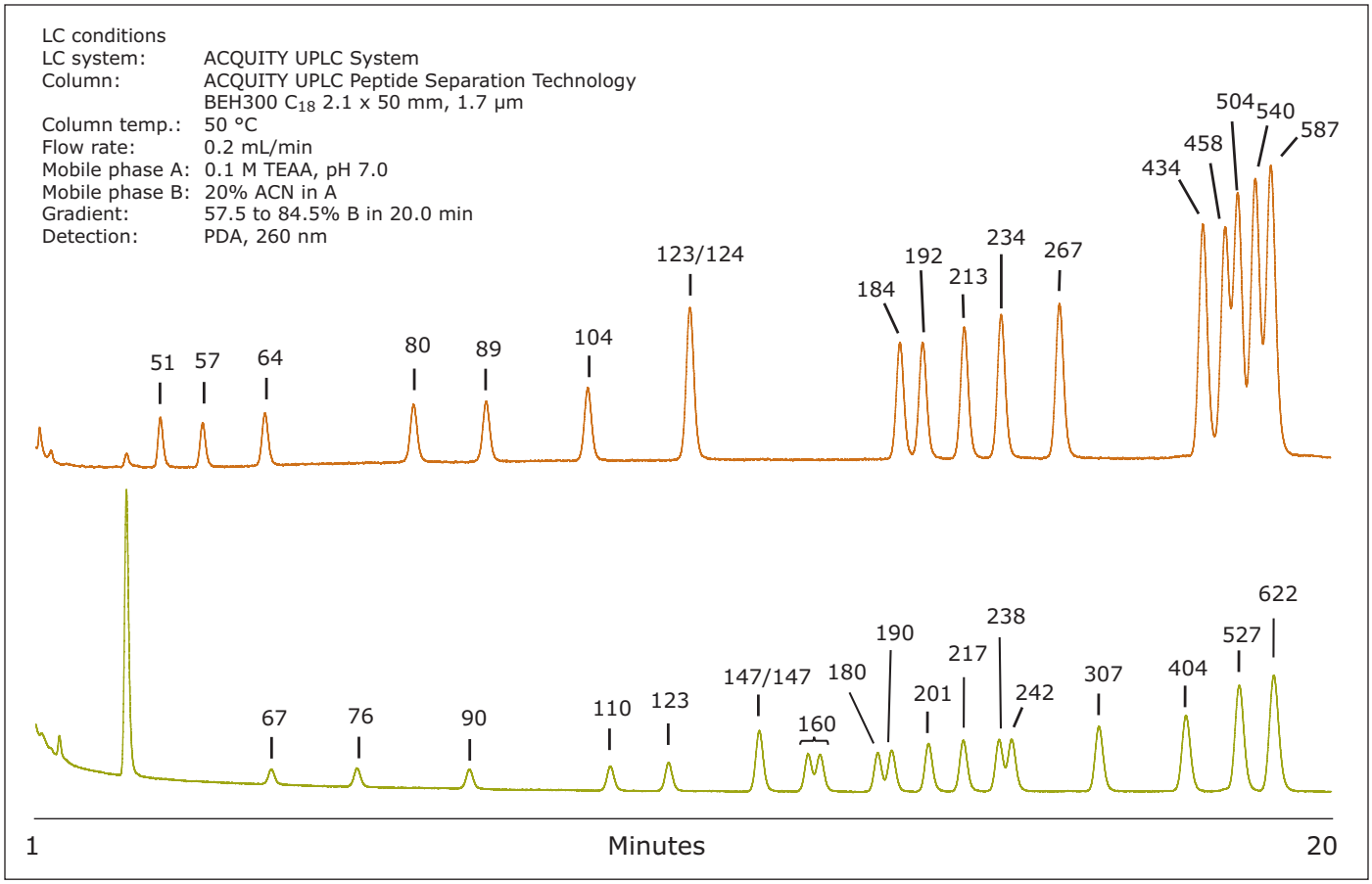

Figure 1. Separation of HaeIII (top trace) and MspI (bottom trace) digests of the plasmid pBR322.

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.

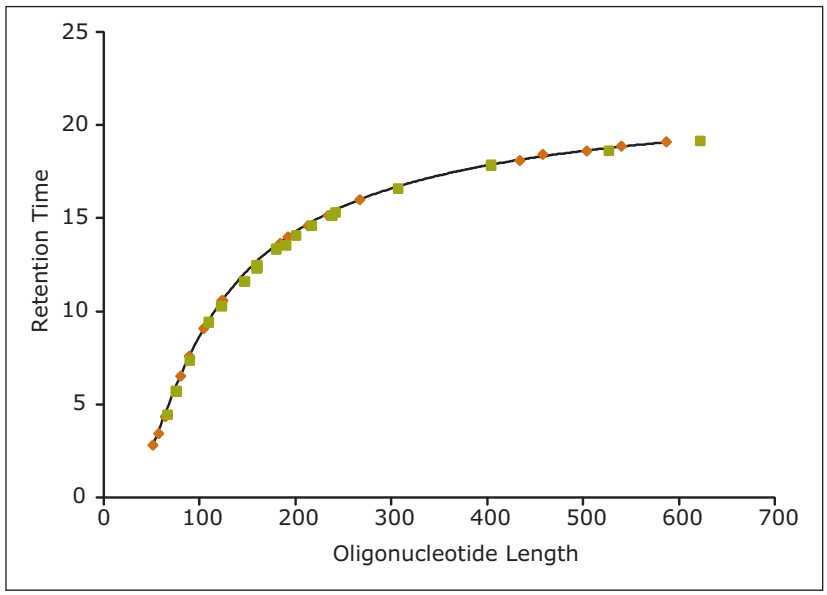

Figure 2. Fit of data between dsDNA fragment length (bp) and observed retention time. The fit was constructed for HaeIII pBR322 restriction digest. Red dots represent data for HaeIII pBR322 digest used for fitting. Green squares represent MspI pBR322 digest data points (not used for curve fitting).

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.

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.

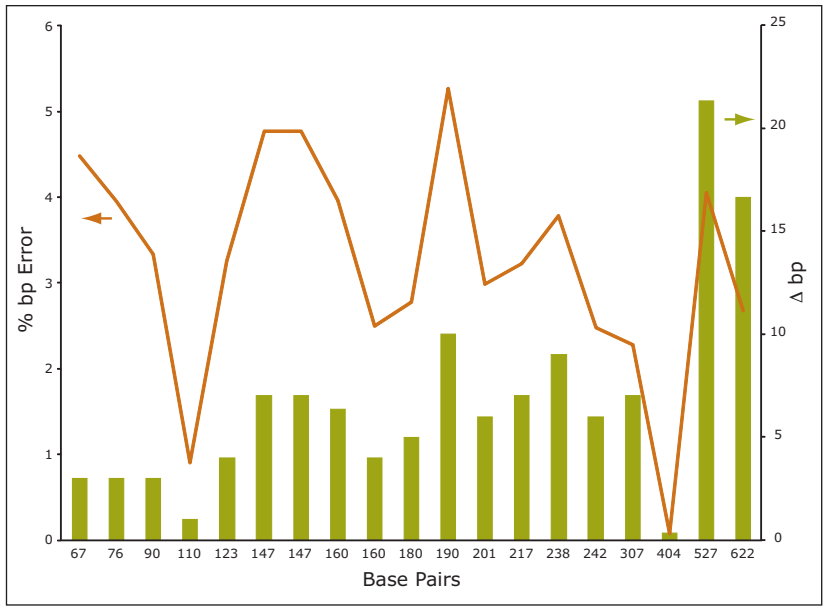

Figure 3. Absolute (green bars) and relative (red line) errors between predicted and measured retention of dsDNA fragments in ion-pair UPLC method.

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.

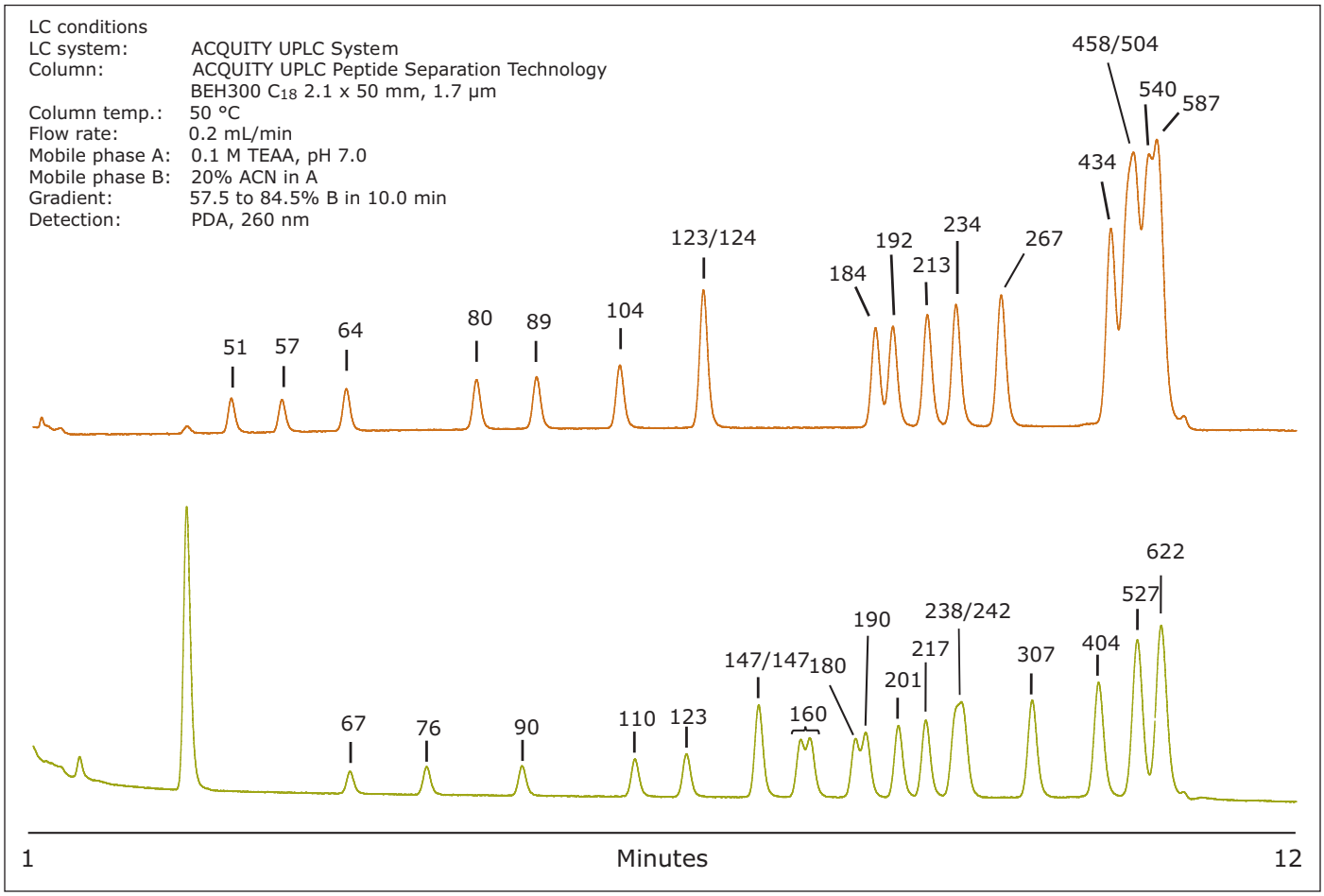

Figure 4. Separation of HaeIII (top trace) and MspI (bottom trace) digests of the plasmid pBR322.

CONCLUSIONS

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

1. Gilar M, Yang H, Bouvier ESP, Warren B. HPLC and UPLC Columns for the Analysis of Oligonucleotides. Waters Application Note. 2007; 720002376en.

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UPLC Analysis of Phosphorothioate Oligonucleotides: Method Development

Martin Gilar
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

In this application note, we illustrate and provide guidelines for fast, high-resolution, efficient analysis of therapeutic phosphorothioate oligonucleotides in a single analysis method.

WATERS SOLUTIONS

ACQUITY UPLC® System

Oligonucleotide Separation Technology

ACQUITY UPLC PDA Detector

KEYWORDS

Oligonucleotides, ACQUITY UPLC, method development, OST

INTRODUCTION

Antisense phosphorothioate oligonucleotide therapies are a promising treatment for a number of diseases, including cancer, diabetes, high cholesterol, and AIDS. The inherently unique characteristics of phosphorothioate 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 a regulated QC laboratory.

The ACQUITY UltraPerformance LC® (UPLC®) System with Oligonucleotide Separation Technology (OST) Columns, packed with 1.7 µm sorbent, offer superior analytical performance for phosphorothioate 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.

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 ten 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 and Gilar described a novel ion-pairing buffer suitable for efficient analysis of therapeutic phosphorothioate oligonucleotides.[1,2] 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).

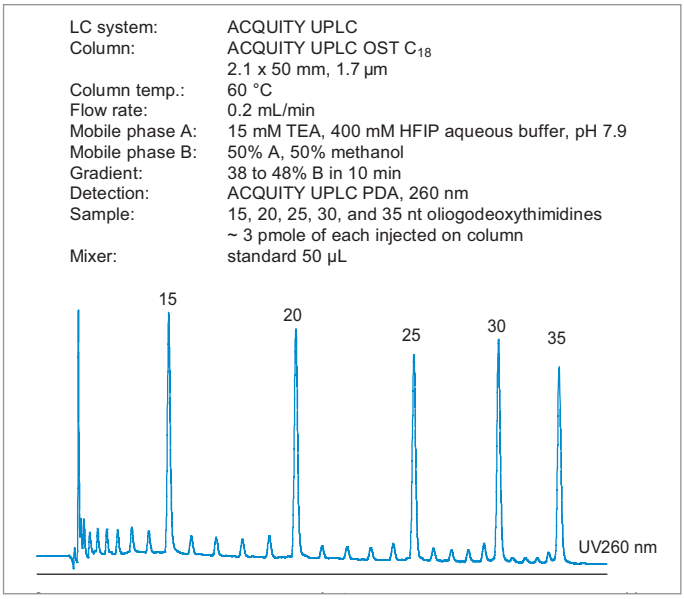

Figure 1. Separation of synthetic oligodeoxythymidines phosphorodiester oligos on a 2.1 x 50 mm, 1.7 µm UPLC OST C_{18} Column.

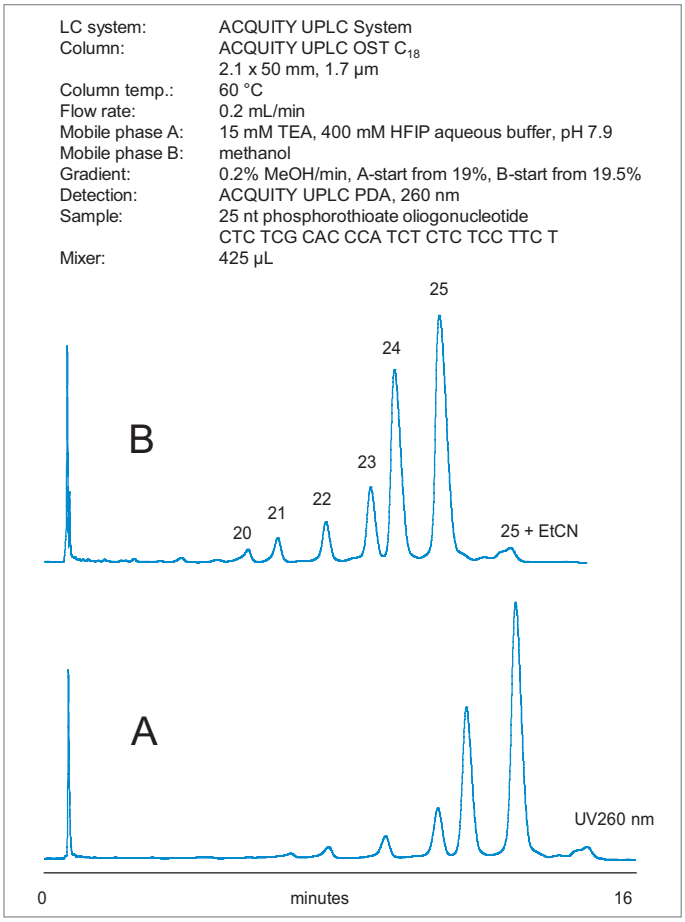

Figure 2. Separation of 25 nt phosphorothioate oligonucleotide from 3' truncated metabolites (3'-exonuclease digested sample). Gradient slope was 0.2% MeOH/min. Gradient started at 19% (A) or at 19.5% MeOH (B).

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 recom-mended. 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.

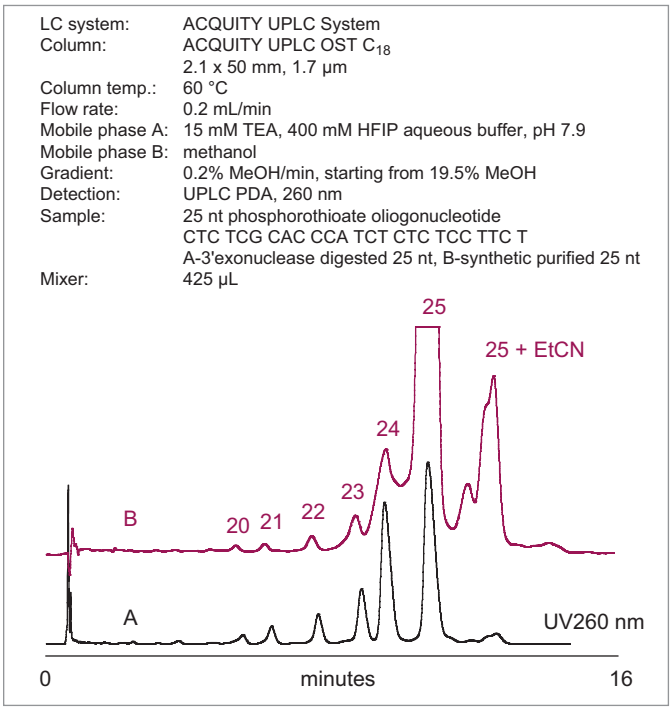

Figure 3. Separation of 25 nt phosphorothioate oligonucleotide from shorter species. (A) 3'exonuclease digested 25 nt, (B) synthetic 25 nt oligo contaminated with a trace amounts of N-x peaks and EtCN 25 nt synthetic by-products.

CONCLUSION

The ACQUITY UPLC System with Oligonucleotide Separation Technology Columns enable high-resolution, high-throughput analysis of phosphorothioate oligonucleotides in a single method. The development of fast analytical methods for native and modified oligonucleotides can be achieved quickly, increasing the overall operational efficiency of a laboratory. UPLC technology will increase the productivity of any laboratory developing LC and LC-MS methods and performing analysis of oligonucleotides.

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2. Gilar M, Fountain KJ, Budman Y, Holyoke JL, Davoudi H, Gebler JC. Characterization of Therapeutic Oligonucleotides Using Liquid Chromatography with On-line Mass Spectrometry Detection. *Oligonucleotides*. 2003; 13 (4): 229–43.
3. UPLC Separation of Oligonucleotides: Method Development. Waters Application Note. 2007. 720002383EN.
4. UPLC-MS Separation of Oligonucleotides in Less Than 5 Minutes: Method Development. Waters Application Note. 2007. 720002387EN.

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UPLC Separation of Oligonucleotides: Effect of Increased Flow Rate and Faster Run Time

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

We highlight the utility of the ACQUITY UPLC System when used with a 1.7-µm column. The platform provides accurate, reproducible, and rapid separation of oligonucleotides. The method also illustrates that analysis times can be dramatically reduced by scaling both the gradient slope and flow rate proportionately, thus yielding constant gradient slope per volume of mobile phase.

WATERS SOLUTIONS

ACQUITY UPLC System

Oligonucleotide Separation Technology Column

KEY WORDS

Oligonucleotide analysis, RNAi, DNA, method development

INTRODUCTION

There is an increased desire to perform rapid and accurate analysis of oligonucleotides. The ACQUITY Ultra Performance LC® (UPLC®) System combined with Waters Oligonucleotide Separation Technology (OST) Columns packed with 1.7-µm sorbent as shown here allows for both rapid and accurate analysis that is not possible using conventional HPLC.

Conventional HPLC methods for the analysis of oligonucleotides generally suffer from low resolution of oligonucleotides, particularly when analysis times are reduced. The UPLC method presented here expands on the topic discussed previously in application note, "UPLC-MS Separation of Oligonucleotides in Less Than Five Minutes: Method Development,"[1] which provides guidelines for the rapid analysis of RNAi and DNA in less than 2.5 minutes in most cases.

RESULTS AND DISCUSSION

Ion-pairing reversed phase (IP-RP) liquid chromatography is a commonly accepted separation strategy for the analysis of oligonucleotides. Of the known ion-pairing systems, triethylamine hexafluoroisopropanol (TEA/HFIP) is commonly used due to its MS compatibility and impressive resolving power. Additionally, we utilized hexylammonium acetate (HAA), which also offers exceptional resolution.

We developed a method for the analysis of up to 35-mer oligonucleotides in under 2.5 minutes and oligonucleotides up to 25 mer in under 2 minutes with impressive resolution. This was accomplished by establishing appropriate UPLC conditions to successfully elute all species in under 10 minutes at a flow rate of 0.2 mL/min, which corresponds to a total flow of 2 mL. We then increased the flow rate incrementally while maintaining a constant gradient slope. By scaling the overall analysis time so that the gradient was completed within the same pumped volume, we achieved a constant gradient slope per column volume. As shown in Figure 1, the ACQUITY UPLC coupled with an ACQUITY UPLC OST Column offers exceptional resolution at all flow rates tested.

EXPERIMENTAL

Samples

OST MassPREP™ Standard was dissolved in 100 mM TEAA. RNAi and DNA samples (IDT Technologies) were prepared by partial digestion of purified samples with phosphodiesterase II (Sigma).

LC conditions

LC system:	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 to 0.8 mL/min
Mobile phase A:	100 mM hexylammonium acetate, pH 7.0
Mobile phase B:	Acetonitrile
Gradient:	28 to 39 %B
Detection:	PDA UV 260 nm

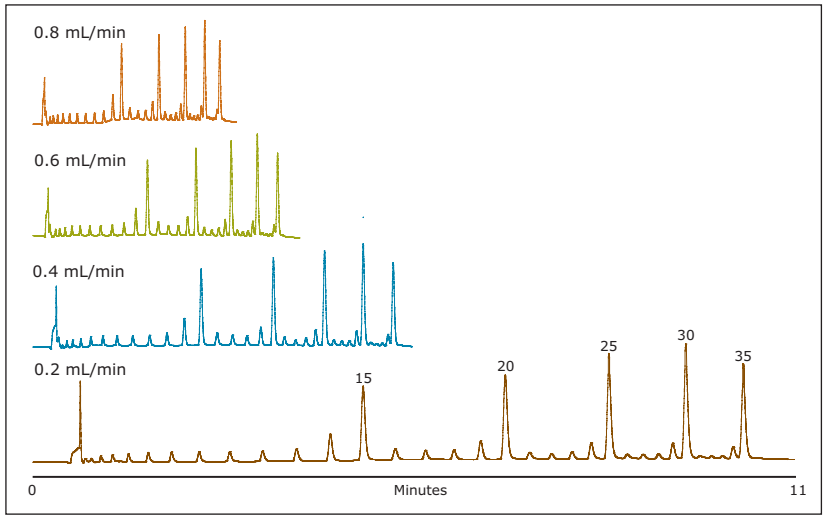

Figure 1. UPLC chromatograms for the separation of deoxythimidine sequences (OST MassPREP Standard) at various flow rates with constant gradient slope.

Additionally, system pressure increased significantly as the flow rate increased, ranging from 3200 psi at 0.2 mL/min to 12,000 at 0.8 mL/min. These pressures far exceed those possible with conventional HPLC but are accomplished routinely using the ACQUITY UPLC System.

To determine the reproducibility of retention time as a function of gradient slope, we determined the volume of eluent necessary to elute each oligonucleotide at the flow rates tested. We found exceptional reproducibility of delivered volume to oligonucleotide elution as shown in Figure 3. This highlights the selectivity of our system for oligonucleotides, regardless of flow rate.

We tested our method with a more challenging sample, a heteromolecular DNA sequence, Figure 4. Our rapid separation method provided regular retention based on oligo length with impressive resolution from failure sequences.

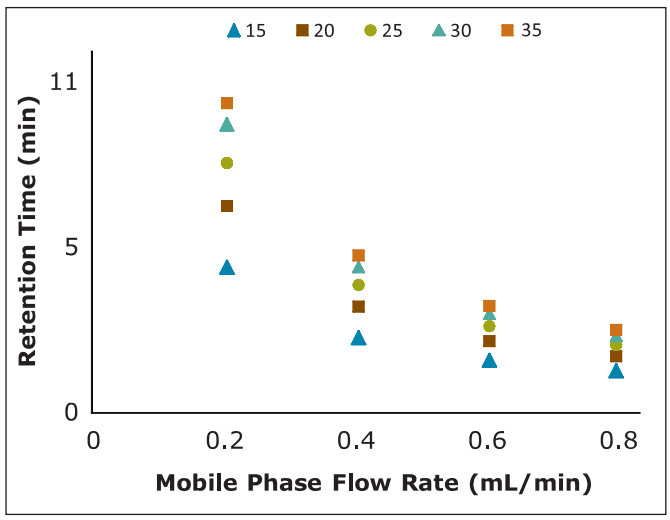

Figure 2. Comparison of mobile phase flow rate and oligo retention time at various flow rates.

CONCLUSION

The method presented in highlights the utility of the ACQUITY UPLC System when used with an ACQUITY UPLC BEH Column. The combined system provides accurate, reproducible, and rapid separation of oligonucleotides. The method also illustrates that analysis times can be dramatically reduced by scaling both the gradient slope and flow rate proportionately, thus yielding constant gradient slope per volume of mobile phase. This is accomplished by using sub-2-micron particles and the high pressure capabilities of the ACQUITY UPLC. The method provides accurate and predictable retention of both homo- and hetero-molecular oligonucleotides.

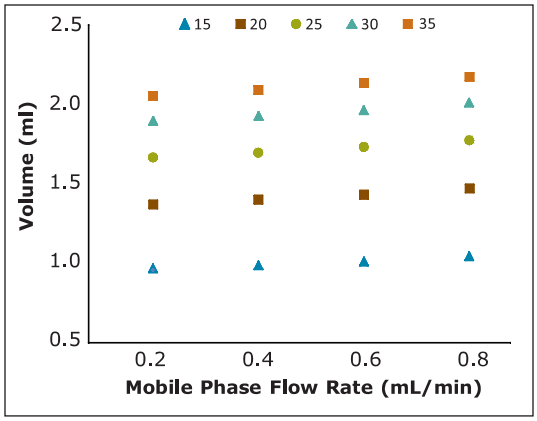

Figure 3. Comparison of mobile phase volume pumped at various flow rates for the retention of various oligonucleotide sequences.

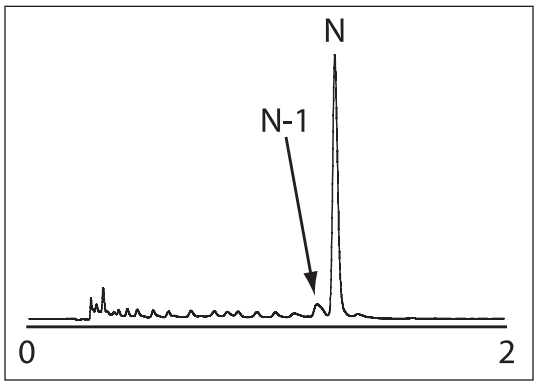

Figure 4. UPLC separation of RNA (top trace) and DNA (bottom trace) in under 2 minutes. N-x sequences are 5' truncated.

Reference

1. UPLC-MS Separation of Oligonucleotides in Less than Five Minutes: Method Development. Waters Application Note. 2007; 720002387en.

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[TECHNOLOGY BRIEF]

Successful Analysis of siRNA Using the ACQUITY UPLC H-Class Bio System

GOAL

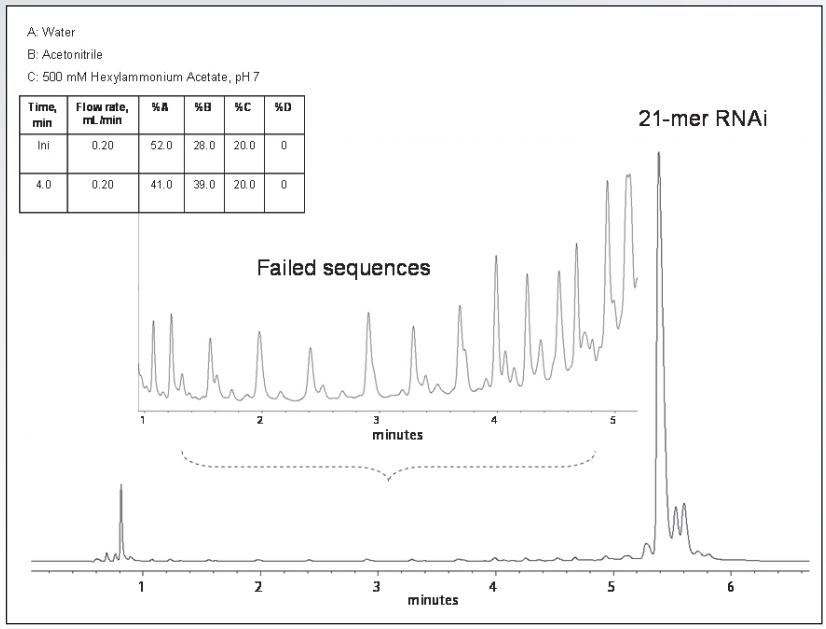
To separate a heterogeneous mixture of a siRNA oligonucleotide and its failed sequences using the ACQUITY UPLC® H-Class Bio System with Auto•Blend Plus™ Technology.

BACKGROUND

Synthetic oligonucleotides are now widely applied in molecular biology, clinical diagnosis, and the development of new therapeutic agents. RNAi therapeutics have attracted significant attention since the discovery of the RNA interference mechanism, prompting the development of analytical methods for short RNA oligonucleotides, typically 21 nucleotides (nt) in length.

RNA oligonucleotides are produced by a step-wise solid-phase synthesis. The product released from the resin contains the desired sequence contaminated with truncated sequences and synthesis-related impurities. These contaminants need to be quantitated and characterized to ensure drug safety and efficacy.

The ACQUITY UPLC H-Class Bio System is used to efficiently separate the targeted 21 nt siRNA from multiple by-products of the synthesis.



UPLC separation of 21 nt siRNA targeted product from its failed sequences serves as control of oligonucleotides synthetic quality. The Auto•Blend function of the ACQUITY UPLC H-Class Bio System allows an automatic mixing of three solvents resulting in fast and accurate analysis procedure.

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Ion-pair reversed-phase UPLC is an efficient method for characterizing the full range of impurities in synthetic oligonucleotides. Several ion-pair reagents are available for the separation of siRNA, including some that are suitable for LC-MS applications and for non-denaturing conditions that preserve siRNA duplexes. In addition, modified oligonucleotides may require special mobile phase compositions. Optimization of the ion-pairing system for a particular sample can require the preparation of multiple formulations of mobile phases for testing. This tedious, iterative process can be simplified by using an instrument that accurately and precisely blends mobile phase mixtures from pure solvents and concentrated stocks as required by the experiments. The four-solvent blending feature of the ACQUITY UPLC H-Class Bio System was developed for this purpose.

THE SOLUTION

Binary gradients are commonly used for the separations of synthetic oligonucleotides. With the Auto•Blend function of the ACQUITY UPLC H-Class Bio System, a ternary gradient was used for the analysis of a 21 nt siRNA sample. Solvent C was chosen to contain the ion-pairing reagent, hexylammonium acetate, pH 7, at 500 mM concentration. Solvent A and solvent B are 100% water and 100% acetonitrile, respectively. In this way, mobile phase preparation is straight forward with reduced risk of mixing errors. The concentration of the ion-pairing modifier was kept constant at 20% throughout the separation while applying 28% to 39% gradient of the organic component. The siRNA sample was separated on a UPLC® Oligonucleotide Separation Technology (OST) C_{18} column followed by UV detection on a tunable UV detector (TUV).

With UPLC, the undesirable impurities are successfully resolved from the targeted 21 nt siRNA sequence. The main peak is a full-length product, while the small peaks are failed sequences. These multiple contaminants are efficiently resolved within 6 minutes of separation. Several of the oligonucleotides detected eluting past the target peak have longer sequences than the full-length RNA product. These are longer oligonucleotides with nucleotides added during a step-wise synthesis, or the full-length product with incompletely removed protection groups.

SUMMARY

We have demonstrated the ability of an ACQUITY UPLC H-Class Bio System to separate the targeted 21 nt siRNA from multiple by-products of the synthesis in 6 minutes. Auto•Blend Plus Technology provides flexibility for the method development because the manipulation of solvents composition is simplified. This highly efficient and cost-effective separation satisfies regulatory requirements and is expected to provide more robust and reliable characterization of the oligonucleotides.

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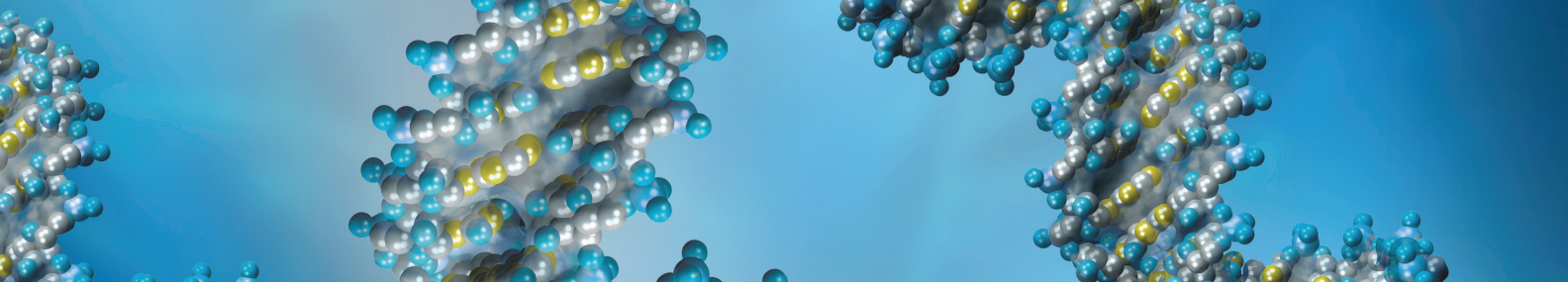

[UPLC-MS]

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RNAi Duplex Analysis and Purification

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

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, offtarget 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

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 MW water to yield concentrations of approximately 2 nmol/µL.

An aliquot of one set of complementary strands was purified as ssRNA1 and one strand was partially digested with the exonuclease phosphodiesterase II to generate a 5' truncated ladder or RNAi.

Analytical LC conditions

LC system:	ACQUITY UPLC
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:	SQ Detector
Mode:	ESCapillary: 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

Purification LC conditions

LC system:	Alliance® Bio HPLC
Column:	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

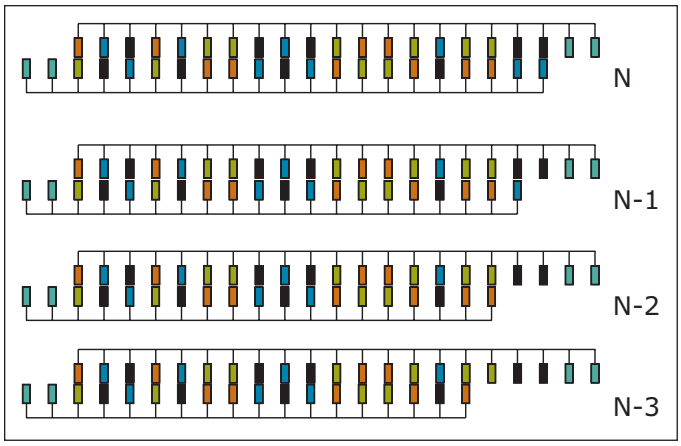
Fraction analysis by UPLC

LC system:	ACQUITY UPLC
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

RESULTS AND DISCUSSION

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.

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 singlestranded 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.

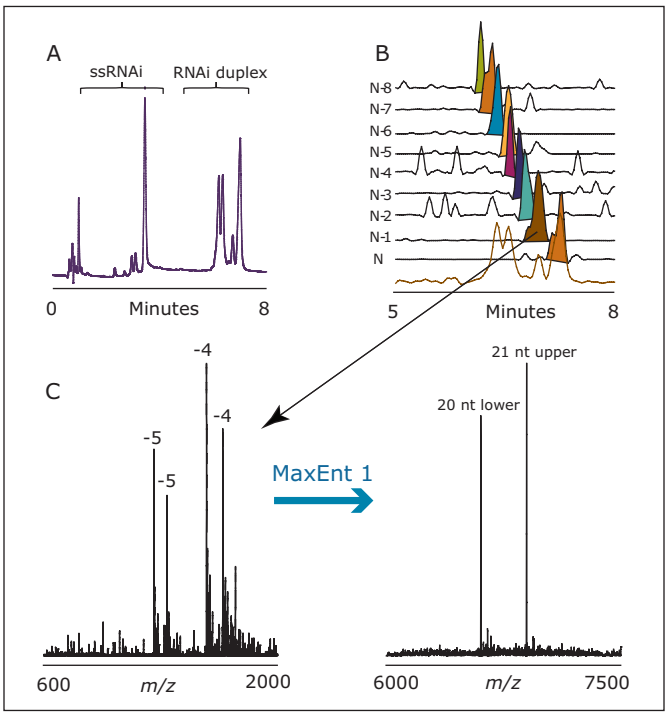


Figure 1. UPLC analysis of RNAi duplex mixture.

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.

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 semipreparative 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.

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 thepossibility 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 singlestranded 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.

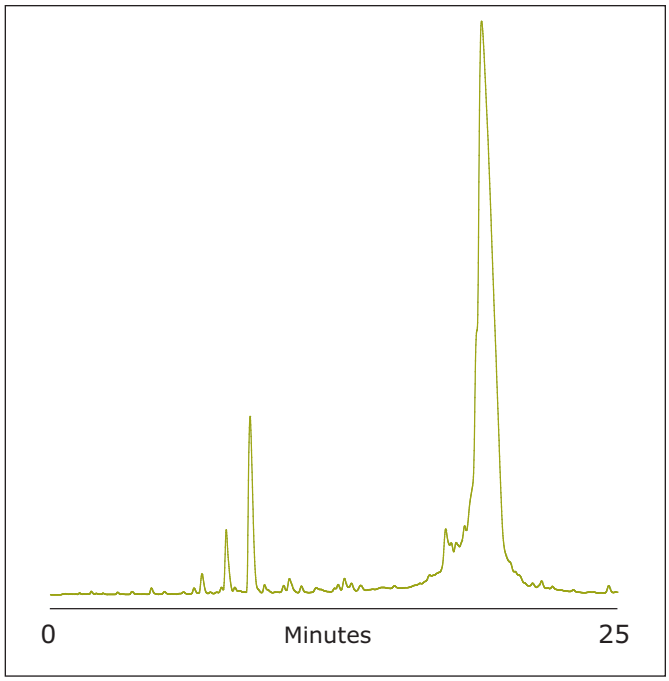


Figure 2. HPLC purification of RNAi prepared by separate annealing of complementary single stranded RNA.

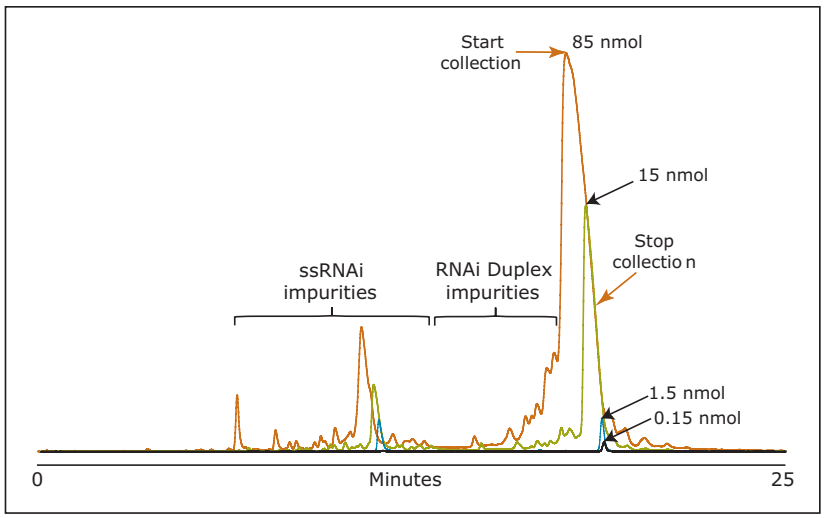


Figure 3. HPLC traces for the purification of RNAi duplexes generated by oncolumn annealing of ssRNA injected sequentially. Collection window indicated for 85 nmol injection only were generally collected from peak apex to 30% of the peak height.

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%.

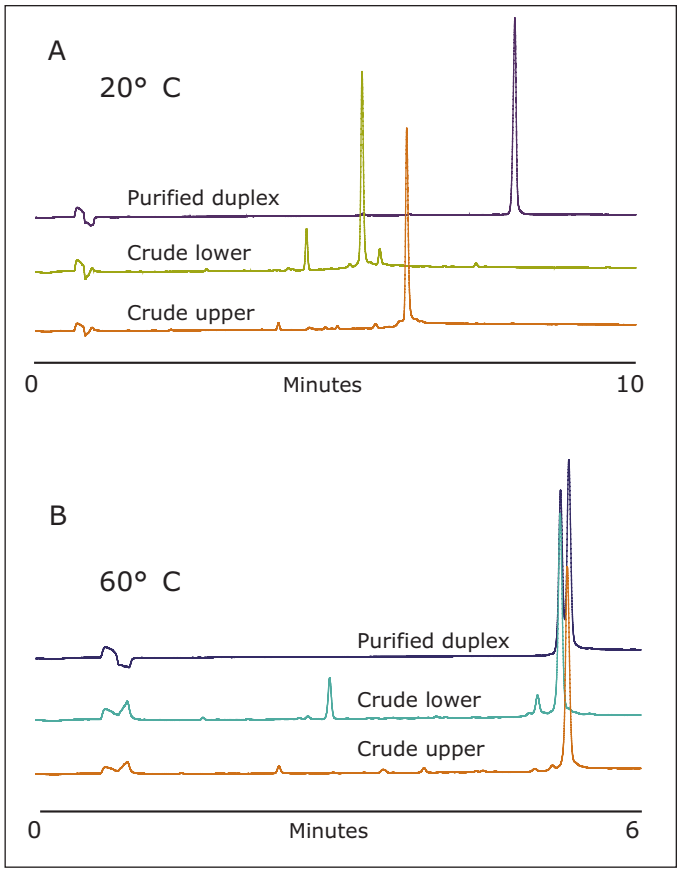


Figure 4. UPLC verification of RNAi duplex purity and composition.
Panel A: Analysis of single stranded and duplex RNAi at 20 °C.
Panel B: Analysis of single stranded and duplex RNA at 60 °C, which denatures the duplex.

CONCLUSIONS

In this application note, we have described a novel method for analysis and purification of double stranded siRNA. Using the Alliance Bio HPLC and ACQUITY UPLC systems with ACQUITY UPLC and XBridge OST columns, we were able to efficiently resolve fulllength 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.

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Adding Mass Detection to Synthetic Oligonucleotide Analyses with the ACQUITY QDa Detector

Robert E. Birdsall and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Addition of mass data to synthetic oligonucleotide assays using MassLynx® enabled with MaxEnt™ deconvolution algorithm
- Complementary mass information using traditional IP-RPLC mobile phases
- Increased productivity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

ACQUITY UPLC BEH Column

MassLynx SCN 9.25 with MaxEnt1

KEY WORDS

Oligonucleotide, mass detection, H-Class, ACQUITY, QDa

INTRODUCTION

The Waters® ACQUITY QDa Detector has been established as a cost-effective means for obtaining mass spectral data within existing optically-based LC workflows in the biopharmaceutical manufacturing environment when used as an orthogonal detection technique.[1-3] Research into therapeutic oligonucleotides has received steadily increasing attention from the pharmaceutical industry due to potential applications using deoxyribonucleic acid (DNA) sense/antisense oligonucleotides and interfering ribonucleic acid- (iRNA) based therapies.[4-5] IP-RPLC has become a prevalent technique in the analysis of synthetic oligonucleotides in part due to the selectivity offered by such techniques as well as its ability to incorporate MS-friendly reagents and buffers as first demonstrated by Apffel and colleagues.[6-7] Mass information afforded by MS detection offers an efficient means of identifying challenging base modifications for improved productivity in synthetic therapeutic oligonucleotide workflows. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability as an orthogonal detection technique in the analysis of synthetic oligonucleotides.

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution for detecting oligonucleotides across a wide molecular weight range and can be readily integrated into existing UV-based workflows. A set of polyT standards ranging from 15 nt to 35 nt in length were used in this study to evaluate the accuracy and compatibility of the ACQUITY QDa Detector in oligonucleotide analyses using traditional IP-RPLC mobile phases comprised of triethylamine (TEA) and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP).

EXPERIMENTAL

Triethylamine (99.5% purity) and 1,1,1,3,3,3-Hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. LC-UV grade solvents (Optima series) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were purchased from Waters (p/n 186004135) and prepared at a concentration of 10 pmol/uL. Mass loads on column were kept constant at 50 pmol or 5 µL injections.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC Oligonucleotide BEH C_{18} Column, 130Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186003949)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

*mobile phases prepared gravimetrically

Gradient:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	81.0	19.0	0	0
15.00	0.200	73.5	26.5	0	0
16.00	0.200	50.0	50.0	0	0
17.00	0.200	81.0	19.0	0	0
21.00	0.200	81.0	19.0	0	0

QDa Detector settings

Sample rate:	2 points/sec
Mass range:	410–1,250 Da
Mode:	negative
Collection mode:	continuum
Cone voltage:	20 V
Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt1

RESULTS AND DISCUSSION

IP-RPLC has become the prevalent separation technique in the analysis of oligonucleotides in part due to the high separation efficiency afforded by such methods when compared to alternative charge-based separations.[5] The use of MS-friendly buffers such as TEA/HFIP – as first demonstrated by Apffel and colleagues – provides an efficient means in the identification of challenging base modifications of oligonucleotides based on orthogonal mass spectrometry data.[6-7] To demonstrate that the ACQUITY QDa is compatible with such IP-RPLC methods, a separation of five polyT standards of increasing length were separated using a TEA/HFIP buffer. As shown in Figure 1, the optical chromatogram (TUV) and response from the mass detector (QDa) – which was in a serial configuration post optical detector – show a high degree of correlation. From this data, it is evident that the ACQUITY QDa Detector is capable of providing mass spectral data using traditional IP-RPLC methods that incorporate ion pairing agents such as TEA buffered in HFIP. As shown in Figure 1, the intensity profile of the ACQUITY QDa Detector is similar but not identical to the UV profile. Interrogation of the raw MS spectrum was performed to gain insight into intensity profile differences.

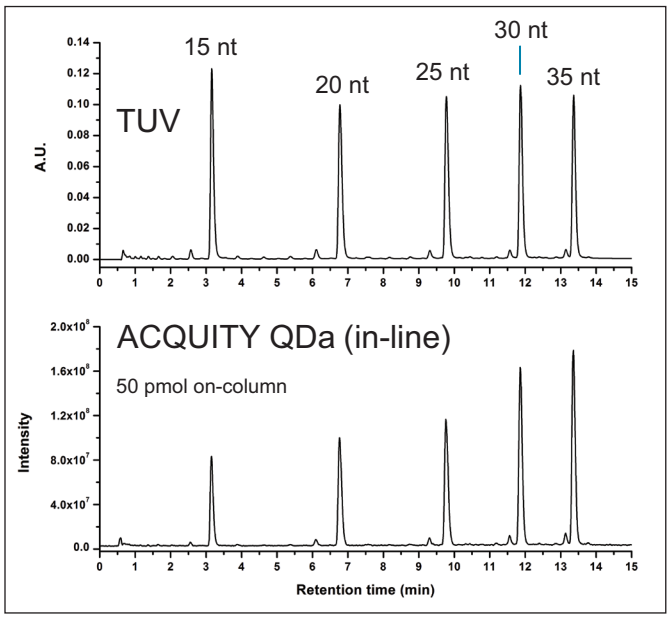

Figure 1. Oligonucleotide analysis with QDa. An IP-RPLC separation of five polyT standards with the ACQUITY QDa in a serial configuration post UV detection.

Table 1 shows that multiple charge states are observed (green highlight) for each of the oligonucleotide standards. Closer inspection of the 35 nt standard indicates at least nine charge states were observed. The shorter sequences, which had greater disparity in intensities, were observed with fewer charge states, indicating potential lower charge states may not be observed as they are outside the detector scan range (>1250 *m/z*). This phenomena does not affect mass data for therapeutic oligonucleotides, as even the shortest standard (nt 15) had five charge states observed.

green = observable charge state

nt	Avg. MW	$[M-4H]^{-4}$	$[M-5H]^{-5}$	$[M-6H]^{-6}$	$[M-7H]^{-7}$	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$	$[M-16H]^{-16}$	$[M-17H]^{-17}$
15	4,500.9	1124.2	899.2	749.1	642.0	561.6	499.1	449.1	408.2	374.1	345.2	320.5	299.1	280.3	263.8
20	6,021.9	1504.5	1203.4	1002.6	859.3	751.7	668.1	601.2	546.4	500.8	462.2	429.1	400.5	375.4	353.2
25	7,542.9	1884.7	1507.6	1256.1	1076.5	941.9	837.1	753.3	684.7	627.6	579.2	537.8	501.9	470.4	442.7
30	9,063.8	2265.0	1811.8	1509.6	1293.8	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2	565.5	532.2
35	10,584.8	2645.2	2116.0	1763.1	1511.1	1322.1	1175.1	1057.5	961.2	881.1	813.2	755.0	704.6	660.5	621.6

Table 1. Oligonucleotide m/z table. A charge state table based on average molecular weight of the polyT standards was used to identify the observed charge states detected with the ACQUITY QDa. Green highlight indicates charge states observed for each standard.

As shown in Table 1, the number of charge states observed across the polyT standards gives rise to the question of instrument accuracy across such a diverse range. An assessment of charge state accuracy of the ACQUITY QDa Detector was thus performed using the observed charge states for the oligonucleotide standards. For this assessment, the difference between the observed and theoretical charge state *m/z* value was determined using the average molecular weight of each oligonucleotide. The results for a technical triplicate of the 30 nt standard are listed in Table 2 as a representative sample. It can be seen from Table 2 that the derived masses for the observed charge states are within the instrument specification of ± 0.2 Da with a high degree of method repeatability demonstrated by the low RSD of 0.02% or lower for each charge state. Similar results were obtained for observed charge states for the remaining standards (data not shown) demonstrating the ACQUITY QDa is capable of providing accurate mass information for oligonucleotide analyses.

30 nt, N=3	$[M-8H]^{-8}$	$[M-9H]^{-9}$	$[M-10H]^{-10}$	$[M-11H]^{-11}$	$[M-12H]^{-12}$	$[M-13H]^{-13}$	$[M-14H]^{-14}$	$[M-15H]^{-15}$
Expected	1132.0	1006.1	905.4	823.0	754.3	696.2	646.4	603.2
Observed	1132.1	1006.1	905.4	823.1	754.3	696.2	646.5	603.4
S.D.	0.07	0.12	0.04	0.00	0.04	0.04	0.14	0.08
%R.S.D.	0.01	0.01	0.00	0.00	0.01	0.01	0.02	0.01

Table 2. m/z accuracy evaluation. Expected charge states based on average molecular weight of the 30 nt standard were compared to the observed charge states and were within instrument specification of ± 0.2 Da.

The increased number of charge states observed with oligonucleotides as shown in Figure 2A result in mass spectrums that are not straightforward to interpret. Workflows that can deliver accurate mass information in an efficient manner are highly desirable in the analysis of therapeutic oligonucletoides. To this end, deconvolution algorithms such as MaxEnt1 can be incorporated to provide mass data of oligonucleotide spectra for improved productivity. To evaluate this functionality, a one minute window centered across the peak apex of the 30 nt standard was used to combine the MS spectrum data acquired by the QDa. Deconvolution was performed with a peak width of approximately 0.7 Da and a binning resolution of 0.5 Da. As shown in Figure 2B, the spectrum of the 30 nt standard was deconvoluted to a zero charge state mass of 9,064.5 Da, which was within 0.7 Da of the expected average molecular weight. A minor sodium (Na^+) adduct was also observed with a relative intensity of 6%. Trace salts are routinely encountered in LC-based separations because of their high affinity towards the phosphodiester backbone of oligonucleotides. Similar to the charge state evaluation, an assessment of the mass accuracy of the deconvolution algorithm for the complete set of standards was performed.

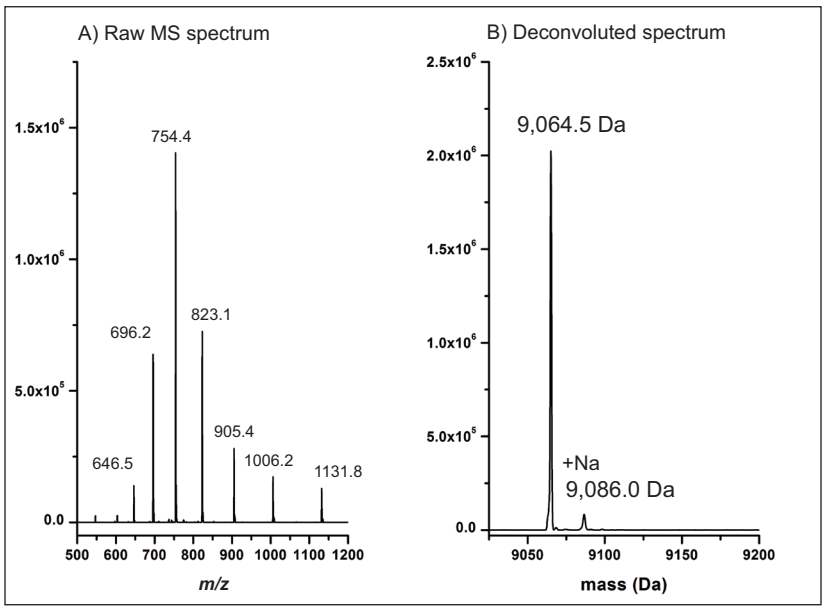

Figure 2. MaxEnt1 Deconvolution of MS spectrum. A) Multiple charge states associated with the raw spectrum of the 30 nt standard when acquiring continuum data using the ACQUITY QDa. B) Deconvolution of the raw MS spectrum of the 30 nt standard resulted in a parent peak mass of 9,064.5 Da (+0.7 Da) and a minor sodium (Na^+) adduct peak with a relative intensity less than 6%.

Using the same methodology as before, the difference between the observed and theoretical deconvoluted mass value was determined using the average molecular weight of each oligonucleotide. An identical number of scans and *m/z* range was used to combine MS spectrum for the polyT standards. Deconvolution parameters were kept constant for the standards with the results listed in Table 3. As shown in Table 3, the deconvolution results were observed to be highly reproducible across a technical triplicate with no deviation observed in the deconvoluted mass. Mass accuracy was observed from +0.0 Da to +0.7 Da across the polyT standards with increasing deviation associated with increasing sequence length. This is not entirely unexpected, as instrument error increases with higher charge states. In light of this, a mass accuracy of ±1.0 Dalton for oligonucleotides ranging from 15–35 nt demonstrates that the ACQUITY QDa is capable of providing adequate mass information in an efficient manner for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

N=3	nt 15	nt 20	nt 25	nt 30	nt 35
Expected	4500.9	6021.9	7542.9	9063.8	10584.8
Trial 1	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 2	4500.9	6022.5	7543.5	9064.5	10585.5
Trial 3	4500.9	6022.5	7543.5	9064.5	10585.5
Average	4500.9	6022.5	7543.5	9064.5	10585.5
Δ Mass (Da)	0.0	0.6	0.6	0.7	0.7

Table 3. Mass accuracy evaluation of MaxEnt1 deconvoluted mass. Expected mass based on average molecular weight of the polyT standards were compared to the average deconvoluted mass. Mass accuracy ranged from +0.0 Da to +0.7 Da across the set of standards.

CONCLUSIONS

Cost-effective techniques that add value and can be implemented into existing workflows with minimal effort are highly desirable in the pharmaceutical industry. The addition of complementary mass information in a single workflow afforded by the ACQUITY QDa Detector provides analysts an efficient means to improve productivity in routine assays. A natural extension of the ACQUITY QDa portfolio is to evaluate its applicability with biopharmaceuticals beyond that of mAbs. From this work it has been demonstrated that the ACQUITY QDa is compatible with IP-RPLC mobile phases and is able to detect and report mass information for oligonucleotides over a wide molecular weight range. Collectively, these results establish the ACQUITY QDa as an ideal addition to an analyst's lab for increased productivity and confidence of data analysis for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

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High-throughput Screening of Oligonucleotides for Identity and Purity Assessment Using the ACQUITY QDa Detector and ProMass for MassLynx

Robert E. Birdsall and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Addition of mass data to synthetic oligonucleotide assays using MassLynx® Software, enabled with MaxEnt™ deconvolution algorithm
- Increased productivity with automated high-throughput batch processing and analysis of synthetic oligonucleotides
- Readily interpretable results with interactive web-based format for increased confidence in data analysis

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultra-Violet (TUV) Detector

Waters® BEH Column

MassLynx Software with MaxEnt1

KEY WORDS

oligonucleotide, batch processing, mass detection, H-Class, ACQUITY, QDa

INTRODUCTION

Research into therapeutic oligonucleotides has received steadily increasing attention from the pharmaceutical industry. This is due to potential applications using deoxyribonucleic acid (DNA) sense/antisense oligonucleotides and interfering ribonucleic acid- (RNAi) based therapies.[1,2] The production of oligonucleotides with high yields via automated stepwise synthetic methods is well established. As part of the production process, purification and desalting steps are incorporated to remove byproducts of synthesis, such as failed sequences and production impurities. Characterization of purified synthetic products must be carried out prior to use in therapeutic applications to ensure product identity and purity. Ion Pairing Reversed Phase Liquid Chromatography (IP-RPLC) has become a prevalent technique in the analysis of synthetic oligonucleotides in part due to the selectivity offered by such techniques, as well as its ability to incorporate mass spectrometry-friendly reagents and buffers as first demonstrated by Apffel and colleagues.[3,4]

Mass information afforded by MS detection offers an efficient means of identifying challenging base modifications for improved productivity in synthetic therapeutic oligonucleotide workflows. Pharmaceutical companies engaged in oligonucleotide research are often investigating numerous potential biotherapeutic candidates, which can negatively impact productivity as the characterization process of synthetic oligonucleotides often requires manual processing of LC-MS data. Previous work demonstrated that incorporation of ProMass (Novatia, LLC) for MassLynx Software enables automated spectrum deconvolution and data analysis for high-throughput screening of UPLC-MS data generated on Waters MS instruments, such as the ACQUITY SQD Detector and SYNAPT®.[5] As one of the newest mass detectors from Waters, the ACQUITY QDa has been established as an efficient means for obtaining mass information within existing, optically-based LC workflows in the biopharmaceutical manufacturing environment when used as an orthogonal detection technique.[6-8]

The objective of this application note is to demonstrate that the ACQUITY QDa Detector provides a simple and cost-effective solution in the assessment of identity and purity of synthetic oligonucleotides and that mass spectral data acquired can be readily processed with ProMass for MassLynx Software in an automated fashion.

EXPERIMENTAL

Chemicals and reagents

Triethylamine (99.5% purity) and 1,1,1,3,3,3-hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. Mass spectrometry grade solvents (Optima series) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were from Waters (P/N 186004135). siRNA upper strand 5'-UCGUCAAGCGAUUACAAGGTT-3' and its complementary lower strand 5'-TTCCUUGUAAUCGCUUGACGA-3' were ordered from Integrated DNA Technologies. All samples were prepared at a concentration of 10 pmol/µL. Mass loads on column were kept constant at 50 pmol or 5 µL injections.

LC conditions

LC system:	ACQUITY UPLC H-Class
Detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC OST BEH C_{18}, 1.7 µm, 2.1 mm x 50 mm (P/N 186003949)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

**mobile phases prepared gravimetrically*

Gradient table: (polyT standards)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	81.0	19.0	0	0
15.00	0.200	73.5	26.5	0	0
16.00	0.200	50.0	50.0	0	0
17.00	0.200	81.0	19.0	0	0
21.00	0.200	81.0	19.0	0	0

Gradient table: (high-throughput screening)

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.200	82.0	18.0	0	0
4.00	0.200	80.0	20.0	0	0
4.01	0.200	50.0	50.0	0	0
6.00	0.200	50.0	50.0	0	0
6.01	0.200	82.0	18.0	0	0
10.00	0.200	82.0	18.0	0	0

Detector settings

Sample rate:	2 points/sec
Mass range:	410–1250 Da
Mode:	ESI negative
Collection mode:	continuum
Cone voltage:	20 V
Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt1

RESULTS AND DISCUSSION

Recently, it was demonstrated that the ACQUITY QDa Detector is capable of providing mass spectral data using traditional IP-RPLC methods, which incorporate ion pairing agents such as TEA buffered in HFIP, as shown in Figure 1A.[9] In the previous study, it was also shown that the ACQUITY QDa is capable of providing accurate mass information within its operating specifications. In addition, it was shown that MS spectra processed within MassLynx using the MaxEnt1 deconvolution algorithm provided analysts with straightforward data interpretation of complex spectra (Figure 1B).

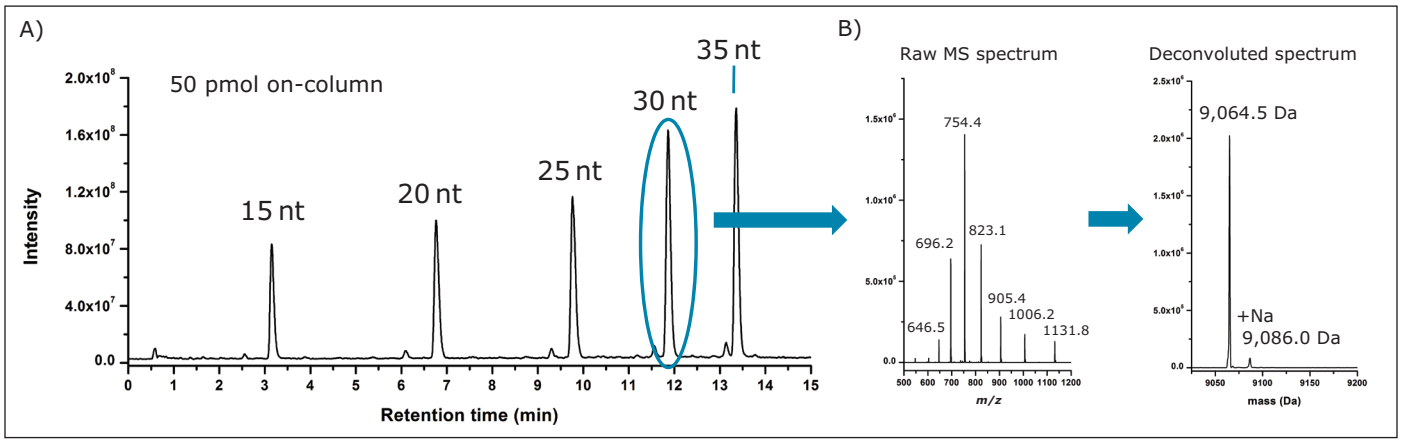


Figure 1. Oligonucleotide analysis with the ACQUITY QDa. A) An IP-RPLC separation of five polyT standards with the ACQUITY QDa in a serial configuration post-UV detection. B) Deconvolution of the raw MS spectrum of the 30 nt standard resulted in a parent peak mass of 9,064.5 Da (+0.7 Da) and a minor sodium (Na^+) adduct peak with a relative intensity less than 6%.

As aforementioned, pharmaceutical companies engaged in oligonucleotide research often incorporate software such as ProMass that enable automated data analysis in a high-throughput manner for improved productivity. A natural extension of the previous study is to evaluate the compatibility of ProMass with average mass data acquired using the ACQUITY QDa Detector. As with the previous work, a one minute window centered across the peak apex of the polyT standards was used to combine the MS spectrum data acquired by the ACQUITY QDa, as shown in the left panel of Figure 2. ProMass, which uses the ZNova deconvolution algorithm, offers a panel of deconvolution parameters for method flexibility in the deconvolution of components/peaks in the mass chromatograms, as shown in the right panel of Figure 2. For this experiment, the default settings used were: Peak Width=3, Merge Width=0.2, Minimum Score=2, and Normalize Scores=1. Spectrum for each polyT standard was copied to the operating system clipboard and processed using the manual processing feature of ProMass, as illustrated in Figure 2.

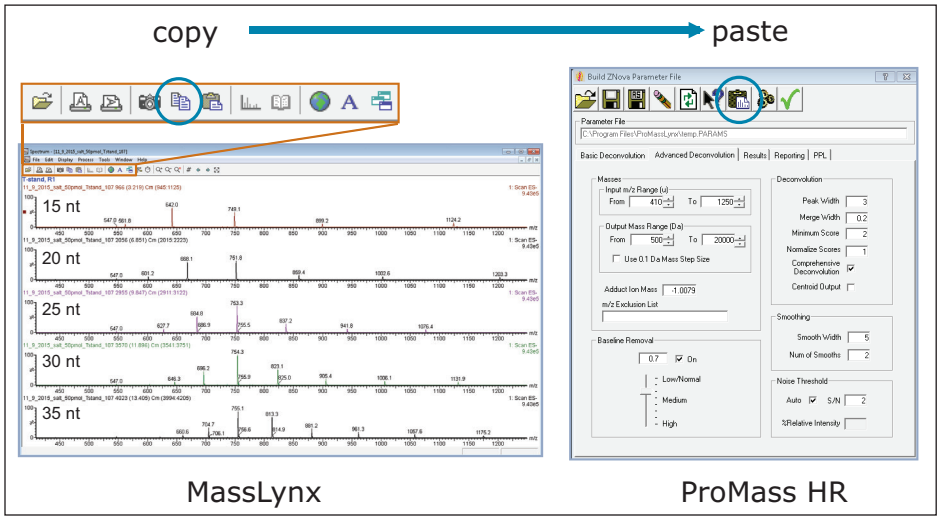
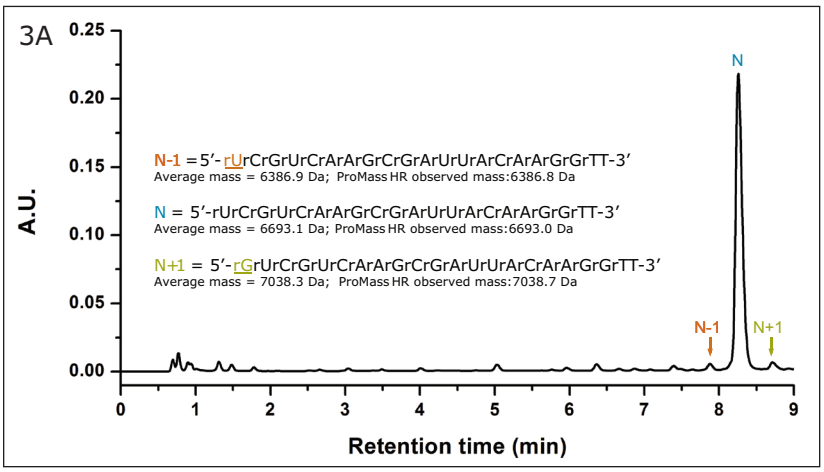


Figure 2. Data compatibility with ProMass HR and ProMass HR compatibility with the ACQUITY QDa. A one minute window of combined spectra acquired with the ACQUITY QDa data was evaluated with the manual processing feature (copy/paste) of ProMass HR using the default deconvolution settings.

N=3	15 nt	20 nt	25 nt	30 nt	35 nt
Expected	4500.9	6021.9	7542.9	9063.8	10584.8
Observed average	4500.8	6022.0	7543.3	9063.8	10585.3
Δ mass	-0.1	0.1	0.4	0.0	0.5

Table 1. ProMass HR deconvolution mass accuracy. Average mass accuracy was observed from -0.1 Da to +0.5 Da for a technical triplicate of the polyT standards based on nominal mass-to-charge ratio data acquired with the ACQUITY QDa Detector.

The deconvolution results were observed to be highly reproducible across a technical triplicate with mass accuracy observed between -0.1 Da to +0.5 Da across the polyT standards (Table 1). To further evaluate the mass accuracy of ProMass with a sample more representative of one found in a therapeutic setting, a siRNA sample with a sequence length of 21 nucleotides – exhibiting a double thymine overhang – was separated using a high resolution separation gradient (Figure 3A). The pure oligonucleotide (N) was resolved from the base deletion (N-1) and base insertion (N+1) forms in under ten minutes. Using a one minute integration window from 7.7 minutes to 8.8 minutes, ProMass successfully identified the deletion and insertion as shown in the sequence ladder summary report (Figure 3B). Similar to the polyT analysis, spectrum acquired with the ACQUITY QDa was combined from 7.5 to 9.5 minutes for deconvolution. Mass accuracy of the associated spectrum was observed to range between +0.1 Da to +0.4 Da. This demonstrates that ProMass is compatible with mass spectral data acquired with the ACQUITY QDa and is capable of providing adequate mass information for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.



3B

Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Abundance (in Spectrum)	%Purity (Estimate)	Identity	Result Code
8.25	6693.1	6693.2	0.1 Da (0.001 %)	2.20E+008	97.69	96.07	Target Mass	

Sequence Ladder Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
7.88	6386.9	6385.9	-1.0 Da (-0.016 %)	3.57E+006	6693.1 (Minus rU)
8.25	6693.1	6693.2	0.1 Da (0.001 %)	2.20E+008	rU1-T21
8.71	7038.3	7037.9	-0.4 Da (-0.006 %)	2.89E+006	6693.1 (Plus rG)

Chromatogram Summary

RT (min)	Base Peak Mass (Da)	Intensity	Spectral Quality	LC/MS Peak Area	LC/MS Area Percent
7.88	6385.9	3.57E+006	ok	2.87E+005	1.58
8.25	6693.2	2.20E+008	ok	1.75E+007	96.07
8.71	7037.9	2.89E+006	ok	4.29E+005	2.36

Figure 3. High-resolution siRNA chromatogram. A) A siRNA sequence 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang was separated from the base deletion (N-1) and base insertion (N+1) forms using a ten minute, high resolution separation gradient from 13% B to 21% B and B) analyzed using ProMass.

With the performance confirmed of ProMass utilizing mass spectral data acquired with the ACQUITY QDa, the ability to process data in a high-throughput manner was then evaluated. The software package provides a formatted MassLynx sample list, which is designed to allow UPLC-MS data to be processed immediately after data acquisition while the next sample in the queue is analyzed or batch processed offline post-acquisition in an automated fashion (Figure 4). The user provides the raw data, defines the target sequence, and provides details about the target product search (Target Info) – correct configuration of which is essential for successful data processing. Two columns for defining parameter files required by ProMass for processing the LC-MS chromatograms are also provided in the sample list format and are defined by the analyst as shown in Figure 4. The ProMassBridge Parameter File, which is part of the ProMassBridge application, facilitates a connection between MassLynx raw files and ProMass, and contains the user-defined parameters (e.g. retention time range, smoothing and subtracting settings) for integration of the MS chromatograms. The ProMass ZNova Parameter File contains user-definable settings for MS data analysis such as the input and output masses, ion polarity, deconvolution settings, target mass, impurities tolerance thresholds, and options for the data report.

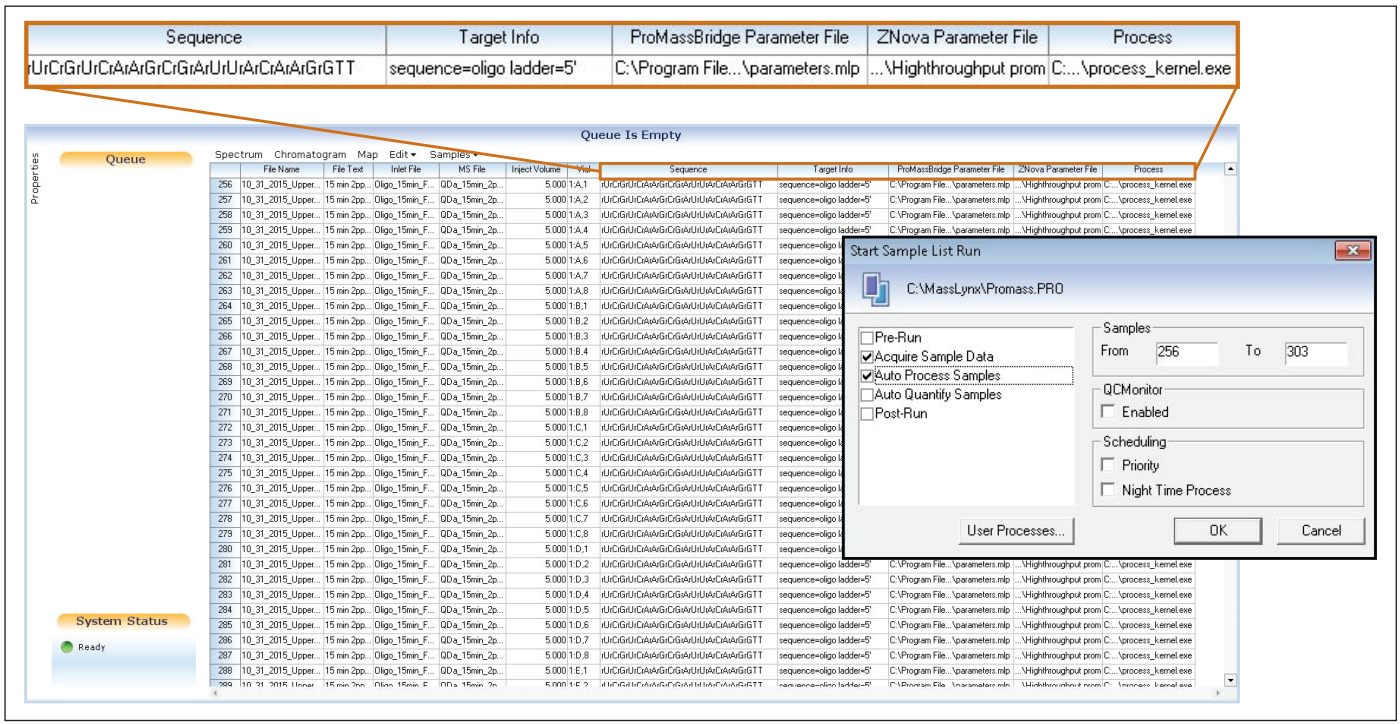


Figure 4. MassLynx sample list format. A unique, formatted MassLynx sample list designed for UPLC-MS data to be processed following data acquisition or batch processed offline post-acquisition in an automated fashion.

The siRNA sample previously used (upper strand) and its complementary sequence (lower strand) were used to evaluate the batch processing capability of ProMass. A ten minute high-throughput separation method was used for the analysis (Figure 5). The ProMassBridge Parameter File was configured to integrate MS chromatograms between 2.0 and 3.5 minutes with lockspray functionality disabled (this function is used for HRMS data; e.g., QToF MS). All other parameters were left at their default settings. For high-throughput analysis mode, ProMass software has the ability to display the data summary in an interactive sample plate format or Excel sheet.

To test the interactive sample plate format display option, upper siRNA samples were loaded in the standard 48-well plate format with every 7th vial position loaded with the lower siRNA complementary sequence as a negative control. As shown in Figure 4, samples from line 256 to line 303 were selected for batch processing with the upper siRNA sequence targeted for identification. After batch processing, the data is displayed in a color-coded, internet browser-based format (Figure 6). The colors, which represent the result of the targeted mass search, are defined in the legend of Figure 6. The plate format display is interactive; the analysis results can be displayed by selecting the actual vial position. Information such as a summary report of the analysis with target peak identification, spectral abundance, and peak purity can be accessed in this manner along with the corresponding spectrum and chromatogram. Additional information includes deconvolution results, identified impurities, and details of associated spectra.

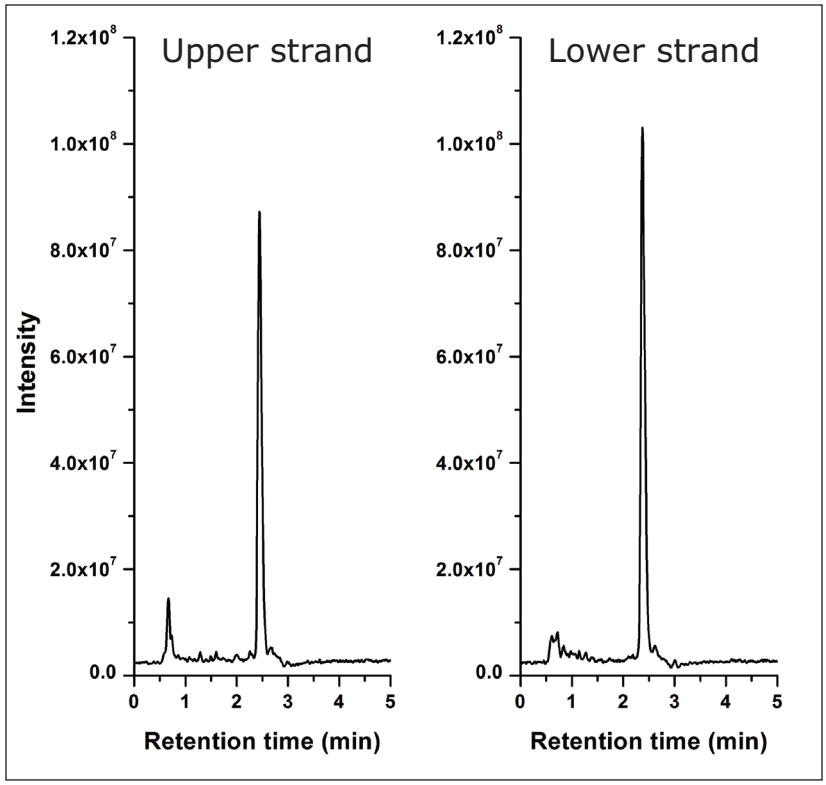


Figure 5. siRNA chromatogram. A siRNA sequence 5'-UCGUCAAGCGAUUACAAGGTT-3' (upper strand) with a double thymine overhang and its complementary sequence 5'-TTCCUUGUAAUCGCUUGACGA-3' (lower strand) were separated using a ten minute high-throughput screening gradient.

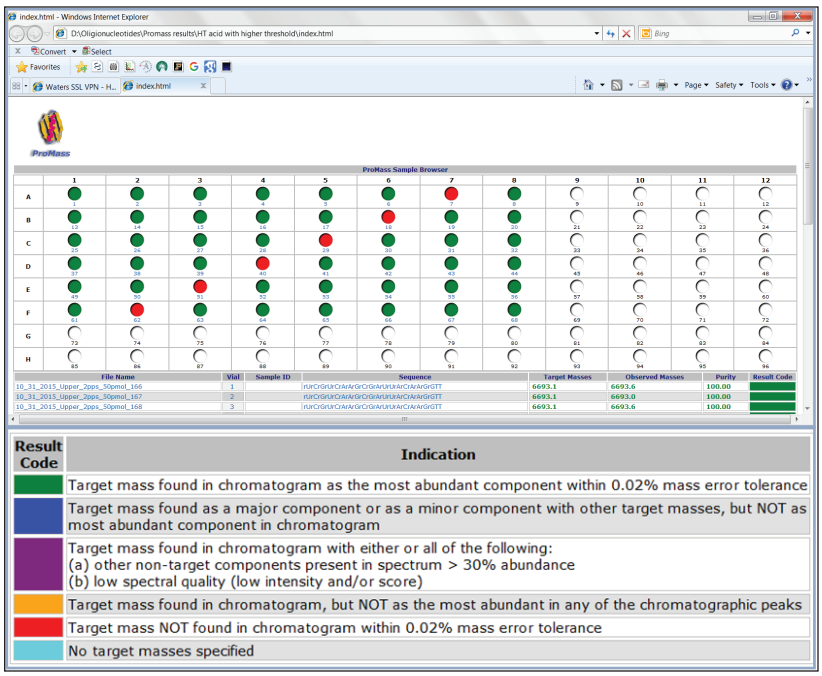


Figure 6. Interactive data summary. ProMass HR provides the ability to display data results in a color-coded, internet browser-based format for efficient analysis review.

CONCLUSION

Mass information afforded by MS detection offers an efficient means of identifying challenging base modifications for improved productivity in synthetic therapeutic oligonucleotide workflows. Methods that can be rapidly deployed and automated in the assessment of product identity and purity in the production of synthetic oligonucleotides are highly desirable. In this study it was demonstrated the ProMass software can be adopted into an ACQUITY QDa based MassLynx project for confirmation of analyte mass and yield results consistent with the molecular weight of the target sequence within the default mass tolerance settings.

Through the use of ProMass, assignment of target peaks, base modifications, and process impurities can be performed in a high-throughput automated fashion with significantly reduced analysis time for improved productivity. The interactive color-coded results in web browser-based format are user-friendly and enable straightforward data interpretation. This work demonstrates the ACQUITY QDa is an ideal addition to an analyst's lab for increased productivity and confidence of data analysis for routine identification and purity assessments in the manufacturing process of synthetic oligonucleotides.

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Evaluation of Alternative Ion-pairing Reagents in the Analysis of Oligonucleotides with the ACQUITY QDa Detector

Robert E. Birdsall and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Method compatibility with alternative ion-pairing reagents
- Minimizing costs through low volume use of IP-RPLC reagents
- Increased productivity through the use of on-line orthogonal detection techniques

WATERS SOLUTIONS

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class System

ACQUITY UPLC Tunable Ultraviolet (TUV) Detector

Waters® Oligonucleotide Separation Technology (OST)

MassLynx® Mass Spectrometry Software, SCN 9.25

KEYWORDS

Oligonucleotide, mass detection, H-Class, ACQUITY, QDa

INTRODUCTION

Electrospray ionization- (ESI) based analyses of oligonucleotides are often performed with buffers comprised of triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). This is in part due to the increased mass spectrometry (MS) sensitivity afforded by the HFIP buffering reagent as demonstrated by Apffel and colleagues.[1,2] The benefit of mass information in identifying challenging base modifications is well established.[3] However, as oligonucleotides with novel base modifications come to market with varied physicochemical properties, achieving optimal chromatography conditions may require alternative ion-pairing reagents other than TEA, which can be buffered with HFIP. Recently, it was demonstrated that amines other than TEA could be used with HFIP and produce adequate MS response using an IP-RPLC/MS-based technique.[4] As shown in previous work using the ACQUITY QDa Mass Detector, mass information afforded by MS detection offers improved productivity in synthetic therapeutic oligonucleotide workflows.[5]

A natural extension of this work is to evaluate the applicability of alternative IP reagents in the analysis of oligonucleotides using the ACQUITY QDa. A set of polyT standards ranging from 15 nt to 35 nt in length as well as a ssRNA sequence (5'-UCGUCAAGCGAUUACAAGGTT-3') were used in this study to assess method repeatability, column longevity, and assay comparability. Triethylamine (TEA), butylamine (BA), and dibutylamine (DBA) were evaluated in this study as alternative IP reagents.

EXPERIMENTAL

Sample Description

Triethylamine (99.5% purity), butylamine (99.5% purity), dibutylamine (99.5% purity), and 1,1,1,3,3,3-hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. Optima series solvents were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. PolyT oligonucleotide standards were purchased from Waters (P/N 186004135) and prepared at a concentration of 10 pmol/µL. A ssRNA upper strand (5'-UCGUCAAGCGAUUACAAGGTT-3') was purchased from Integrated DNA Technologies. Mass loads on column were kept constant at 50 pmol.

LC conditions

LC system:	ACQUITY UPLC H-Class
LC detectors:	ACQUITY UPLC TUV w/Ti flow cell, ACQUITY QDa Detector
Absorption wavelength:	260 nm
Column:	ACQUITY UPLC Oligonucleotide BEH C_{18}, 1.7 µm, 2.1 mm x 50 mm (P/N 186003949)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	5 µL

Mobile phase

TEA:HFIP

Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

BA:HFIP

Mobile phase A:	15 mM BA, 50 mM HFIP prepared in H_2O, pH 9.0
Mobile phase B:	15 mM BA, 50 mM HFIP prepared in MeOH

DBA:HFIP

Mobile phase A:	15 mM BA, 25 mM HFIP prepared in H_2O, pH 9.5
Mobile phase B:	15 mM BA, 25 mM HFIP prepared in MeOH

Mobile phases prepared gravimetrically.

Detector conditions

Detector:	ACQUITY QDa
Sample rate:	2 points/sec
Mass range:	410–1250 Da
Mode:	Negative
Collection mode:	Continuum
Cone voltage:	20 V
Capillary voltage:	0.8 kV
Probe temp.:	600 °C

Data management

MassLynx SCN 9.25 with MaxEnt™1

RESULTS AND DISCUSSION

Developing chromatographic methods is a process that involves decisions based on a myriad of factors including assay robustness, resolution, and selectivity. For many analysts, developing a method typically starts with a literature search to determine if there is precedence for the desired separation to establish a baseline for comparison. In the case of IP-RPLC/MS-based oligonucleotide analyses, buffers comprised of TEA:HFIP have become the "gold standard" for comparison due to its high separation efficiency and MS compatibility.[1,2] In this regard, a comparability test was initially performed to see if alternative amines could produce similar chromatographic profiles with adequate MS response as in the case of mobile phases comprised of TEA:HFIP.

Previous work has shown that a gradient of 0.5% B/min with a mobile phase comprised of 15 mM TEA and 400 mM HFIP is sufficient in separating a set of polyT standards ranging from 15 nt to 35 nt in length.[6] Using this separation as a baseline, a comparability study was performed using butylamine and dibutylamine as shown in Figure 1. Concentration of the alternative amines was held constant at 15 mM while the HFIP was adjusted for optimum MS response as previously published by Gong, et al.[4] In the case of butylamine and dibutylamine, the concentration of HFIP used was 50 mM and 25 mM, respectively.

As shown in Figure 1, comparable selectivity was achieved with the two alternative amines. Furthermore, butylamine was able to achieve similar selectivity as the TEA:HFIP with a gradient of 0.46% B/min, but with a lower starting organic composition; whereas dibutylamine required doubling the gradient to 0.80% B/min with similar starting organic composition as TEA:HFIP. These results suggest the concentration of HFIP and hydrophobicity of the amine impact oligonucleotide retention on the column. However, "tuning" of the separation conditions can be performed to achieve the desired separation, which in this case was to match the selectivity of the TEA:HFIP separation.

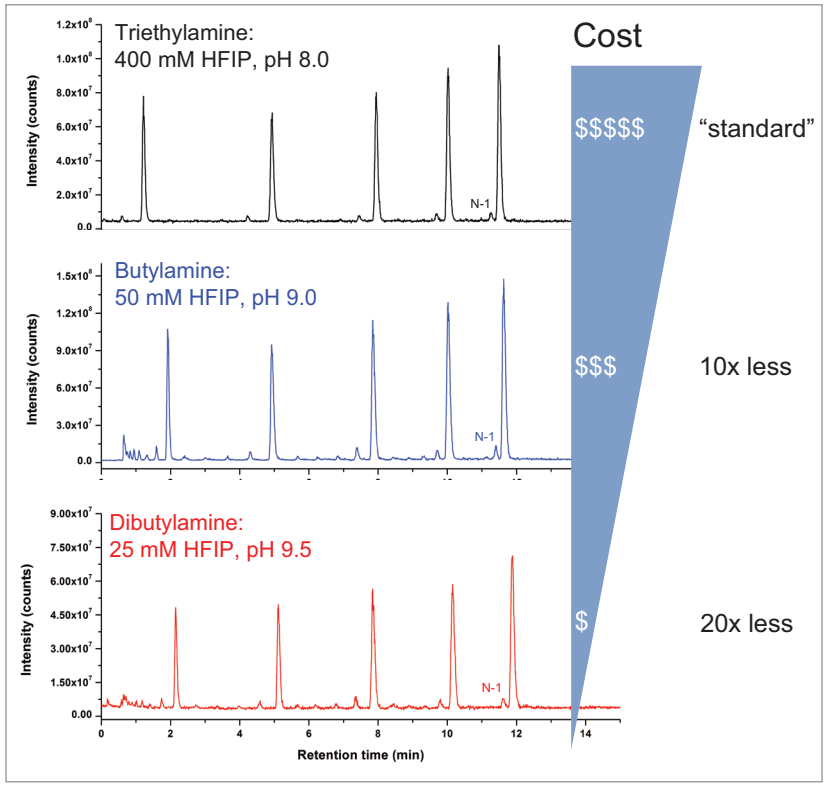

Figure 1. Evaluation of alternative IP reagents. ACQUITY QDa response for separation of the MassPREP™ OST polyT standard mixture using trimethylamine, butylamine, and dibutylamine buffered with 400 mM, 50 mM, and 25 mM HFIP, respectively.

Mass detector response was shown to be similar between TEA:HFIP and BA:HFIP, with BA:HFIP showing marginally better signal response. This was in contrast to the DBA:HFIP run, which showed approximately a 2-fold drop in signal intensity, despite having an identical mass load. The reduced signal intensity in the DBA:HFIP separation does not hinder detection of the failed sequence peak (N-1, Figure 1), indicating alternative amines such as butylamine and dibutylamine can produce adequate MS signal response for analysis when buffered with HFIP.

Interestingly, an unintended corollary with assay cost was observed during the comparability test. Cost-prohibitive MS-grade purity reagents such as HFIP are often cited as a concern in oligonucleotide assay development. Figure 1 demonstrates that IP reagents such as butylamine and dibutylamine can reduce assay costs through reduced HFIP use while maintaining assay selectivity when compared to TEA:HFIP, making them an appealing alternative in IP-RPLC/MS-based techniques. As indicated in Figure 1 though, lowering the concentration of HFIP results in a mobile phase with a pH >8.0, which can be a concern with respect to column longevity.

To test the impact of a modest increase in pH on column longevity, the mobile phase prepared with butylamine (15 mM BA:50 mM HFIP, pH 9.0) was selected for evaluation. For this study the gradient was extended to 30 minutes with a slope of 0.5% B/min. Mobile phases were prepared in 200 mL batches and refreshed every 24 hours over the course of the time study. To conserve samples, four water blanks were injected in succession using the method followed by an injection of the mixture of polyT standards. As shown in Figure 2, the PolyT standards were separated with a high degree of repeatability over 400 injections with nearly identical chromatographic profiles. Selectivity was calculated for the first four peaks relative to the last peak as a means to probe column robustness in the presence of elevated pH and temperature. Calculated values based on the experimental data were averaged over 24-hour time blocks and reported with corresponding error bars as shown in Figure 3. Selectivity values were determined to have less than 1% RSD across 400 injections over 200 hours.

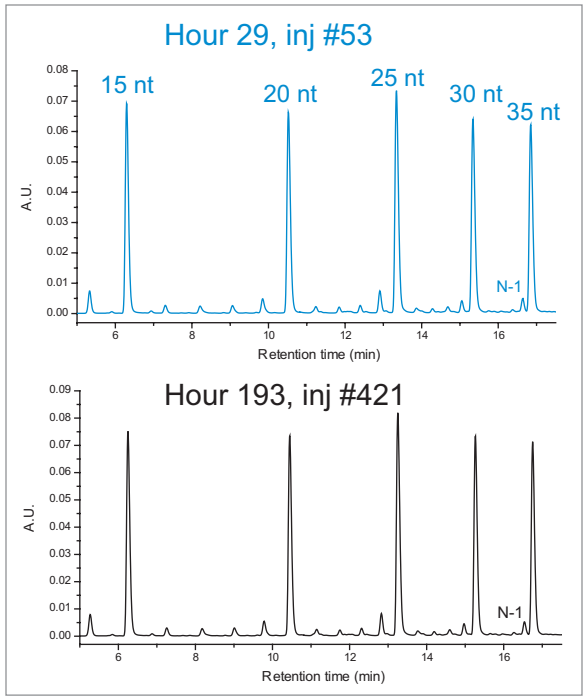

Figure 2. Column longevity evaluation at elevated pH. The MassPREP OST polyT standard mixture was separated using a 30-minute method across 400 injections over 200 hours. The PolyT standards were separated with a high degree of repeatability observed in the chromatographic profile of the standards, as well as the failed sequences (N-X).

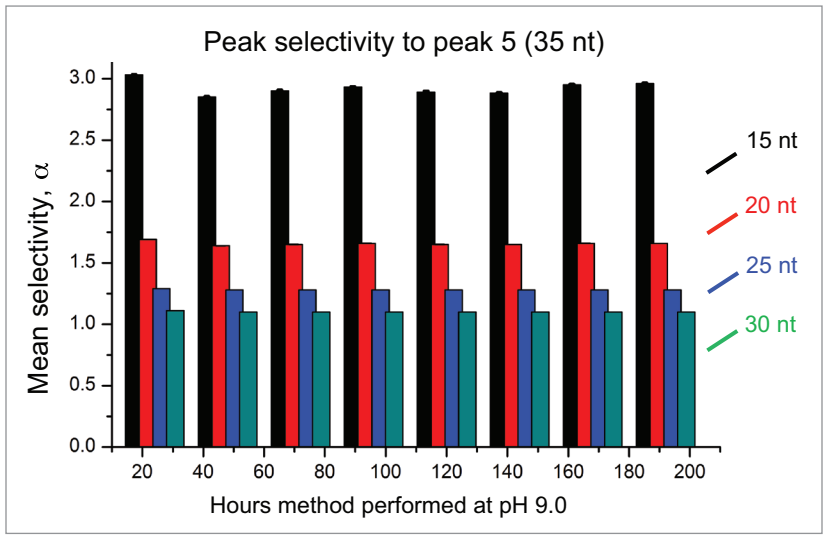

Figure 3. Column selectivity evaluation. Peak selectivity was calculated for the first four standard peaks (15–30 nt) relative to the last peak (35 nt) from a separation using elevated pH and temperature. Calculated values based on the experimental data were averaged over 24-hour time blocks and reported with corresponding error bars. Selectivity values were determined to have less than 1% RSD.

To further probe column performance, inter-assay variability was evaluated for retention time and peak width at half height. As shown in Table 1, retention times were stable across the injection series with RSDs ≤1.66%. Peak width was also highly stable with average peak width at half height ≤0.08 minutes with RSDs ≤1.49%. Collectively, this data confirms that the Waters OST column exhibits a high degree of ruggedness when using methods that require high pH and elevated temperatures over extended time periods. With comparability and column longevity confirmed when using alternative IP mobile phase compositions, a sample more representative of an oligonucleotide was used to test the applicability of the proposed method.

A comparison was made between TEA:HFIP and BA:HFIP mobile phases in the separation of a 21 nt ssRNA as shown in Figure 4. Using a high-resolution gradient, the N-1 and N+1 impurities were separated from the target peak using both mobile phase compositions with a high degree of comparability. Similar to before, the initial starting %B was lower for the BA:HFIP mobile phase in comparison to the TEA:HFIP (7% vs. 13%). The gradient slope for the BA:HFIP was slightly shallower at 0.6% B/min in comparison to the 0.8% B/min for TEA:HFIP, most likely due to the butylamine being less hydrophobic and requiring less eluent to desorb from the ligand surface.[7,8] In addition, the ACQUITY QDa response was observed to be highly comparable across both mobile phases for the ssRNA (1.68×10^8 TEA:HFIP vs. 1.43×10^8 BA:HFIP). The ability to separate the impurity sequences from a more representative oligonucleotide using BA:HFIP further corroborates the notion that IP reagents other than TEA:HFIP offer analysts the ability to explore alternative IP-RPLC/MS solutions for oligonucleotide analyses that enable high MS sensitivity and potentially reduce assay costs.

Inter-assay variability					
Peak	Avg. MW	RT (min)	RSD (%)	W_{50}	RSD (%)
15 nt	4500.9	6.15	1.66	0.077	0.90
20nt	6021.9	10.34	0.97	0.078	0.70
25 nt	7542.9	13.16	0.72	0.079	0.68
30 nt	9063.8	15.16	0.65	0.078	1.49
35 nt	10584.8	16.65	0.59	0.078	0.83

Table 1. Inter-assay variability was evaluated for retention time and peak width at half height (W_{50}) across the injection series. Mean retention times were observed with RSDs ≤1.66%. Mean peak widths at half height were observed to be ≤0.08 minutes with RSDs ≤1.49%.

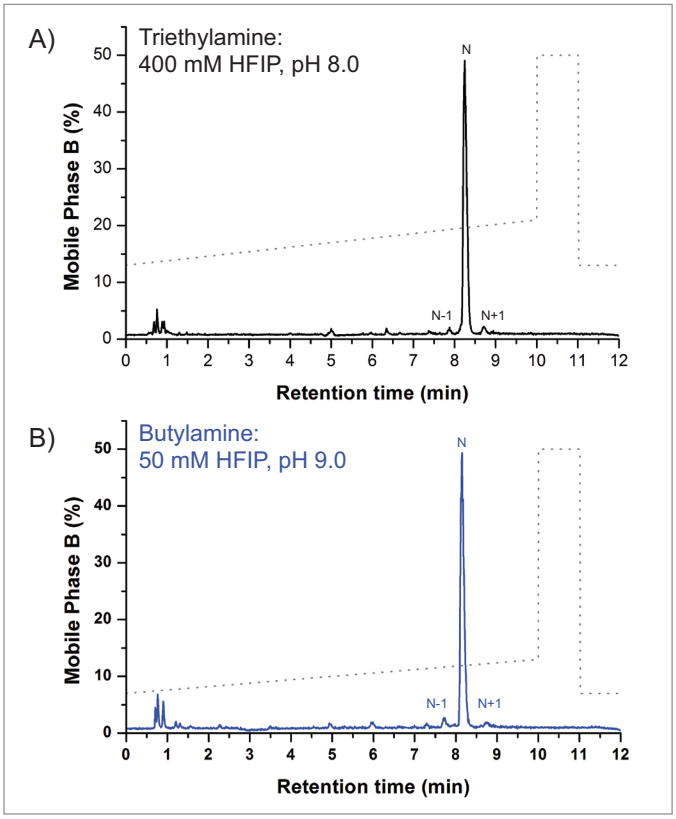

Figure 4. Impurity profiling of ssRNA. A 21 nt ssRNA (5'-UCGUCAAGCGAUUACAAGGTT-3') was separated from its N-1 and N+1 impurities using a high-resolution gradient with mobile phases comprised of A) 15 mM TEA : 400 mM HFIP, and B) 15 mM BA : 50 mM HFIP.

CONCLUSION

As new therapeutic oligonucleotides come to market with novel physicochemical properties, IP-RPLC/MS-based techniques that incorporate alternative IP buffers to TEA:HFIP may be required to achieve optimal chromatography conditions. The Waters OST columns exhibit a high degree of ruggedness when using alternative methods that require high pH and elevated temperatures over extended time periods, and can be incorporated into existing ACQUITY QDa workflows. Collectively, this work demonstrates that Waters offers a cost-effective solution to the challenges facing today's analysts in oligonucleotide analyses that is flexible, robust, and readily deployable.

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Hexylammonium Acetate as an Ion-pairing Agent for IP-RP LC Analysis of Oligonucleotides

Sean M. McCarthy and Martin Gilar
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

In this application note we describe the preparation of HAA and the benefits to its use for a variety of applications compared to TEAA and/or TEA-HFIP including:

- Homomolecular oligonucleotides
- Long oligonucleotides
- Heteromolecular oligonucleotides
- Phosphorothioates
- Labeled oligonucleotides
- Oligonucleotide duplexes

WATERS SOLUTIONS

ACQUITY UPLC® System

Oligonucleotide Separation Technology

MassPREP™ OST Standard

ACQUITY UPLC PDA Detector

KEYWORDS

oligonucleotides, TEAA, TEA-HFIP, HAA, ion-pairing, reversed-phase, ACQUITY UPLC

INTRODUCTION

Analysis of oligonucleotides via ion-pairing reverse-phase liquid chromatography requires efficient ion-paring systems that yield chromatographic separation primarily based on charge/length.[1] The most commonly used ion-pairing agents are triethylammonium acetate (TEAA) and triethylamine hexafluoroisopropanol (TEA-HFIP). TEAA is commonly used due to its low cost and duplex compatability, but suffers from limited resolution of oligonucleotides. TEA-HFIP offers benefits over TEAA, such as increased resolution and predictable retention.

The benefits are the result of increased ion-pairing and a decrease in reversed-phase behavior resulting from the nucleobases, evident in single-stranded oligonucleotide separations. Despite these benefits, the utility of a TEA-HFIP system is somewhat limited. In particular limitations arise for separation of duplex oligonucleotides, because it is denaturing and contributes significantly to melting. The costs associated with the use of TEA-HFIP are significantly higher than those associated with using acetate-buffered mobile phases when considering purchase and disposal.

To overcome some of these issues, we investigated an ion-paring system which uses hexylammonium acetate (HAA). HAA, when used with the Waters® ACQUITY UPLC System, provides the same or better resolution of a variety of oligonucleotides, and is non-denaturing for duplexes. The cost associated with the use of HAA is significantly lower than that of HFIP-based mobile phases.

EXPERIMENTAL

Mobile phase preparation

Hexylamine and acetic acid were purchased from Sigma Aldrich. The concentration of HAA in this example is 100 mM single-stranded; however, we have prepared 50 and 10 mM for increased MS-compatibility with little loss in resolution.

1 L of 100 mM HAA is prepared as follows:

It is highly recommended that mobile phase components be measured by mass instead of volume. Significant variation in retention times may result from inaccurate mobile phase preparation.

1. Weigh 981 g of water and place in a beaker with a Teflon™ coated magnetic stir bar.
2. Add 5.99 g (5.71 mL) of acetic and stir the mixture.
3. Add 10.12 g (13.21 mL) of hexylamine.
4. Stir the resulting solution so there is a significant vortex for 5 minutes.
5. The pH of the solution should be approximately 7.0. If the pH needs to be adjusted, carefully add acetic acid or hexylamine as appropriate in ~1 µL aliquots and stir until dissolved. This solution is used as MP A.
6. MP B is prepared as 50% ACN in MP A. This should be prepared by mass to ensure reproducible results. The density of MP A is assumed to be the same as water for the purpose of MP B preparation.

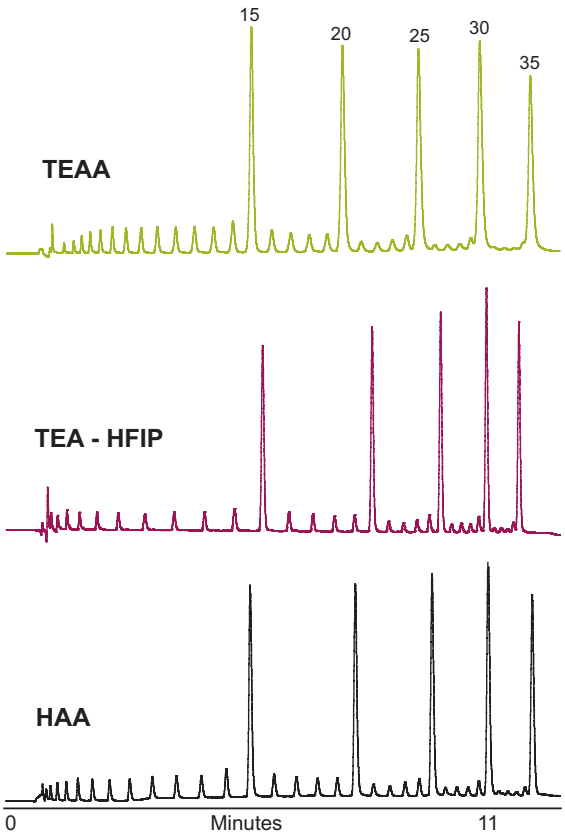
The mobile phase can be used for several days without any significant loss in oligonucleotide resolution.

Oligonucleotide samples should be dissolved in either 100 mM HAA or 100 mM TEAA, pH 7.0. There is strong affinity of hexylamine for the particle sorbents; therefore, it is highly recommended that a column be dedicated for use with HAA due to its high affinity for the stationary phase.

RESULTS AND DISCUSSION

HOMOMOLECULAR OLIGONUCLEOTIDES

The Waters Oligonucleotide Separation Technology (OST) standard demonstrates the resolving capabilities of HAA compared to traditionally-used mobile phases. The separations shown in Figure 1 were normalized by adjusting each gradient so that the 15-mer and 35-mer peaks eluted at the same time with each mobile phase system. As shown, HAA offers a significant increase in resolution compared to the commonly-used, non-denaturing TEAA mobile phase. The increase in resolution is attributed to the increased ion pairing ability of HAA. Comparing the separation with HAA to the separation with TEA-HFIP clearly indicates that HAA provides similar or better resolution than HFIP-based mobile phase. In fact, our data indicated that the resolution of longer oligonucleotides is better with HAA.



LC system:	ACQUITY UPLC with ACQUITY UPLC PDA
Sample:	MassPREP OST Standard
Column:	ACQUITY UPLC OST C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Detection:	260 nm, 20 points/second
Mobile phases:	
Top trace	A: 100 mM TEAA, pH 7.0 B: 20% ACN in A Gradient: 45–55% B in 10 min
Middle trace	A: 15 mM TEA, 400 mM HFIP, pH 7.9 B: 50% MeOH in A Gradient: 30–47% B in 10 min
Bottom trace	A: 100 mM HAA, pH 7.0 B: 50% ACN in A Gradient: 56–78% B in 10 min

Figure 1. Separation of MassPREP OST standard with different ion-pairing systems.

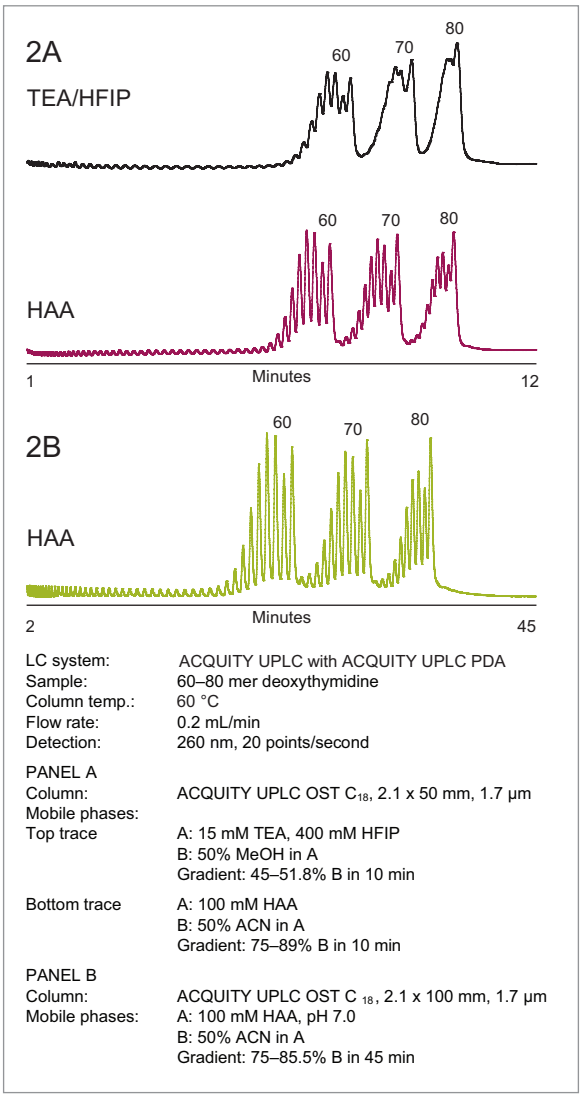
LONG OLIGONUCLEOTIDES

We investigated the increased resolution of longer oligonucleotides with HAA using a sample of 60 to 80 mer deoxythymidines. Figure 2A illustrates the separation of a 60 to 80 mer deoxythymidine ladder performed with TEA-HFIP, top trace, and with HAA, bottom trace. The two separations were normalized by adjusting the gradient of each system so that the retention of the 60 and 80 mer occur at approximately the same time, which allows direct comparison of their resolving power. As shown, there is a significant increase in resolution for long oligonucleotides with HAA compared to TEA-HFIP. Resolution can be increased further by increasing the column length (Figure 2B), but the gradient time must also be scaled proportionally.

HETEROMERIC OLIGONUCLEOTIDES

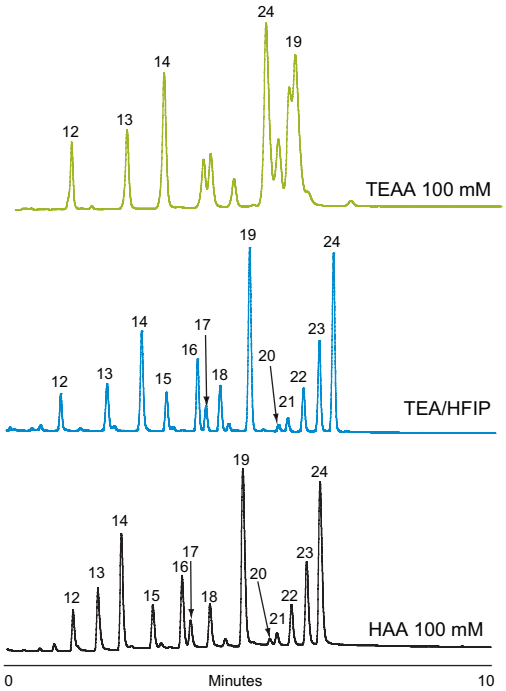
Since most synthetic oligonucleotides used for therapeutic and diagnostic purposes are composed from a variety of bases, we investigated the separation of a more realistic sample of a heteromeric oligonucleotide. For this purpose we used the same chromatographic conditions used in Figure 1 to provide the most fair comparison.

As shown in Figure 3, TEAA does not effectively separate the heteromeric oligonucleotides. In contrast, both HAA and TEA/HFIP give impressive separations. Careful inspection of the data reveals that the separations are virtually identical. The increased chromatographic resolution obtained with HAA and TEA-HFIP compared to TEAA is the result of increased ion-pairing behavior and decreased hydrophobic contribution of the nucleobases. For applications where MS analysis is not required, the use of HAA is a cost-effective alternative that provides the same or better resolution.



LC system:	ACQUITY UPLC with ACQUITY UPLC PDA
Sample:	60–80 mer deoxythymidine
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Detection:	260 nm, 20 points/second
PANEL A	
Column:	ACQUITY UPLC OST C_{18}, 2.1 x 50 mm, 1.7 µm
Mobile phases:	
Top trace	A: 15 mM TEA, 400 mM HFIP B: 50% MeOH in A Gradient: 45–51.8% B in 10 min
Bottom trace	A: 100 mM HAA B: 50% ACN in A Gradient: 75–89% B in 10 min
PANEL B	
Column:	ACQUITY UPLC OST C $_{18}$, 2.1 x 100 mm, 1.7 µm
Mobile phases:	A: 100 mM HAA, pH 7.0 B: 50% ACN in A Gradient: 75–85.5% B in 45 min

Figure 2. Separation of long oligonucleotides.



LC system:	ACQUITY UPLC with ACQUITY UPLC PDA
Sample:	5'-CCC CTT GGT TAA CCA AGG TTC CAA-3'
Column:	ACQUITY UPLC OST C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Detection:	260 nm, 20 points/second
Mobile phases:	
Top trace:	A: 100 mM TEAA, pH 7.0 B: 20% ACN in A Gradient: 45–55% B in 10 min
Middle trace:	A: 15 mM TAE, 400 mM HFIP, pH 7.9 B: 50% MeOH in A Gradient: 30–47% B in 10 min
Bottom trace:	A: 100 mM HAA, pH 7.0 B: 50% ACN in A Gradient: 56–78% B in 10 min

Figure 3. Separation of heteromeric oligonucleotides.

PHOSPHOROTHIOATES

The separation of phosphorothioates can be very challenging. This is largely due to the inherent chirality of the phosphorothioate linkage, which often leads to broad peaks and incomplete resolution of trunca ted sequences due to isomeric distribution. Figures 4 and 5 show that HAA adequately resolves truncated phosphorothioate sequences from the full-length sequence. The ability to resolve N-1 phosphorothioates indicates that HAA partially suppresses diastereomeric resolution. While the MS sensitivity resulting from the use of HAA is lower than that of TEA-HFIP, useful MS data is easily obtained and confirms the separation of the full-length phosphorothioate from truncated sequences as shown in the selected ion chromatograms in Figure 5.

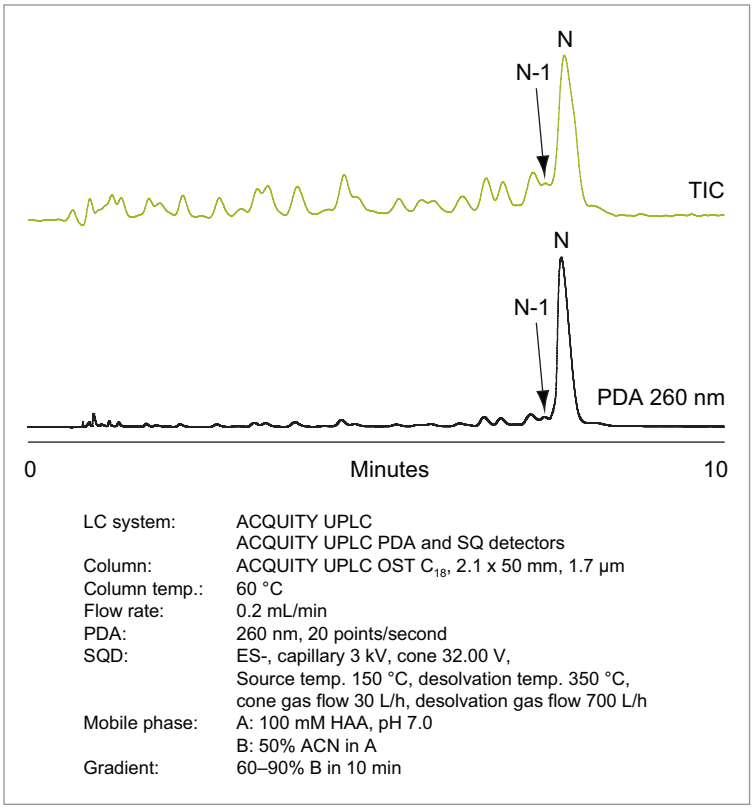


Figure 4. Separation of phosphorothioate with HAA.

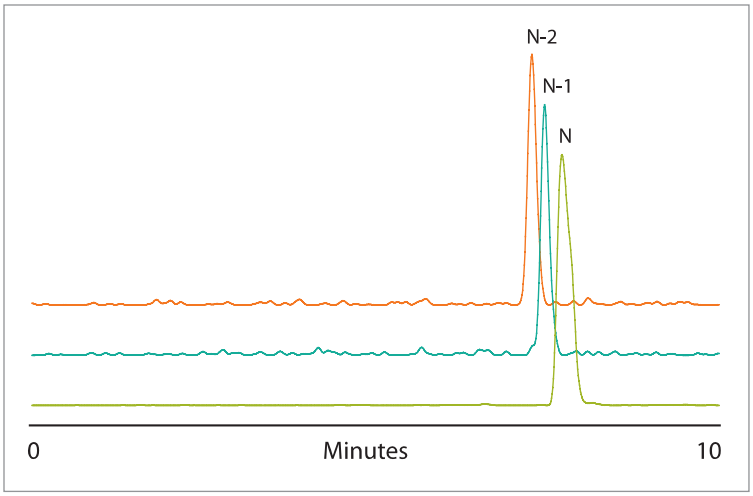


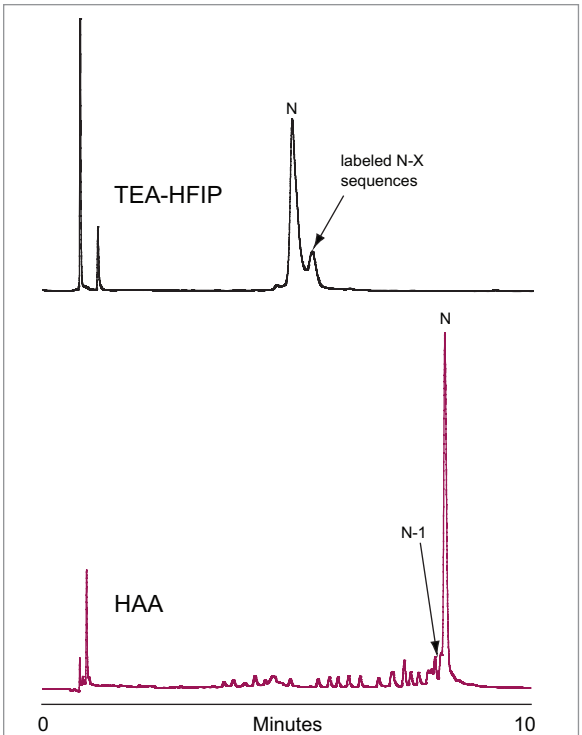
Figure 5. Selected ion chromatograms of phosphorothioate.

LABELED OLIGONUCLEOTIDES

Many oligonucleotides are labeled with fluorescent tags for a variety of reasons. These tags are generally large aromatic and poly-aromatic groups that impart significant hydrophobicity to the oligonucleotide. For this reason, inefficient ion pairing often leads to poor resolution of labeled species as hydrophobic forces overcome the ion-pair mechanism. Figure 6 illustrates the analysis of a hydrophobically-labeled oligonucleotide with TEA-HFIP (top trace) and HAA (bottom trace). TEA-HFIP does not yield the desired elution pattern. Instead, the target peak is followed by truncated labeled species. The reversal in elution order results from increased contribution of the hydrophobic tag to chromatographic behavior as the hydrophilic oligonucleotide size decreases. The use of a more efficient ion-paring agent, such as HAA, results in regular retention of all labeled species in their expected order.

OLIGONUCLEOTIDE DUPLEXES

As mentioned previously, HAA is non-denaturing and is fully compatible with oligonucleotide duplexes. We investigated the ability of HAA to resolve mismatched from full-length RNA duplexes. To demonstrate the ability of HAA to resolve RNA duplexes, we utilized a purified upper RNAi strand and a partially-digested lower strand. Upon annealing, a ladder of siRNA duplexes is formed with partially 5' truncated lower RNAi strands (Figure 7).



LC system:	ACQUITY UPLC with ACQUITY UPLC PDA
Column:	ACQUITY UPLC OST C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
PDA:	260 nm, 20 points/second
Mobile phase:	A: 100 mM HAA, pH 7.0 B: 50% ACN in A
Gradient:	60–90% B in 10 min

Figure 6. Separation of labeled oligonucleotide.

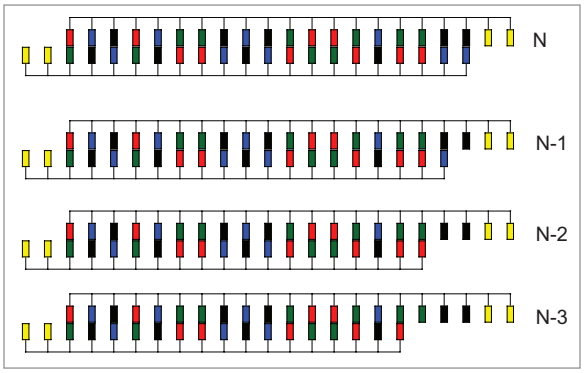
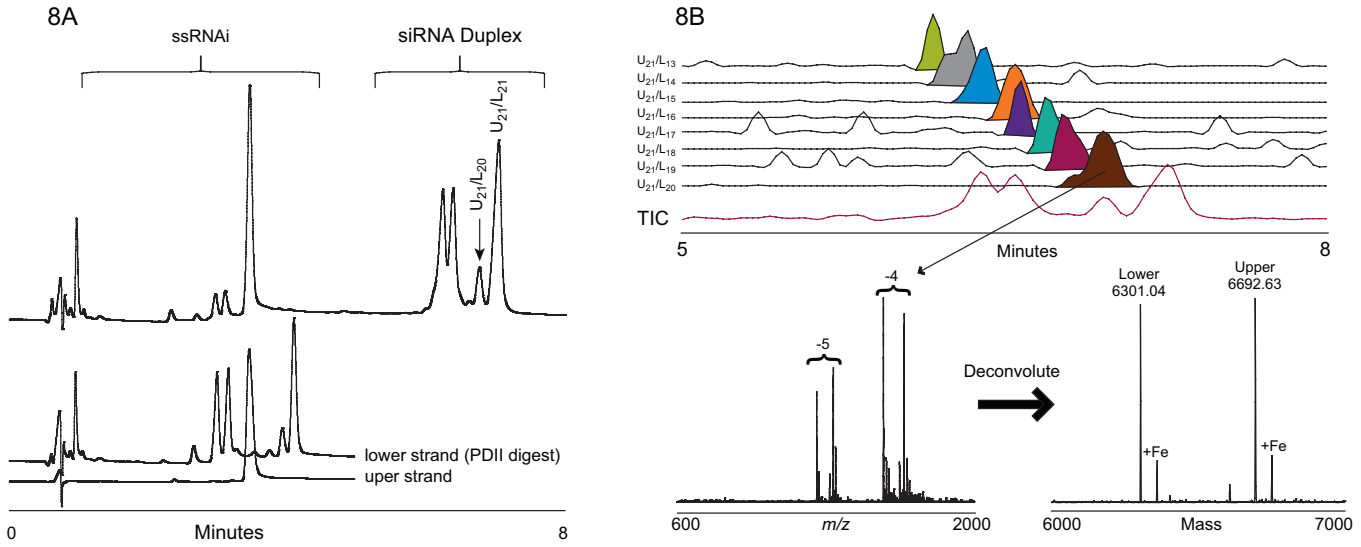


Figure 7. Duplexes formed by annealing full-length upper RNAi strand with partially-truncated lower RNA strand.

As shown in Figure 8A, HAA successfully resolves truncated siRNA duplexes from full-length duplex and single-stranded RNAi species. Using hexylammonium acetate we were also able to identify each eluting duplex peak by the corresponding mass of complementary RNAi strands with MS. In this way we were able to confirm the elution order of the impurities. Extracted selected ion chromatograms shown in Figure 8B indicate resolution of truncated duplexes.



LC system:	ACQUITY UPLC
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% ACN
Gradient:	30–40% B in 10.0 min (1% ACN/min)
Detection:	PDA, 260 nm SQ, 600–2000 Da

SQ detection parameters:	
Mode:	ES-
Capillary:	3.0 kV
Cone:	28 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Cone gas flow:	31 L/h
Desolvation gas flow:	700 L/h

Figure 8. Separation of siRNA duplexes and single stranded RNA.

CONCLUSIONS

In this application note, we outlined the use of HAA for a variety of oligonucleotide chromatographic applications. HAA and TEA-HFIP exhibit similar resolution of moderate length, unmodified homo and heteromolecular oligonucleotides. For separation of longer oligonucleotides, ca. >35-mer, HAA exhibits better resolution which can be further improved with longer columns and correspondingly longer gradient times. HAA is able to adequately resolve phosphorothioates, a particularly difficult separation. The separation of labeled oligonucleotides shows a clear advantage of HAA over TEA-HFIP with HAA giving predictable retention of labeled species and minimal contribution of the hydrophobic label. Finally, the non-denaturing character of HAA allows for its use with oligonucleotide duplexes and offers impressive resolution of mismatches. While HAA does not offer the same MS compatibility as TEA-HFIP, its utility is evident. Taken together this data indicates that the use of HAA with the ACQUITY UPLC System provides exceptional separation of a variety of oligonucleotides at a significantly lower cost than TEA-HFIP.

References

1. Oligonucleotide Separation Technology XBridge® OST C_{18} Columns Method Guidelines. Waters User Manual. 2008. 715001476.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

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ProMass for MassLynx Software for Oligonucleotide Analysis

Vera B. Ivleva, Sean M. McCarthy, Ying Qing Yu, and Martin Gilar
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

ProMass for MassLynx® Software enables batch mass deconvolution and data analysis for UPLC-MS data generated on both single quadrupole and time-of-flight Waters® MS instruments. ProMass for MassLynx is the only software available on the market that performs automatic assignment of the high-complexity chromatograms of an oligonucleotide. The software allows significant time savings in labs that routinely perform quality control and study *in-vitro* metabolism of native and modified samples.

WATERS SOLUTIONS

ACQUITY UPLC®

SYNAPT® HDMS®

Xevo® QTof MS

UPLC® Oligonucleotide Separation Technology Columns

MassLynx 4.1 Software

KEYWORDS

ProMass Software, batch mass deconvolution, peak assignments, oligonucleotide, RNA, SiRNA

INTRODUCTION

Oligonucleotides are an emerging class of biopharmaceutical compounds that includes antisense oligonucleotides (AO) and short interfering RNA oligonucleotides (siRNA), among others. Oligonucleotides are produced via automated stepwise synthetic methods. While synthetic procedures offer high yields, by-products of synthesis need to be removed and the desired product must be characterized prior to use in therapeutic applications.

UPLC-MS methods have been successfully applied to the analysis of the oligonucleotides. Due to the presence of failed products and a variety of modifications, the chromatograms can be complex and difficult to interpret even when LC-MS data are available. Manual processing of LC-MS data is laborious and time-consuming.

ProMass Software, currently used for LC-MS data processing by many laboratories analyzing oligonucleotides, has been recently adopted for MassLynx Software. ProMass for MassLynx enables automated data analysis in a high-throughput manner. This application note describes the utility of the software for characterization of truncated oligonucleotides, modifications, and other impurities.

EXPERIMENTAL

Oligonucleotide samples

MassPREP OST standard (Waters) consisting of 15, 20, 25, 30, and 35 nucleotides (nt) long of oligodeoxythymidines (poly-T), ~ 1 nmole of each oligo per vial.

21 nt: 5'-UUC UGU AAU CUC UUG UCU ATT-3'
20 nt: 5'-UC UGU AAU CUC UUG UCU ATT-3'
(Integrated DNA Technologies, Coralville, Iowa)

All samples were reconstituted in 0.1-M triethylamine acetate (TEAA) to 40 pmole/µL.

LC conditions

LC system:	Waters ACQUITY® SQD
LC column:	Waters ACQUITY UPLC Oligonucleotide Separation Technology (OST) C_{18} 1.7 µm, 135 Å, 2.1 x 50 mm
Column temp.:	60 °C
Sample injected:	2.5 µL
Flow rate:	0.2 mL/min
Weak wash:	Water
Strong wash:	50% methanol in water
Mobile phase A:	aqueous solution of 15 mM trietylamine (TEA, Fluka 23,962-3) and 400 mM hexafluoroisopropanol (HFIP, Fluka 32,522-8), pH 7.9; solutions were prepared by weight
Mobile phase B:	50% solvent A, 50% methanol, v/v

MS conditions

MS system:	ACQUITY SQD
Capillary:	4 kV
Sampling cone:	40 V
Extraction cone:	3 V
Source temp.:	150 °C
Desolvation temp.:	350 °C
Desolv. gas flow:	700 L/hr

SYNAPT HDMS

Capillary:	3 kV
Sampling cone:	35 V
Extraction cone:	3 V
Source temp.:	150 °C
Desolvation temp.:	300 °C
Desolv. gas flow:	500 L/hr
Lockmass calibration:	CsI 10 mg/mL (water/isopropanol, 1:1), 5 µL/min flow rate, set mass = 1685.765

RESULTS AND DISCUSSION

UPLC separation of the components was accomplished in less than 10 minutes using the MS-compatible ion-pairing mobile phase TEA/HFIP system (Figure 1). Manual assignment of the chromatographic peaks can be laborious and time-consuming, since each peak needs to be deconvoluted.

The manual peak assignment is based on two pieces of information: the highly accurate mass and the retention order of oligonucleotides, which depend on their relative length (shorter oligonucleotides elute before the longer ones). Shorter, 5' truncated failure sequences, typical by-products of synthesis, can be rather confidently assigned. Nevertheless, the manual data analysis is a very time-consuming process.

In order to facilitate faster and more convenient data analysis, ProMass for MassLynx Software was adopted for UPLC-MS chromatogram data processing.

Deconvolution of the components/peaks in the mass chromatograms is performed in the background using ZNova deconvolution algorithm. The software then compares deconvoluted masses of analytes to user-provided expected mass/sequence and the corresponding peak(s) are putatively assigned. Oligonucleotide-failed sequences can also be assigned in a similar fashion.

Figure 2 illustrates the software output, after data analysis of chromatogram shown in Figure 1. Two oligonucleotides, 35- and 30-oligodeoxythymidine sequences, were provided by the user. Software calculated their masses and identified two peaks eluting at 10.28 min and 9.64 min, corresponding to the expected masses. Information about mass error and relative abundance is also provided.

When specified in the processing method, 5' or 3' truncated failure sequences (or both) will also be interrogated by the software. Figure 2 illustrates the output of 5' truncated sequences above the chosen threshold. The dominant peaks of 15, 20, 25, 30, and 35 nt were assigned in an LC-MS chromatogram (see table in Figure 2). The retention time, mass error, and sequence assignment in relation to the target sequence are listed in the table. The target sequence in this case was 35 nt oligodeoxythymidine, the last dominant peak in the chromatogram.

ProMass for MassLynx Software typically processes the UPLC-MS chromatograms immediately after the data acquisition while the next sample in the queue is being analyzed by UPLC-MS. Alternatively, the previously acquired data batch can be assigned for off-line post-processing using the existing MassLynx sample list, which contains appropriate information required by ProMass (methods, deconvolution ZNova parameters, and sequence).

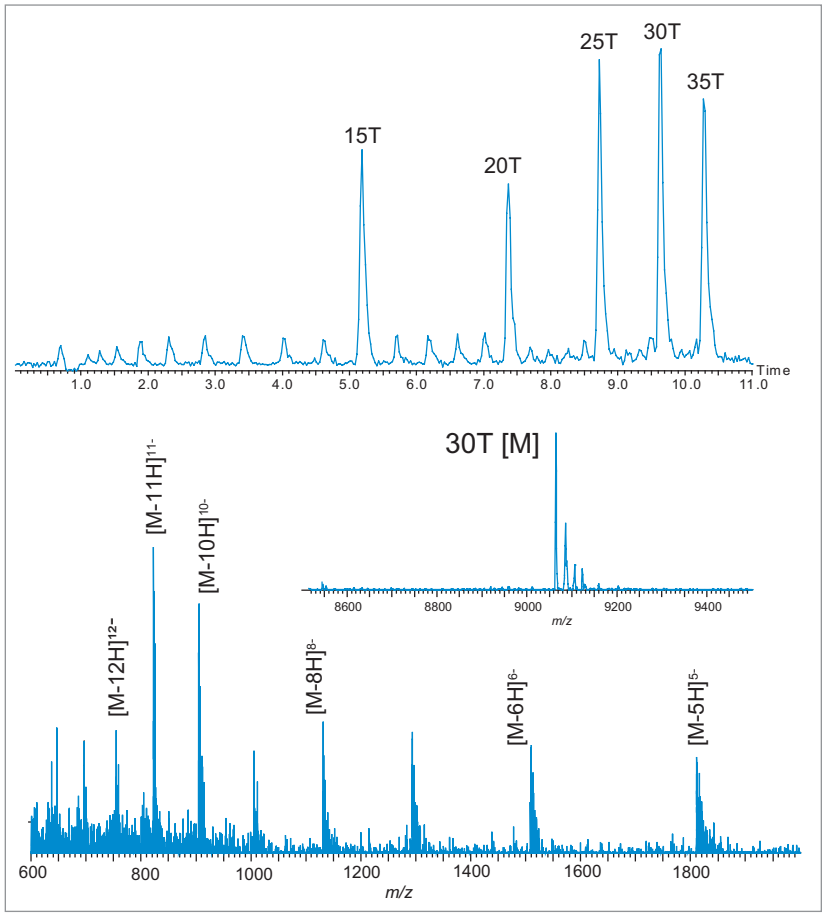


Figure 1. MassLynx chromatogram of OST standards; mass spectrum of 30 T (MaxEnt™1 deconvoluted spectrum on inset).

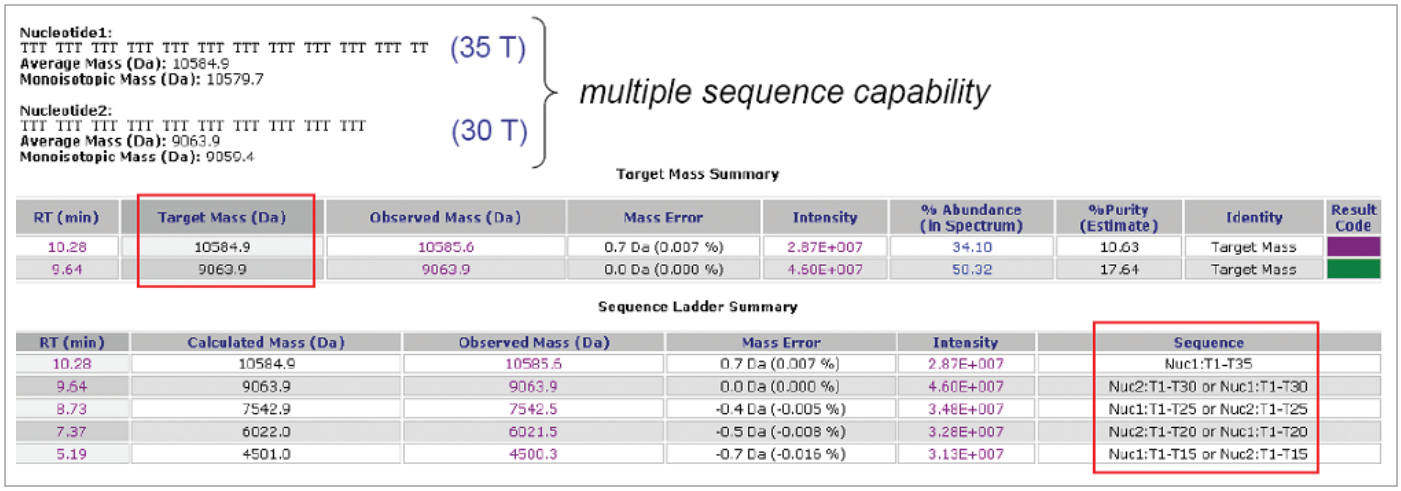


Figure 2. ACQUITY SQD MS data for DNA oligonucleotides.

The workflow of ProMass for MassLynx Software is presented in Figure 3. The software package provides a MassLynx sample list format with explanation of the function of each column field. Correct configuration of the sample list is essential for successful data processing. The user provides the raw data, defines the parameter files, and the optional details about the target product search.

TWO PARAMETER FILES ARE NECESSARY FOR DATA PROCESSING

The parameter file of the application is called ProMassBridge, which provides a connection between MassLynx and ProMass. ProMassBridge takes care of the peak detection and reports the data to ProMass. ProMass software deconvolutes the spectra and reports the assignment of the peaks as an HTML file either in the form of tables, an Excel spreadsheet, or visualized as a 96-well plate.

In the ProMassBridge parameter file, the user defines the desired retention time range, smoothing and subtracting settings, and LockMass correction. The other parameter file is the one of ProMass called ZNova, which defines the input and output masses, ion polarity, deconvolution settings, target mass, impurities tolerance thresholds, and options for the data report.

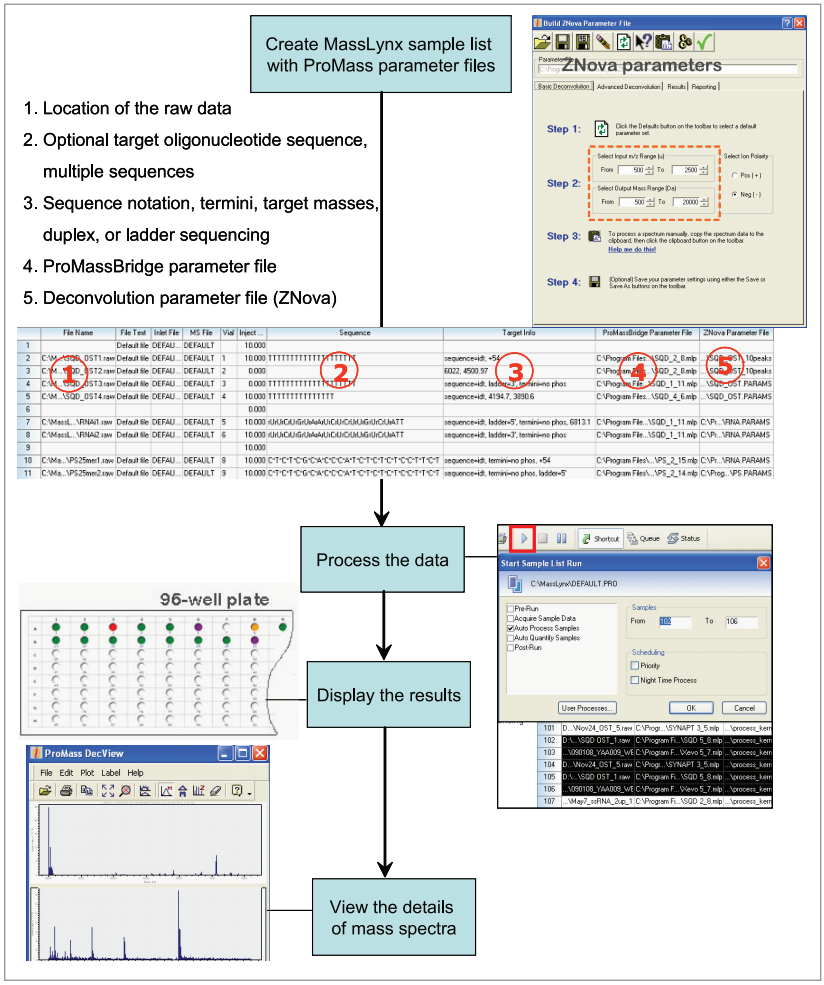


Figure 3. Overview of LC-MS data processing with ProMass for MassLynx.

In the sample set, the user has the option to define the analyte(s) of interest either as target mass, sequence, or combination of both. A variety of nucleotides modifications is supported, such as DNA, RNA, LNA, 2'-O-methylation, 2'-fluorination, or phosphorothioate modificaton. The user can specify any modification according to the Integrated DNA Technology (IDT) notations, or define custom modifications. The target information may include the mass of the targeted product, the adducts, termini modifications, and a request for ladder sequencing or processing of a duplex.

In high-throughput analysis mode, the user can display data summary as a 96-well plate with color-coded entries (Figure 3). The colors represent the result of the targeted mass(s) search. It is offered in interactive format to allow navigation through the chromatograms and the details of mass spectra. There is a general chromatographic summary listing the peaks assigned within the retention time range defined in ProMassBridge parameter file. The mass spectra corresponding to each chromatographic peak can be viewed in detail in the ProMass Viewer window.

In order to test the correctness of ProMass for MassLynx, the data of OST standards acquired on an ACQUITY SQD single quadrupole mass spectrometer were processed (Figure 2). Multisequence capability was investigated using 30 T and 35 T OST homologs as two targeted mass searches. Generally, more than two sequences can be entered. The sequences of both homologs were supplied, and the ProMass for MassLynx calculated the theoretical masses and searched for the corresponding values within the specified time range.

The deconvoluted mass of 30 T species resulted in 0.0 Da mass error, and was marked in green, meaning that the target mass found in chromatogram is the most abundant component within the suggested 0.02% masserror tolerance. The 35 T homolog was marked in purple, which happens when other components present in the spectrum have greater than 30% abundance. The deconvoluted masses of the failed sequences of the targeted 35 T oligonucleotide (30 T, 25 T, 20 T, 15 T) were reported in the sequence ladder summary. ProMass for MassLynx successfully identified the targeted masses and picked the correct picks as truncated sequences. The results correlated with manually deconvoluted masses of OST homologs (Figure 1).

To process the OST chromatogram, the reporting option in the ZNova parameter file was chosen to define the threshold of the chromatographic peaks selected for deconvolution so only the major homologs of the truncated sequences were taken into consideration (Figure 4). Assignment of the entire chromatogram is possible, as was demonstrated in Figure 4 in an example of 21-mer RNAi data obtained on an SYNAPT HDMS instrument. The molecular weight of the intact molecule was calculated based on the provided sequence and deconvoluted by ProMass for MassLynx with 0.011% mass error. In the Target Info column the request for the ladder sequencing was denoted as “ladder=5”.

Generally, the search for the truncated sequences from either 5' or 3' ends is available as are internal cleavages. As a result, 17 truncated sequences were reported in the sequence ladder summary. The structures of these sequences are reported in the summary table rather than assigned directly on the chromatogram.

Using the automatic peak deconvolution, most of the 21-mer sequence of RNAi was confirmed in a matter of a few minutes. The speed of UPLC separation coupled with automated ProMass for MassLynx processing makes this method fast and efficient. The interactive interface covers the vast range of the user's needs: from a quick summary report to the details of the mass spectra.

Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Abundance (in Spectrum)	%Purity (Estimate)	Identity	Result Code
9.00	6506.9	6506.2	-0.7 Da (-0.011 %)	2.21E+004	46.05	38.62	Target Mass	

Sequence Ladder Summary

RT (min)	Calculated Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	Sequence
9.00	6506.9	6506.2	-0.7 Da (-0.011 %)	2.21E+004	rU1-T21
8.73	6200.7	6200.5	-0.2 Da (-0.003 %)	4.95E+002	rU2-T21
8.43	5894.5	5893.7	-0.8 Da (-0.014 %)	5.42E+002	rC3-T21
8.00	5589.4	5588.3	-1.1 Da (-0.020 %)	8.56E+002	rU4-T21
7.65	5283.2	5282.3	-0.9 Da (-0.017 %)	1.08E+003	rG5-T21
7.25	4938.0	4937.4	-0.6 Da (-0.012 %)	7.43E+002	rU6-T21
7.01	4631.8	4631.6	-0.2 Da (-0.004 %)	9.41E+002	rA7-T21
6.22	4302.6	4302.0	-0.6 Da (-0.014 %)	1.37E+003	rA8-T21
5.09	3973.4	3972.5	-0.9 Da (-0.023 %)	9.58E+002	rU9-T21
4.45	3667.2	3666.5	-0.7 Da (-0.019 %)	5.70E+002	rC10-T21
3.79	3362.1	3361.3	-0.8 Da (-0.024 %)	6.58E+002	rU11-T21
3.28	3055.9	3055.5	-0.4 Da (-0.013 %)	3.95E+002	rC12-T21
2.65	2750.7	2750.4	-0.3 Da (-0.011 %)	5.28E+002	rU13-T21
2.12	2444.5	2444.4	-0.1 Da (-0.004 %)	5.03E+002	rU14-T21
1.73	2138.4	2137.4	-1.0 Da (-0.047 %)	7.38E+002	rG15-T21
1.06	1487.0	1486.2	-0.8 Da (-0.054 %)	1.14E+003	rC17-T21
0.97	1181.8	1181.2	-0.6 Da (-0.051 %)	7.97E+002	rU18-T21

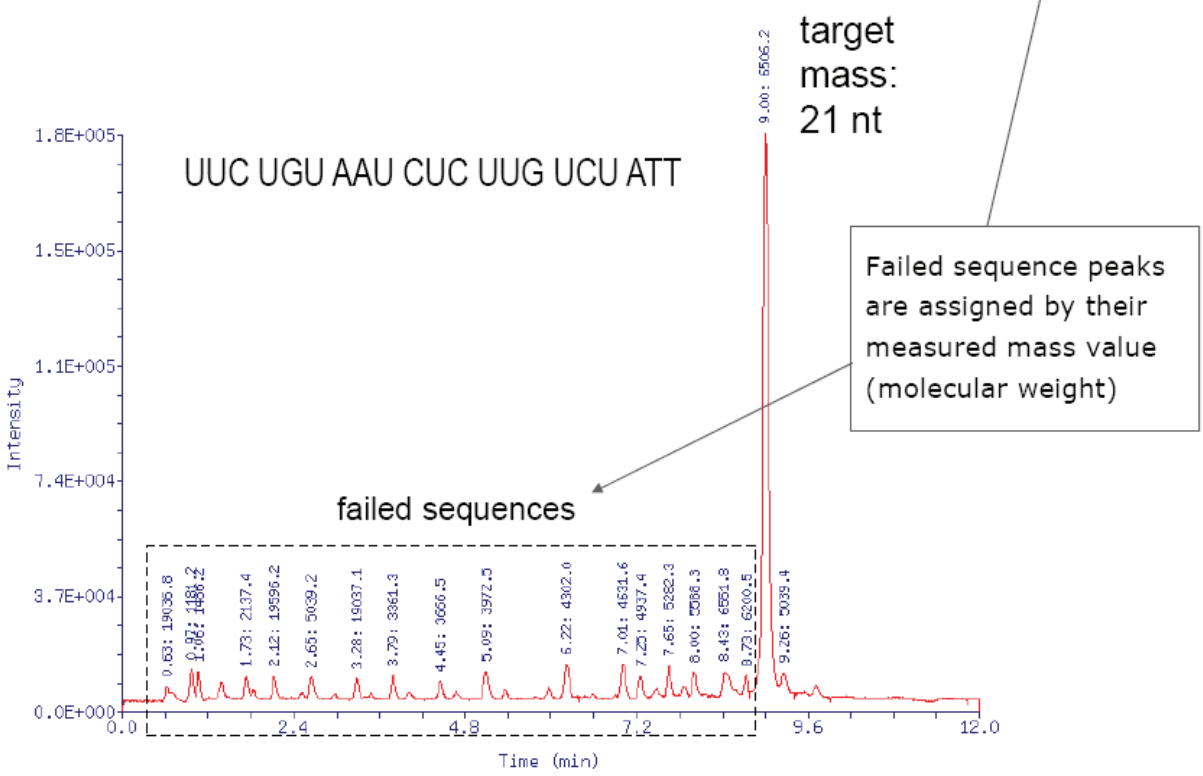
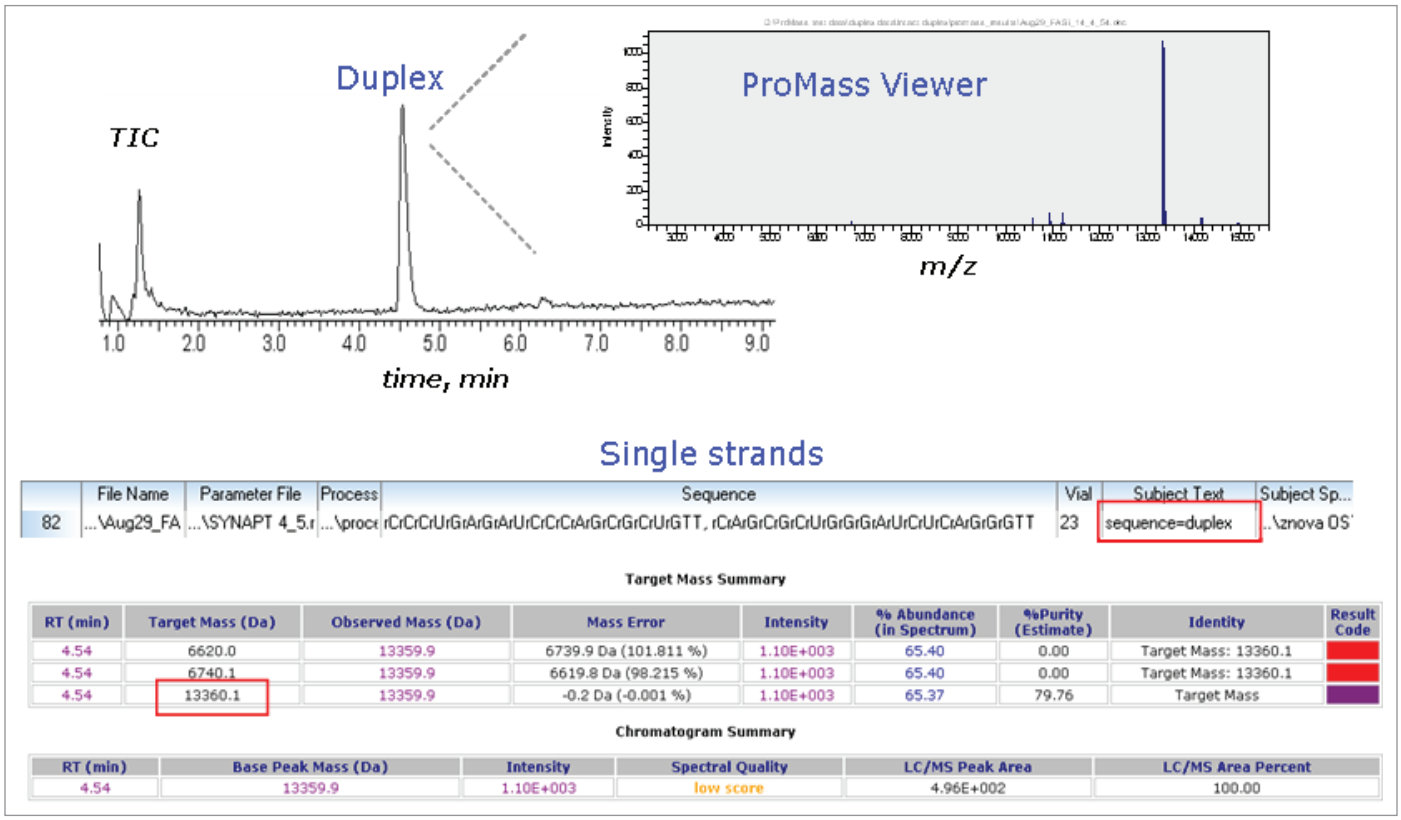


Figure 4. ProMass (for MassLynx) results visualized in browser as tables, chromatograms, and MS spectra. SYNAPT HDMS results for 21 nt siRNA oligonucleotide.

Analysis of the RNAi duplex is feasible if MS conditions allow for detecting the intact duplex peak. Two complementary RNAi strands were injected simultaneously to anneal into a duplex on a column. Consequently, ProMass for MassLynx identified both single RNAi strands and the peak corresponding to the sum of their masses composing the duplex mass (Figure 5). In order to perform this task, the target information should specify "sequence=duplex". In most instances, the duplex partially falls apart onto single RNAi strands upon electrospray ionization, so the same strand can appear twice on the MS chromatogram – once as a part of the decomposed duplex and again as the excess of upper or lower strand during the injection. If there is more than one chromatographic peak with the same mass, ProMass for MassLynx will match the one having highest abundance and report that one in the target mass summary report table. The quantifying aspect of ProMass for MassLynx was verified using 20 T and 25 T OST standards with injected concentration range 1 through 16 pmole.

As an alternative to the automatic high-throughput processing, MassLynx spectra can be deconvoluted manually one at a time simply by copying the spectrum list from MassLynx. It can be useful during the tuning of deconvolution parameters or for quickly processing the spectrum of interest.



Single strands

	File Name	Parameter File	Process	Sequence	Vial	Subject Text	Subject Sp...
82	...\Aug29_FA	...\SYNAPT 4_5.r	...\proce	rCrCrCrUrGrArGrArUrCrCrCrArGrCrGrCrUrGTT, rCrArGrCrGrCrUrGrGrGrArUrCrUrCrArGrGrGTT	23	sequence=duplex	...\znova OS

Target Mass Summary

RT (min)	Target Mass (Da)	Observed Mass (Da)	Mass Error	Intensity	% Abundance (in Spectrum)	%Purity (Estimate)	Identity	Result Code
4.54	6620.0	13359.9	6739.9 Da (101.811 %)	1.10E+003	65.40	0.00	Target Mass: 13360.1	
4.54	6740.1	13359.9	6619.8 Da (98.215 %)	1.10E+003	65.40	0.00	Target Mass: 13360.1	
4.54	13360.1	13359.9	-0.2 Da (-0.001 %)	1.10E+003	65.37	79.76	Target Mass	

Chromatogram Summary

RT (min)	Base Peak Mass (Da)	Intensity	Spectral Quality	LC/MS Peak Area	LC/MS Area Percent
4.54	13359.9	1.10E+003	low score	4.96E+002	100.00

Figure 5. Analysis of intact RNA duplex.

CONCLUSIONS

ProMass for MassLynx Software enables batch mass deconvolution and data analysis for UPLC-MS data generated on Waters MS instruments: single quadruple and QTof. The software was successfully adopted for the data acquired in the MassLynx project. Confirmation of the analyte mass is consistent with the molecular weight of the entered target sequence. ProMass for MassLynx assigns additional peaks on a chromatogram such as failed sequences/fragments and oligonucleotide modifications.

Multiple sequences and/or duplex oligonucleotides can be analyzed, which is a common task for the therapeutics applications.

The software significantly reduces the analysis times and enables automated UPLC-MS data processing in high-throughput mode. The interactively viewed color-coded results in web-based format are user-friendly and allow the researcher to navigate through the chromatographic data and the details of the mass spectra.

ProMass for MassLynx is the only software available on the market that performs automatic assignment of the high-complexity chromatograms of an oligonucleotide. The software allows significant time savings in labs that routinely perform quality control and study *in-vitro* metabolism of native and modified samples.

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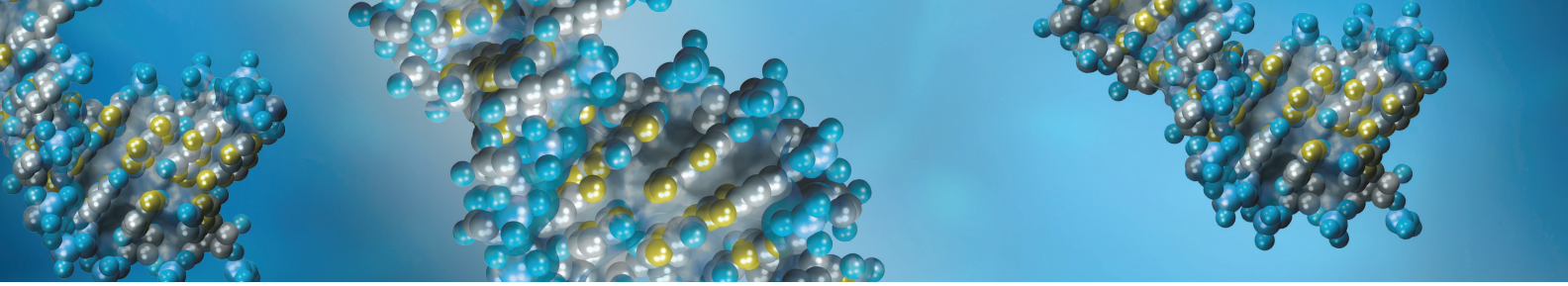

[UPLC-HRMS]

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UPLC-MS Analysis of Interfering RNA Oligonucleotides

Vera Ivleva
Waters Corporation, Milford, MA, USA

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:	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:	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

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.

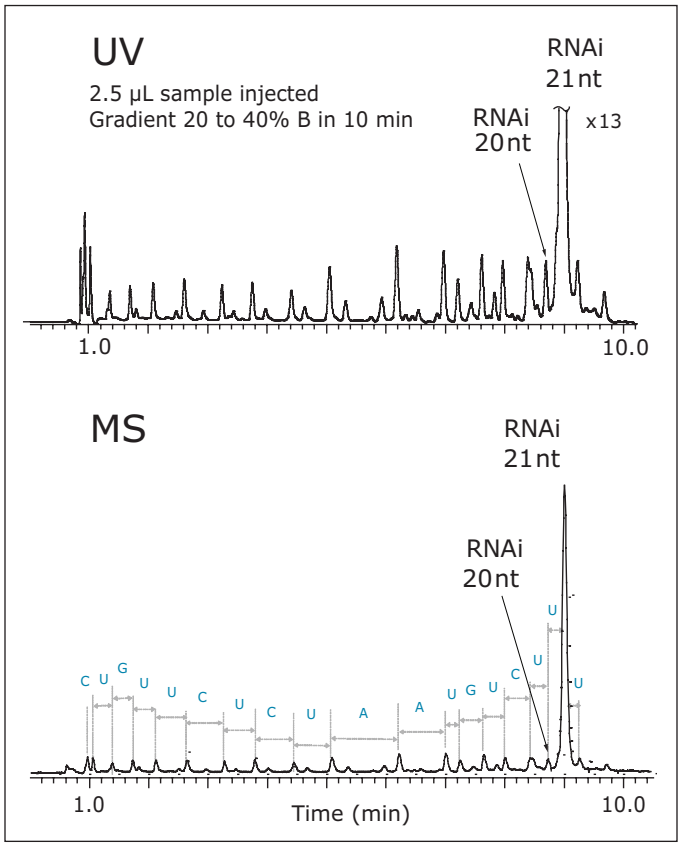

Figure 1. UV chromatogram (260 nm) and total ion chromatogram (TIC) of RNAi 21 nt.

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.

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.

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.

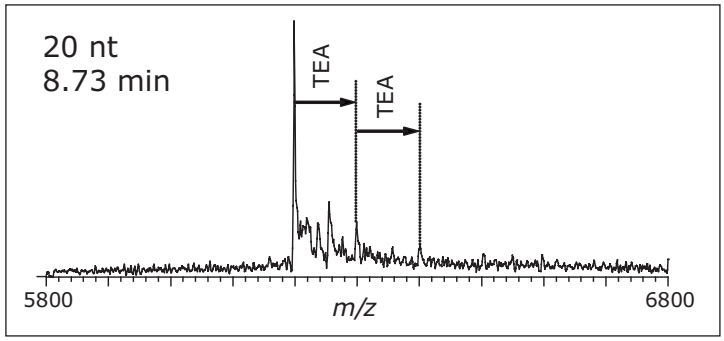

Figure 2. Deconvoluted mass spectrum of RNAi 20 nt using MaxEnt1.

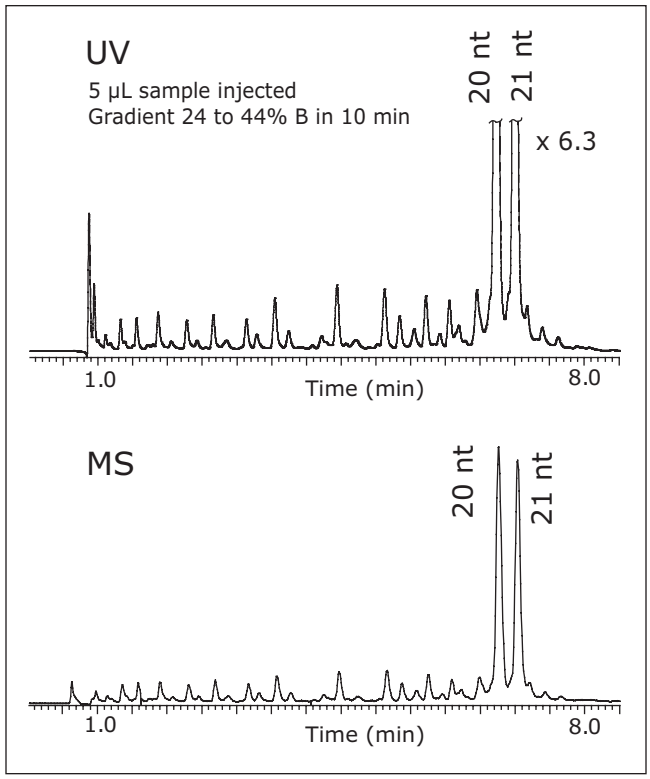

Figure 3. TIC and UV-chromatogram of the resolved oligomers of RNAi.

CONCLUSIONS

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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UPLC/UV-MS Analysis of Oligonucleotides

Vera Ivleva
Waters Corporation, Milford, MA, USA

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® (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.

EXPERIMENTAL

LC conditions

LC system:	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).

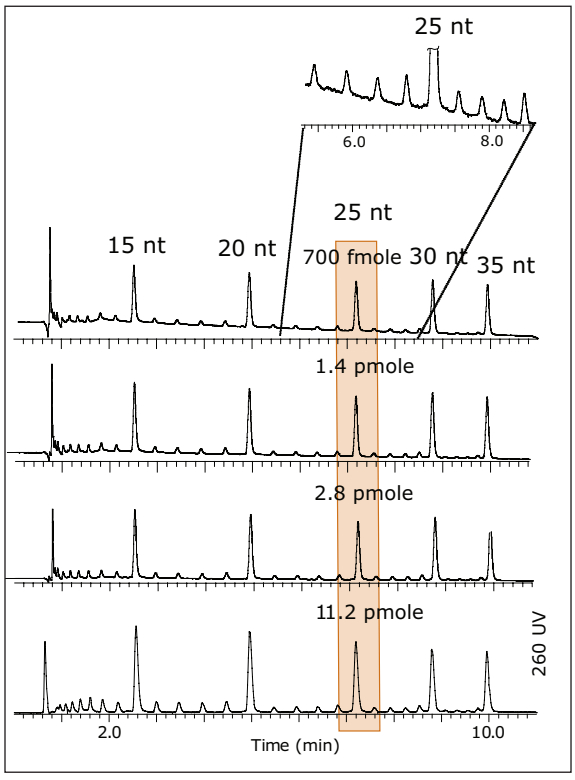

Figure 1. UV chromatograms (260 nm) of 15 to 35-mer oligonucleotide mixture. Injected quantities are highlighted based on 25 nt, which has the lowest concentration in the mixture.

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.

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.

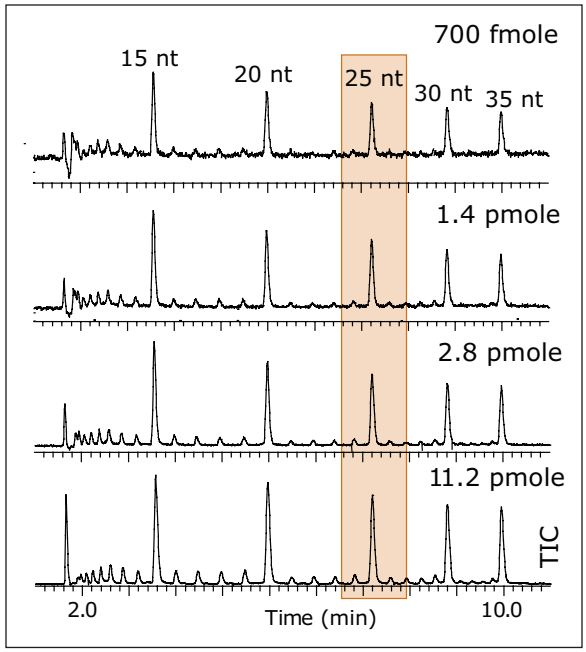

Figure 2. MS chromatograms of oligonucleotide sample (negative ion mode). The indicated injected amounts relate to 25 T oligonucleotide (700 fmole to 11.2 pmole).

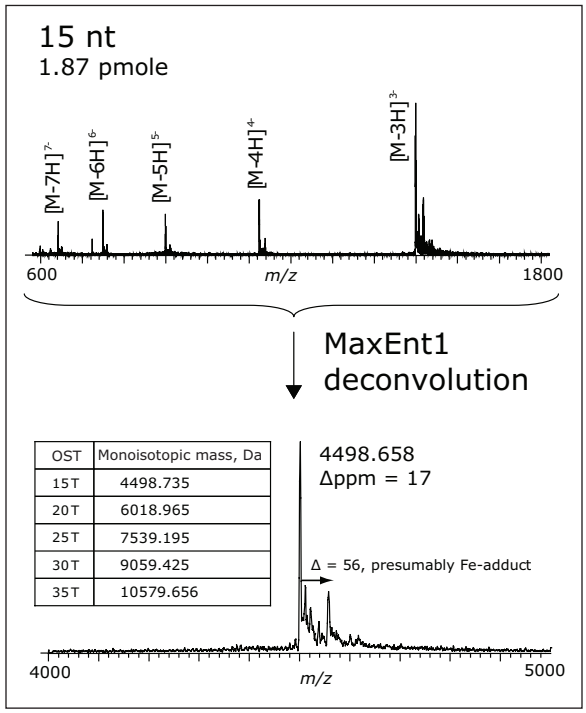

Figure 2. MS chromatograms of oligonucleotide sample (negative ion mode). The indicated injected amounts relate to 25 T oligonucleotide (700 fmole to 11.2 pmole).

CONCLUSIONS

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.

References

1. UPLC analysis of phosphorothioate oligonucleotides: methoddevelopment. Waters Application Note. 2007: 720002405EN.
2. UPLC/MS analysis of interfering RNA oligonucleotides. Waters Application Note. 2007: 720002412EN.

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Optimization of LCT Premier XE MS Settings for Oligonucleotide Analysis

Sean M. McCarthy, Vera Ivleva, Gordon Fujimoto, and Martin Gilar
Waters Corporation, Milford, MA, USA

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:	ACQUITY UPLC®
Column:	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 25-mer)		
Time	%A	Curve
0 min	31%	
5 min	50%	6

RESULTS AND DISCUSSION

Figure 1 shows a typical LC-MS chromatogram of 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).

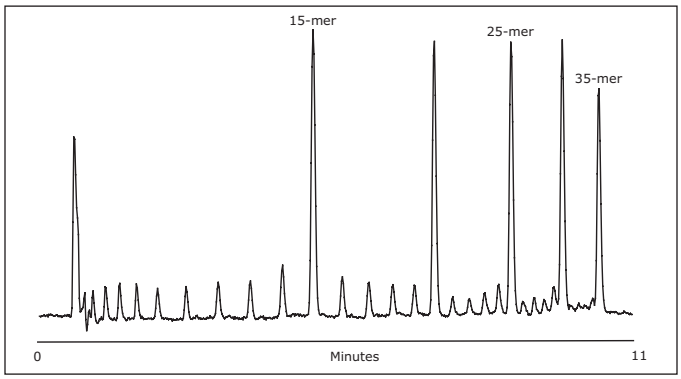

Figure 1. Total Ionic Current (TIC) for the separation of Waters OST standard detected with the LCT Premier XE System.

Polarity	ES-	Puller offset voltage	0.00
Analyzer	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 250 µL	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	Ion guide 1 initial RF	150.0
Y Focus (V)	0.0	Ion guide 1 final RF	150.0
Steering (V)	0.0	Ion guide 2 initial RF	200.0
Tube lens (V)	192.0	Ion guide 2 final RF	200.0
Attenuated Z focus (V)	500.0	Fixed hexapole RF	True
Normal Z focus (V)	65.0	Hexapole RF	180.0
TOF flight tube	5630.0	DRE Mass 0.0000 setting	5.0000
Reflection (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.

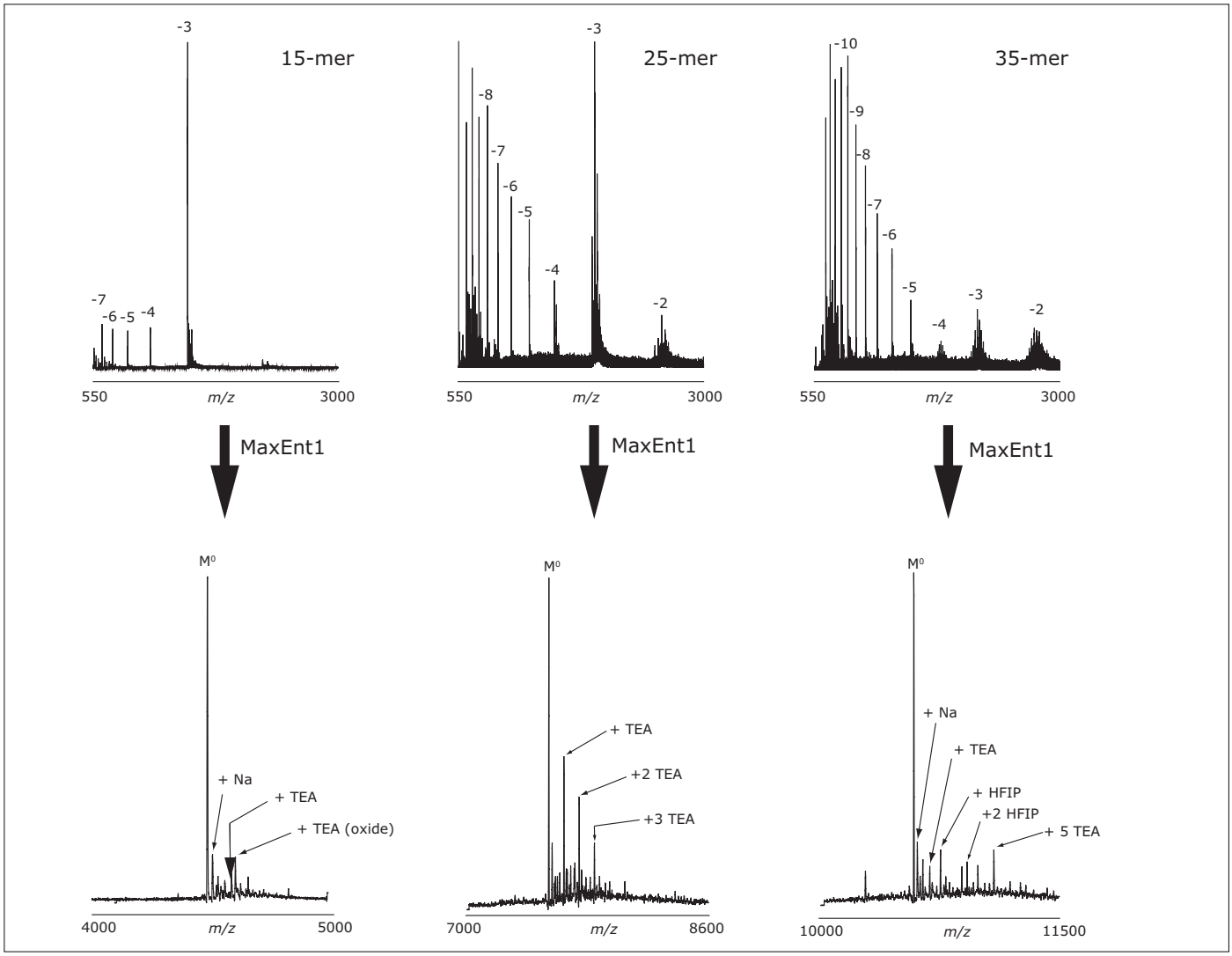

Figure 2. Raw and MaxEnt1 deconvoluted data for 15-, 25-, and 35-mer oligonucleotides of OST standard under normal operating conditions given in Table 1.

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.

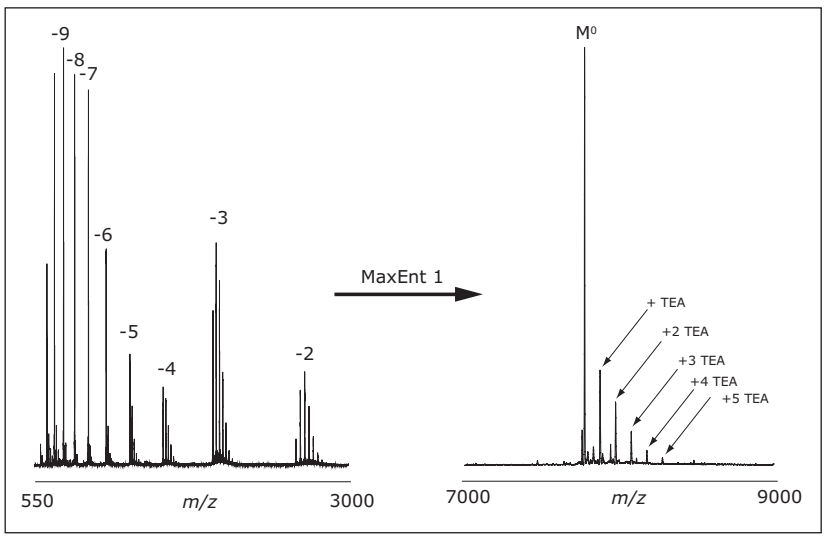

Figure 3. Raw and MaxEnt1 deconvoluted data for 25-mer phosphorothioate under normal operating conditions given in Table 1.

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.

Polarity	ES-	Puller offset voltage	0.00
Analyzer	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 250 µL	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	Ion guide 1 initial RF	150.0
Yfocus (V)	0.0	Ion guide 1 final RF	150.0
Steering (V)	0.0	Ion guide 2 initial RF	200.0
Tube lens (V)	192.0	Ion guide 2 final RF	200.0
Attenuated Z focus (V)	500.0	Fixed hexapole RF	True
Normal Z focus (V)	65.0	Hexapole RF	180.0
TOF flight tube	5630.0	DRE mass 0.0000 setting	5.0000
Reflection (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.

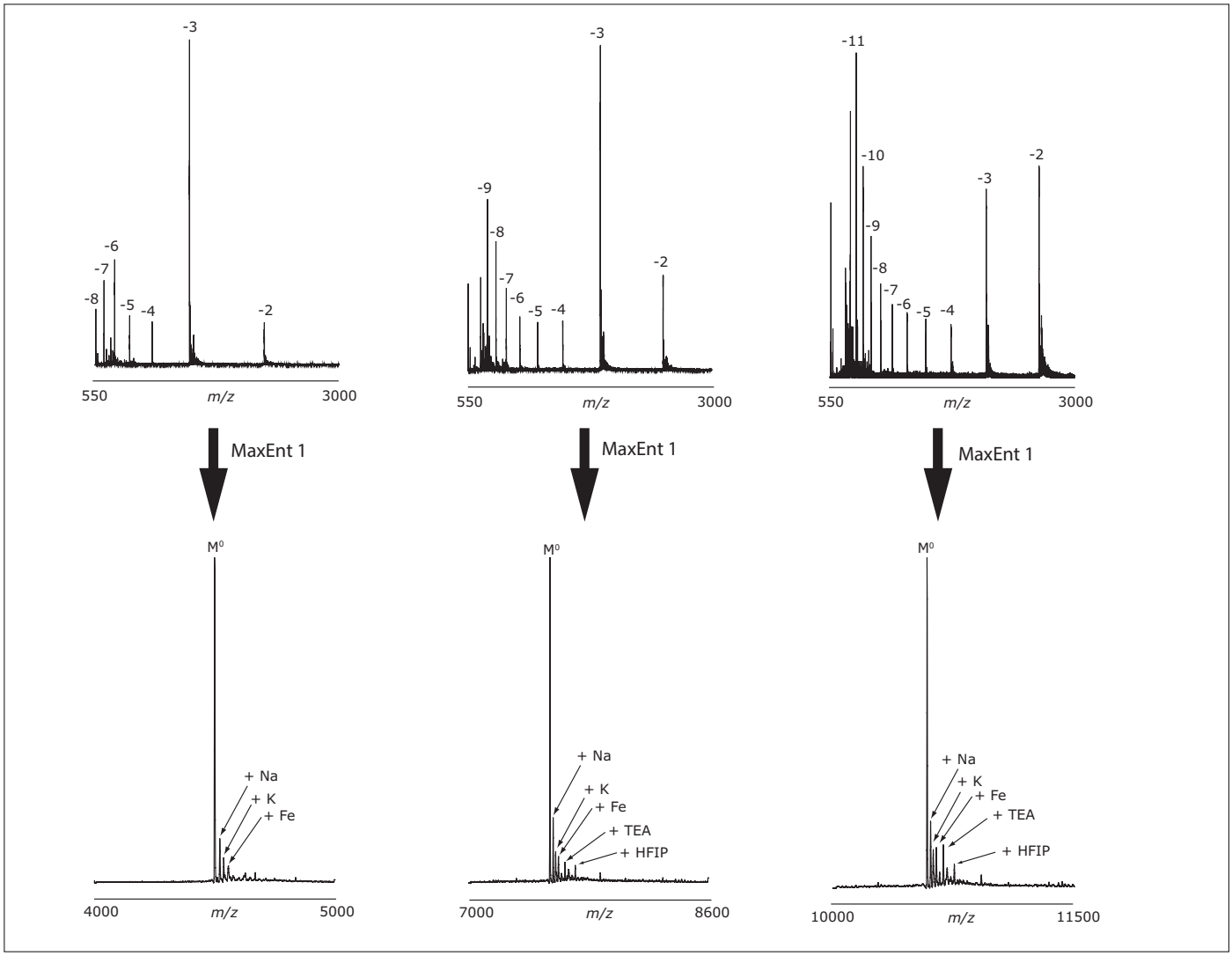

Figure 4. Raw and MaxEnt1 deconvoluted data for 15e, 25e, and 35-mer oligonucleotides of OST standard under optimal operating conditions given in Table 2.

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.

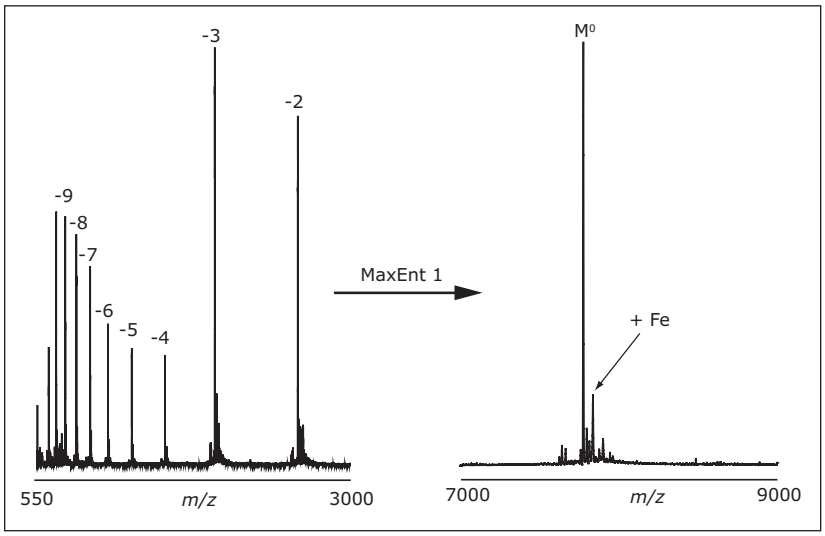

Figure 5. Raw and MaxEnt1 deconvoluted data for 25-mer phosphorothioate under normal operating conditions given in Table 2.

CONCLUSIONS

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/UV-MS Analysis of Phosphorothioate Oligonucleotides

Vera Ivleva
Waters Corporation, Milford, MA, USA

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 (p/n 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 2n, 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 MaxEnt1™ 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.

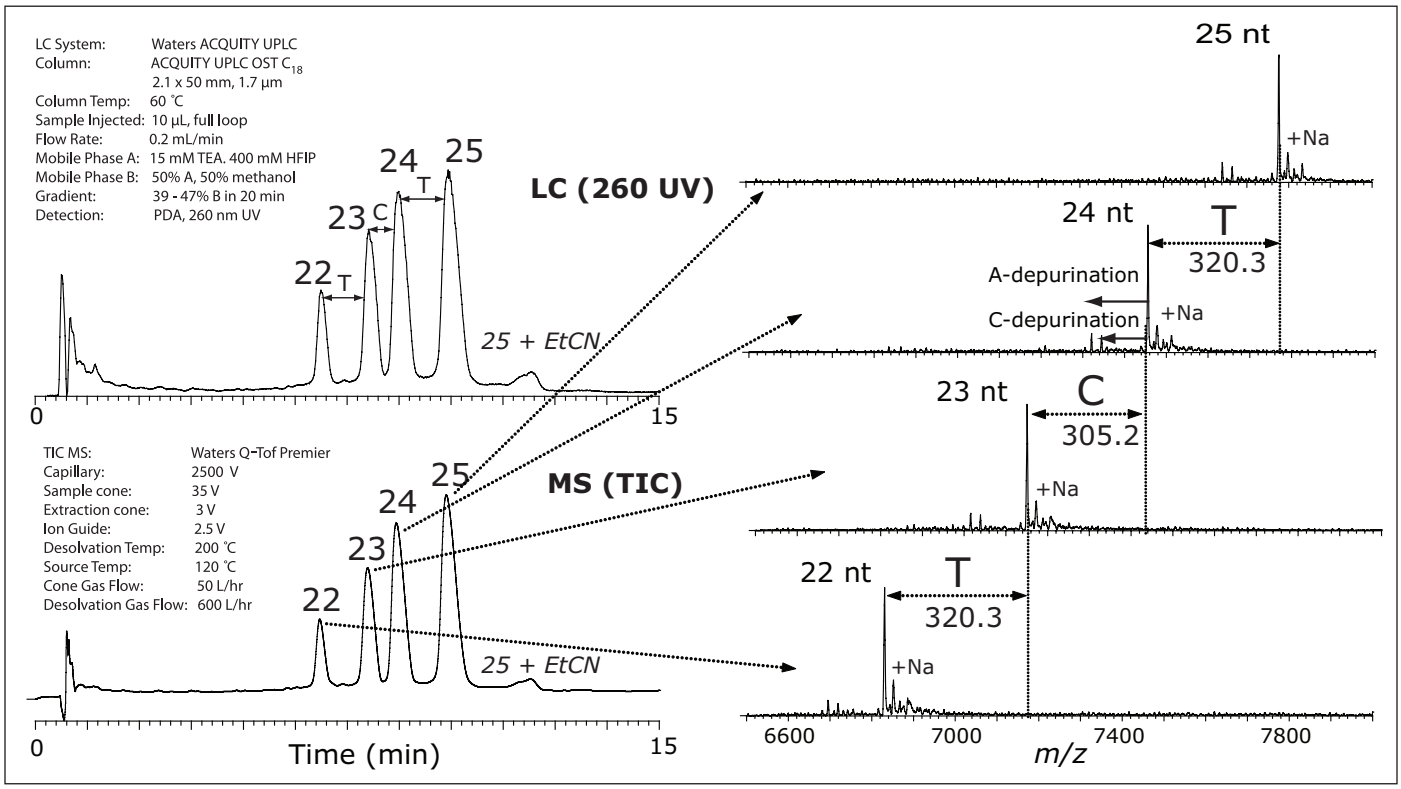

Figure 1. Left: UV and MS chromatograms of 25-mer phosphorothioate oligonucleotide and 3' truncated 24-, 23-, and 22-mer samples. Right: Corresponding MS spectra of the failed sequences (negative ion mode).

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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Developing a Novel, Integrated LC-MS Workflow for High-resolution Monitoring and Characterization of Oligonucleotides

Henry Shion, Robert Birdsall, and Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Optimized RP-UPLC mobile phase compositions for better oligonucleotide separation and lower salt adduct intensity in the MS spectra
- Newly enabled high-resolution, high-throughput data processing capability using ProMass HR integrated with MassLynx® Software
- Enhanced MS/MS fragmentation with the Xevo® G2-XS QTof MS System for full ladder sequencing of oligonucleotide samples

WATERS SOLUTIONS

Xevo G2-XS QTof Mass Spectrometer

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Tunable Ultraviolet (TUV) Detector

Waters® Oligonucleotide Separation Technology (OST)

MassLynx Mass Spectrometry Software

ProMass for MassLynx Software

KEYWORDS

Biotherapeutics, oligonucleotide, DNA, siRNA, Oligonucleotide Separation Technology (OST), MassPREP™ Oligonucleotide Standard, ProMass HR, ACQUITY H-Class Bio, Xevo G2-XS QTof, MassLynx

INTRODUCTION

Methods that enable structural characterization, molecular weight confirmation, and impurity analysis and profiling (e.g. failed sequences and other production-related impurities) are of great importance for therapeutic oligonucleotide development. These assays are often conducted using liquid chromatography coupled with both ultraviolet (UV) and mass spectrometry (MS) detection – enabling quantification via UV absorbance – and structural characterization, mass confirmation, and impurity monitoring via MS detection. The Waters Xevo G2-XS QTof Mass Spectrometer has proven to be an effective tool for characterizing biopharmaceutical drugs through analytical procedures such as intact mass analysis, subunit analysis, peptide mapping, and released glycan analysis.[1–4]

In this study, we describe an integrated LC-MS workflow for identification, impurity profiling, and MS/MS characterization of oligonucleotides using high-resolution mass spectrometry. The complete workflow consists of an ACQUITY UPLC H-Class Bio System coupled to an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector and the Xevo G2-XS QTof Mass Spectrometer. System control and MS data acquisition is accomplished using Waters MassLynx Mass Spectrometry Software. Automated spectral deconvolution, data analysis, and reporting are enabled through an integrated version of ProMass HR (Novatia, Newtown, PA). High-resolution oligonucleotide characterization was demonstrated by performing a full ladder sequencing of a siRNA sample using the MS/MS spectrum within MassLynx.

Figure 1. From left to right: MassPREP Oligonucleotide Standard, Waters Oligonucleotide Separation Technology columns, the ACQUITY UPLC H-Class Bio System, and Xevo G2-XS QTof Mass Spectrometer.

EXPERIMENTAL

Reagents, solvents, and sample preparation

Triethylamine (99.5% purity) and 1,1,1,3,3,3-Hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. Mass spectrometry grade solvents (UHPLC grade) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. MassPREP Oligonucleotide Standard was purchased from Waters (P/N 186004135) and prepared at various concentrations (0.004~5 pmol/µL). siRNAs upper strand 5'-rUrCrGrUrCrArArGrCrGrArUrUrArCrArArGrGrTT-3' and the complementary lower strand 5'-TTrCrCrUrUrGrUrArArUrCrGrCrUrUrGrArCrGrA-3' were purchased from Integrated DNA Technologies (Coralville, Iowa) and prepared at a concentration of 5 pmol/µL in water. Injection volumes were varied depending on the experiments.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV w/Ti flow cell absorption wavelength: 260 nm
Column:	Waters Oligonucleotide BEH C_{18} Column, 1.7 µm, 2.1 mm x 50 mm (P/N 186003949)
Column temp.:	60 °C
Sample temp.:	6 °C
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

Mobile phases were prepared gravimetrically.

MS conditions

MS system:	Xevo G2-XS QTof
Data mass range:	400–3000 Da
Mode:	ESI negative resolution
Cone voltage:	80 V for MS and 120 for MS/MS
Capillary voltage:	2.0 kV
Source offset:	80 V
Source temp.:	125 °C
Desolvation temp.:	500 °C
Desolvation gas low:	800 L/Hr
Lockmass:	Glu Fibrinopeptide B at 100 fmol/µL in 50–50 H_2O-ACN, 0.1% FA

Informatics for data collection and processing

MassLynx v4.1 SCN 9.25 with MaxEnt™1 and MaxEnt3

ProMass HR

ProMass Bridge 1.1

For Waters OST Standard (contains a mix of 5 polyT oligonucleotides, 15–35 nt)					
Time (min)	Flow (mL/min)	%A	%B	%C	%D
Initial	0.20	80.50	19.5	0.0	0.0
15.00	0.20	72.00	28.0	0.0	0.0
16.00	0.20	50.00	50.0	0.0	0.0
17.00	0.20	80.50	19.5	0.0	0.0
21.00	0.20	80.50	19.5	0.0	0.0

Gradient Table 1.

For ssRNA Samples					
Time (min)	Flow (mL/min)	%A	%B	%C	%D
Initial	0.20	87.0	13.0	0.0	0.0
10.00	0.20	77.0	23.0	0.0	0.0
10.10	0.20	50.0	50.0	0.0	0.0
11.10	0.20	50.0	50.0	0.0	0.0
11.20	0.20	87.0	13.0	0.0	0.0
16.00	0.20	87.0	13.0	0.0	0.0

Gradient Table 2.

RESULTS AND DISCUSSION

In this section we review the experimental results generated using the aforementioned high-resolution LC-MS workflow for oligonucleotide analysis; demonstrate the basic performance of the system in terms of mass accuracy, salt adduct reduction, and MS detection sensitivity; and discuss the fit-for-purpose capabilities for high-resolution MS screening and impurity profiling of oligonucleotides, as well as high-resolution characterization via MS/MS ladder sequencing.

Figure 2 shows excellent separation of the polyT oligonucleotides and their truncation sequences (achieved using Waters OST column with BEH C_{18} chemistry). As described in recent studies, LC-MS grade reagents, such as TEA and HFIP, and mobile phase solvents, such as acetonitrile and water, were used to ensure the best LC peak separations.[5,6] The LC-MS grade reagents and solvents also play an important role in helping generate high-quality MS spectra. An MS spectrum example is shown in Figure 3 that displays the multiple charged species distribution/charge envelope of the 25 nt polyT, which demonstrates minimal sodium adduct formation (<5%) and excellent isotopic peak resolution in the zoom-in region of the M^{3-} peak.

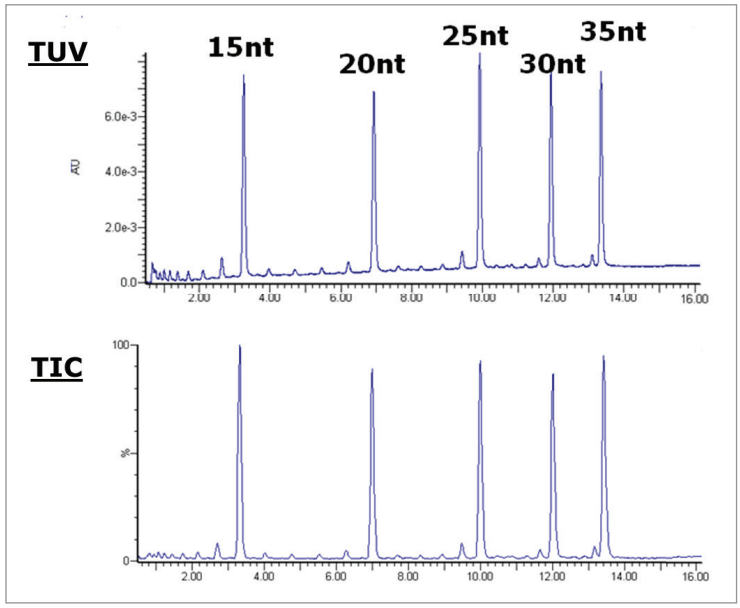

Figure 2. In-line orthogonal TUV and TIC chromatograms of the Waters MassPREP Oligonucleotide Separation Technology Standard, containing a mixture of five polyT oligonucleotides from 15 to 35 nt, with expected synthesis-related impurities.

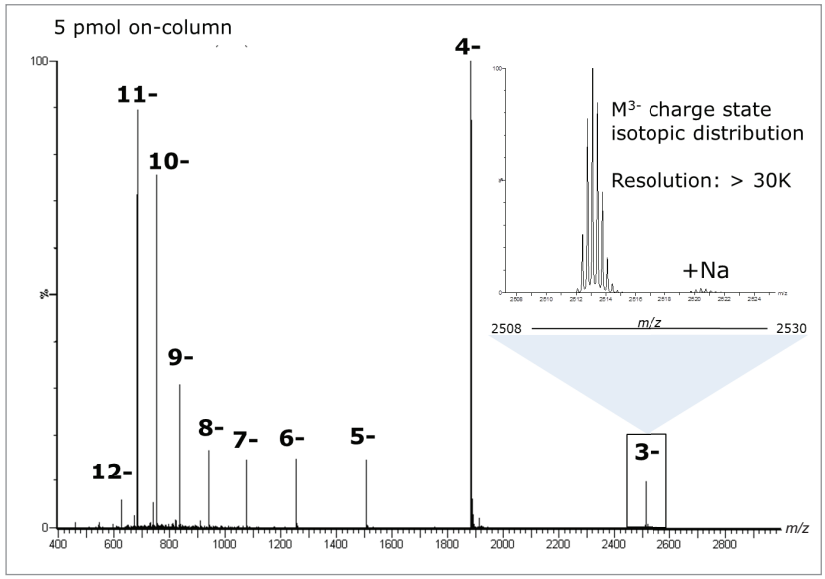

Figure 3. Combined raw ESI spectrum for the 25 nt polyT oligonucleotide from the MassPREP OST Standard.

Using ProMass HR*, data acquired by MassLynx can be automatically processed to obtain the deconvoluted exact monoisotopic masses contained within each sample. Table 1 is a summary from the ProMass HR processed report that displays the monoisotopic mass accuracy for the five polyT oligonucleotides contained within the MassPREP OST Standard. The average mass accuracy across all five polyT oligonucleotides was about 1.25 ppm.

The limit of detection (LOD) of the LC-MS system using the MassPrep OST Standard was determined to be 20 fmol on-column as shown in Figure 4. The comparison between the TUV and TIC chromatograms in the figure suggests the LOD is limited by the optical/TUV signal, and not by the Xevo G2-XS QTof MS instrument.

Using MassLynx in combination with ProMass HR, high-resolution screening and impurity profiling of synthetic oligonucleotides can be performed with automated data acquisition, processing, and analysis for greater productivity. The MassLynx sample list in Figure 5 shows the fields that should be included for this automated process.

**ProMass HR can be used to process high-resolution MS data, such as data generated by time-of-flight (Tof) systems, as well as low-resolution MS data, such as data generated by quadrupole systems. ProMass can only be used to process low-resolution MS data.*

MassPREP OST Standard	Expected mass	Observed mass	Mass accuracy (ppm)
15 nt	4498.7348	4498.7300	-1.07
20 nt	6018.9650	6018.9730	1.33
25 nt	7539.1952	7539.1990	0.50
30 nt	9059.4254	9059.4210	-0.49
35 nt	10579.6560	10579.6260	-2.79
		Average	1.24

Table 1. Summary table of monoisotopic mass accuracy for the MassPREP OST Standard, containing a mixture of five polyT oligonucleotides.

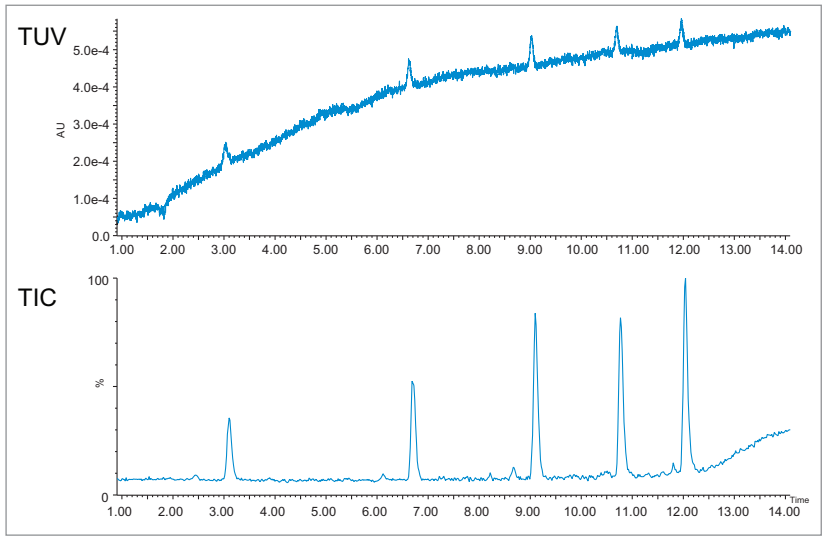

Figure 4. TUV (top) and TIC (bottom) chromatograms for an injection of 20 fmol of the MassPREP OST Standard, containing a mixture of five polyT oligonucleotides.

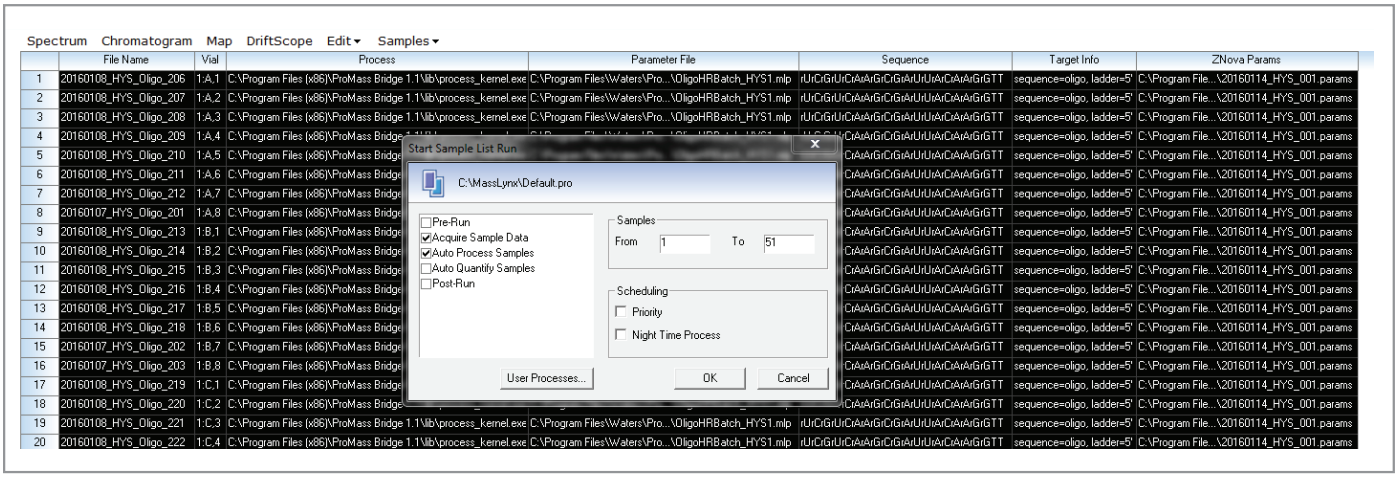

Figure 5. The MassLynx sample list shows the fields required for the automated data acquisition, processing, and analysis process. They include the sample-specific file names where data is stored, the designated injection vial, the process and parameter file locations, the sequence(s) of the targeted molecule(s), the molecule types and their prime information, and the ZNova file that defines ProMass HR parameters.

Figure 6 displays the TUV chromatogram of one of the targeted ssRNA, which has the sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang (upper strand). The chromatogram shows that the targeted ssRNA was well separated from its single base deletion (N-1) and single base insertion (N+1) forms.

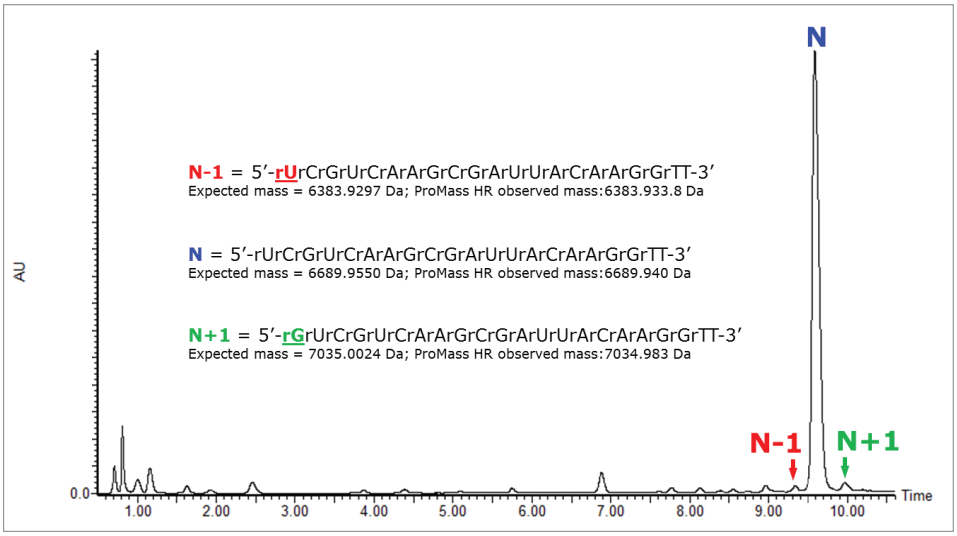

Figure 6. The TUV chromatogram of ssRNA sequence 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang (upper strand of siRNA) shows good separation between the target sequence and the single base deletion (N-1) and single base insertion (N+1) forms using a ten minute LC gradient from 13% B to 23% B.

The batch-processed screening and profiling results for 48 samples, including eight blanks, are reported in Figure 7. The report displays a color-coded LC sample tray where samples containing the target oligonucleotide (upper strand) are marked in green, samples containing a different oligonucleotide (lower strand) are marked in red, and samples containing no oligonucleotide are marked in white. Each well position in the plate format acts as a hyperlink through which one can easily access the detailed experimental results for each sample, such as: raw ESI spectra, chromatographic retention times, variations between expected and observed masses (with mass accuracy in ppm), relative percent area of the targeted oligonucleotide(s), and associated impurities – which, when automatically identified, are labeled.

For instance, as shown in the lower table in Figure 7, the targeted ssRNA (upper strand), its deletion (N-1) of uracil, and the insertion (N+1) of guanine impurities are clearly identified and labeled, and the mass accuracies of 0.5 ppm, 2.2 ppm, and 2.8 ppm and retention times of 9.08, 9.28, and 9.73 minutes are reported for each, respectively. Additionally, from this data an estimate of the percent purity was calculated at 84.26%. The color-coded HTML report interface makes it easy to assess the results.

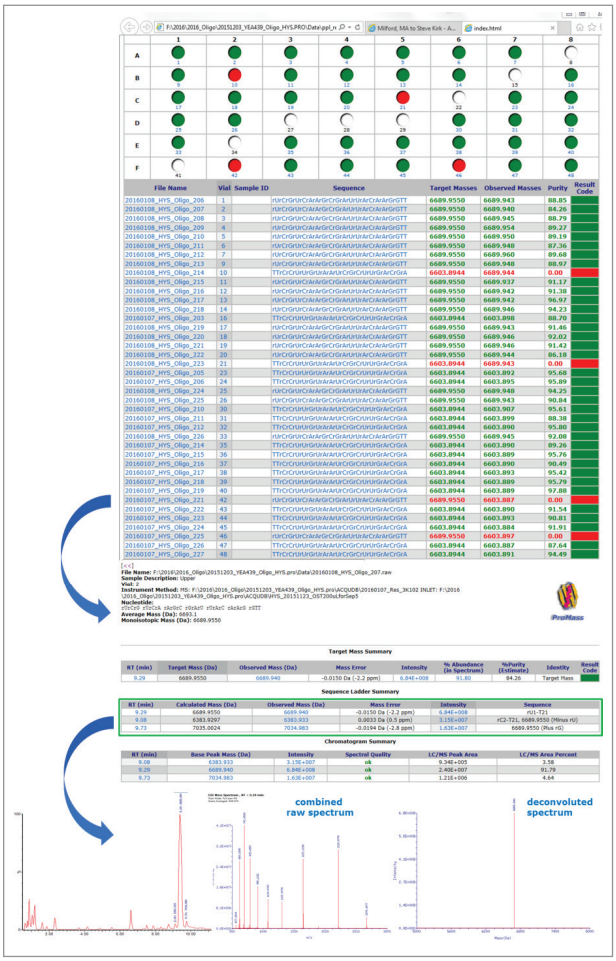

Figure 7. The top image is a high-level summary of batch-processed, experimental results using ProMass HR with MassLynx. Also shown, successive drill-down results for an individual sample, accessed through embedded HTML hyperlinks.

To test the suitability of the LC-MS system for oligonucleotide sequence confirmation, a targeted LC-MS/MS experiment was performed using the siRNA upper strand. Figure 8 shows the MS full scan spectrum of the 21 nt ssRNA sample on the top and the deconvoluted MS/MS spectrum on the bottom. An individual charge state (M^{4-} in this case) was chosen as the precursor ion and then fragmented in the Xevo G2-XS QTof collision cell with collision energy (CE) of 35 V. The MS/MS spectrum was then deconvoluted using MaxEnt3. Formulas composed in an Excel file were used to generate the list of predicted fragment ions based upon the oligonucleotide sequence. Next, the deconvoluted spectrum was manually matched against the list of predicted fragment ions. By finding most of all the predicted ions within the deconvoluted MS/MS spectrum, the sequence of the oligonucleotide can be confirmed. For the siRNA upper strand 21 nt sample and its complementary lower strand (data not shown), all of the C and Y ions were successfully matched to achieve a full ladder sequencing of the samples.

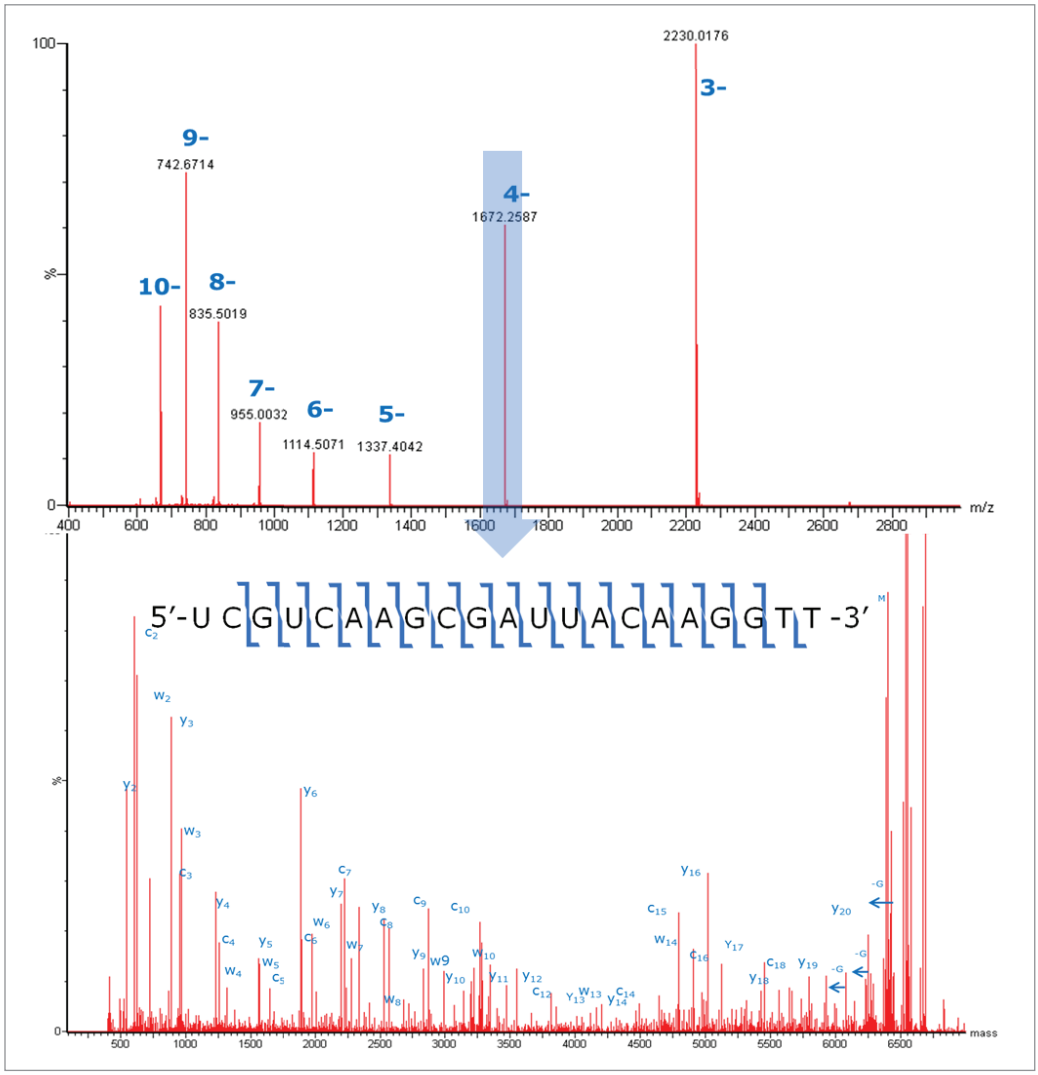

Figure 8. LC-MS/MS full ladder sequence confirmation for the ssRNA sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' was achieved. The experiment was conducted using the M^{4-} peak as the precursor ion. The fragmentation peak assignments were done by matching against the theoretical masses using simple Excel file calculations. MaxEnt3 was used for charge deconvolution and deisotoping.

CONCLUSIONS

A newly-integrated LC-MS workflow for high-resolution mass spectrometry characterization and screening of oligonucleotides has been developed. To ensure the best LC separation and MS detection with the highest mass resolution and lowest salt adduct peaks (less than 5% intensity in the MS spectra), it is recommended to use LC-MS grade TEA, HFIP, and water. The system has been shown to have excellent sensitivity, with a LOD of 20 fmol on-column using the MassPREP Oligonucleotide Standard, containing a mixture of five polyT oligonucleotides. The integration of ProMass HR with MassLynx allows for automated deconvolution of high-resolution LC-MS data as well as automated target ID and impurity analysis of oligonucleotide samples using the Xevo G2-XS QTof MS instrument as demonstrated.

The LC component of the workflow demonstrates excellent and highly reproducible separation of oligonucleotides from their truncation (N-1) and insertion (N+1) sequences, and MS data quality confirm the robustness of the separation and detection workflow. The interactive HTML-based, color-coded summary report generated through ProMass HR makes it easy to review high-throughput experimental results and to navigate/drill down into sample-specific data, including chromatograms, combined raw spectra, deconvoluted spectra, impurity analysis, etc. LC-MS/MS experiments were also performed with the Xevo G2-XS QTof MS instrument, and demonstrate the ability of the system to accomplish full ladder MS-MS sequence confirmation of a 21 nt ssRNA oligonucleotide.

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Impressive Sensitivity and Linearity of Oligonucleotides on a Xevo QTof MS

Sean M. McCarthy, Vera Ivleva, St John Skilton, and Martin Gilar
Waters Corporation, Milford, MA, USA

INTRODUCTION

This work highlights the improved performance characteristics of Waters® biopharmaceutical system solutions for oligonucleotides analysis. The ability to detect at better sensitivity and to provide a linear range for quantitation is demonstrated using UPLC® Technology with the Xevo® QTof Mass Spectrometer. Organizations that are able to simultaneously quantify and identify therapeutic oligonucleotides will realize business advantages because they will have a better characterization of their product, and will be able to get products to customers more rapidly.

Oligonucleotides are generally quantified by UV methods because the absorbance measured at 260 nM is not dependent on sequence or structure. Although quantitation by UV may be preferable, Waters' benchtop mass spectrometer, the Xevo QTof MS, makes comparisons of quantitative and qualitative data more achievable.

In this technical note, we demonstrate that Waters can detect LC-MS oligonucleotides peaks to the low-femtomolar level. The example illustrated shows linear detection in the range of 5 picomolar to 25 femtomolar on-column of a multi-T oligomer.

A standard mix made by Waters that contains a range of oligomers of 15-mer, 20-mer, 25-mer, 30-mer, and 35-mer length (186004135) was used for the analysis. This mixture is used to demonstrate the separation capability of the ACQUITY UPLC® System with a UPLC Oligonucleotide Separation Technology (OST) Column and to demonstrate the ability of the Xevo QTof MS to detect a mixture of oligomers in 15 minutes. This separation and MS detection is challenging to achieve in HPLC mode.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC System
Column:	UPLC Oligonucleotide Separation Technology (OST) Column
Mobile phase A:	15 mM TEA in 400 mM HFIP (pH 7.9)
Mobile phase B:	50% MeOH in A
Gradient:	38 to 48% B in 10 min

MS conditions

MS system:	Xevo QTof MS
Ionization mode:	ESI negative
Capillary voltage:	2.5 kV
Cone voltage:	20 V
Extraction cone:	4 V

RESULTS AND DISCUSSION

Figure 1 shows the calibration curve obtained for the 20-mer oligomer in the range 25 to 500 femtomolar using 12 acquisitions (scans) across the chromatographic peak apex. The signals of the other oligomers in the standard mixture are normalized to that of the 20-mer oligomer and therefore quantities can be extrapolated for the other oligomers.

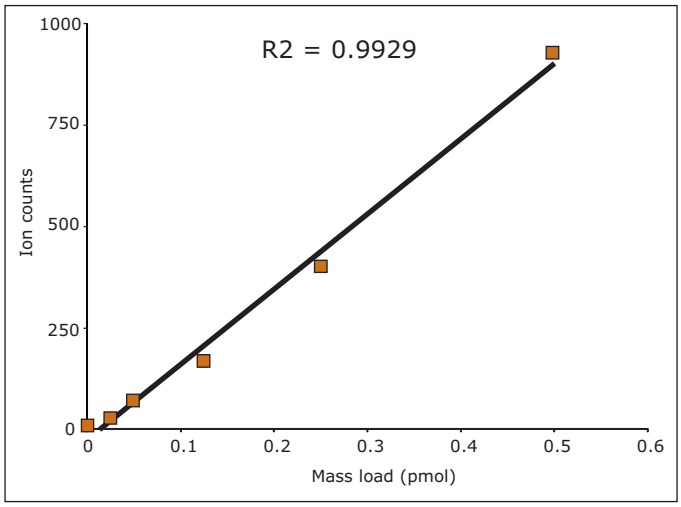

Figure 1. Linearity curve for the 20-mer oligomer at different total column loadings using chromatographic peak areas at the apex.

At the lowest loading level, shown in Figure 2, the most abundant charge state (3-) of the 20-mer is shown to indicate the good signal-to-noise ratio of approximately 10:1 (Raw data with no smoothing in continuum mode).

The raw spectrum of the 3- charge state can be compared to the theoretical data at 10,000 RP. This is shown in Figure 3 where 12 scans at the chromatographic peak apex were averaged to obtain the spectrum. The agreement with the theoretical spectrum for that charge state is 0.016 Da and the deconvoluted data shows agreement with the theoretical isotopic masses. Figure 4 shows the raw and smoothed data compared to the MaxEnt1™ deconvoluted data.

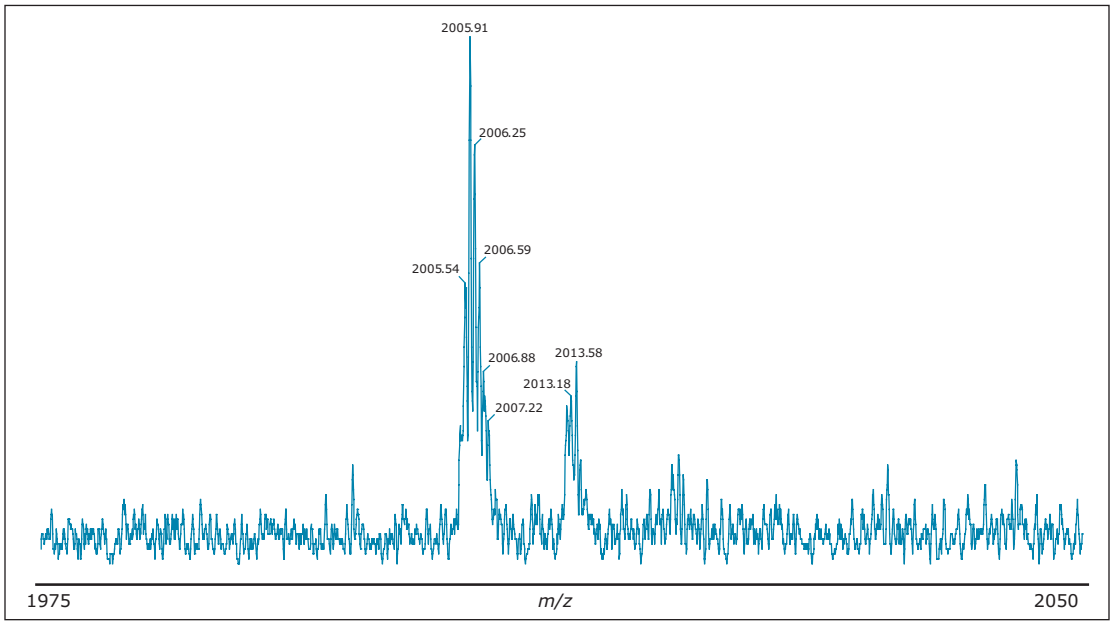

Figure 2. A single spectrum of the multiply-charged (3-) 20-mer oligomer (2005 m/z) with a sodium adduct at 2013 m/z. The resolution of the Xevo QTof MS allows partial isotopic separation at 10,000 RP FWHM.

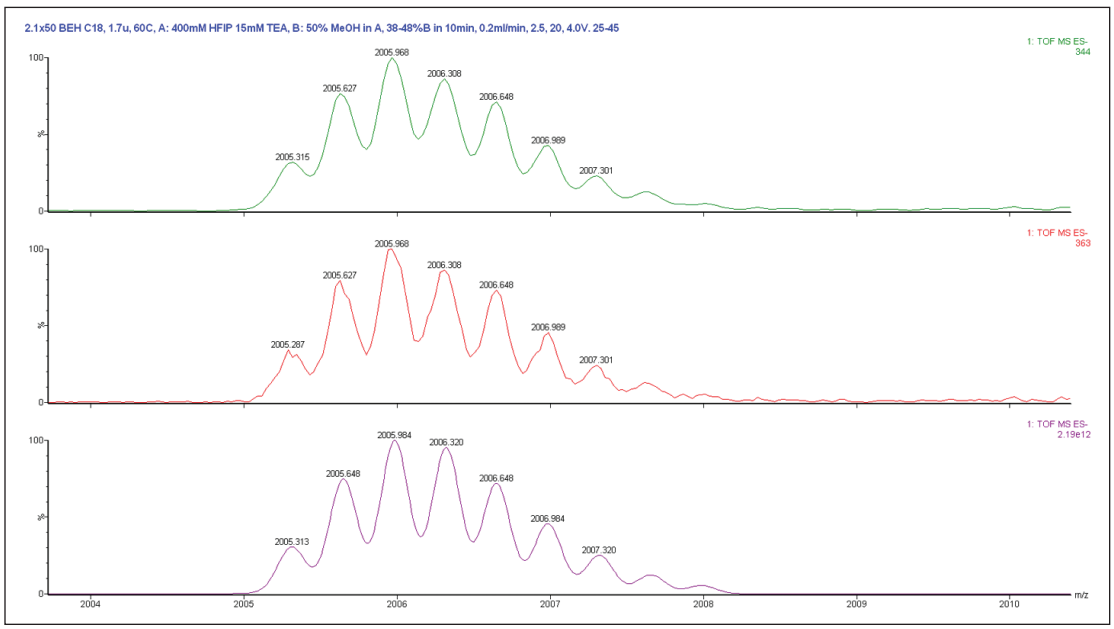

Figure 3. Comparison of actual versus theoretical traces of the 3- charge state of the 20-mer oligomer at 10,000 resolution showing the match to the data obtained on Waters Xevo QTof MS. The top trace shows the smoothed data (1,3 Savitsky-Golay), the middle trace shows the raw continuum data for the 250 femtomol sample and the bottom trace shows the theoretical isotope model for that elemental composition available in MassLynx® 4.1. Note the excellent agreement in masses at peak top between theoretical, and raw and smoothed data.

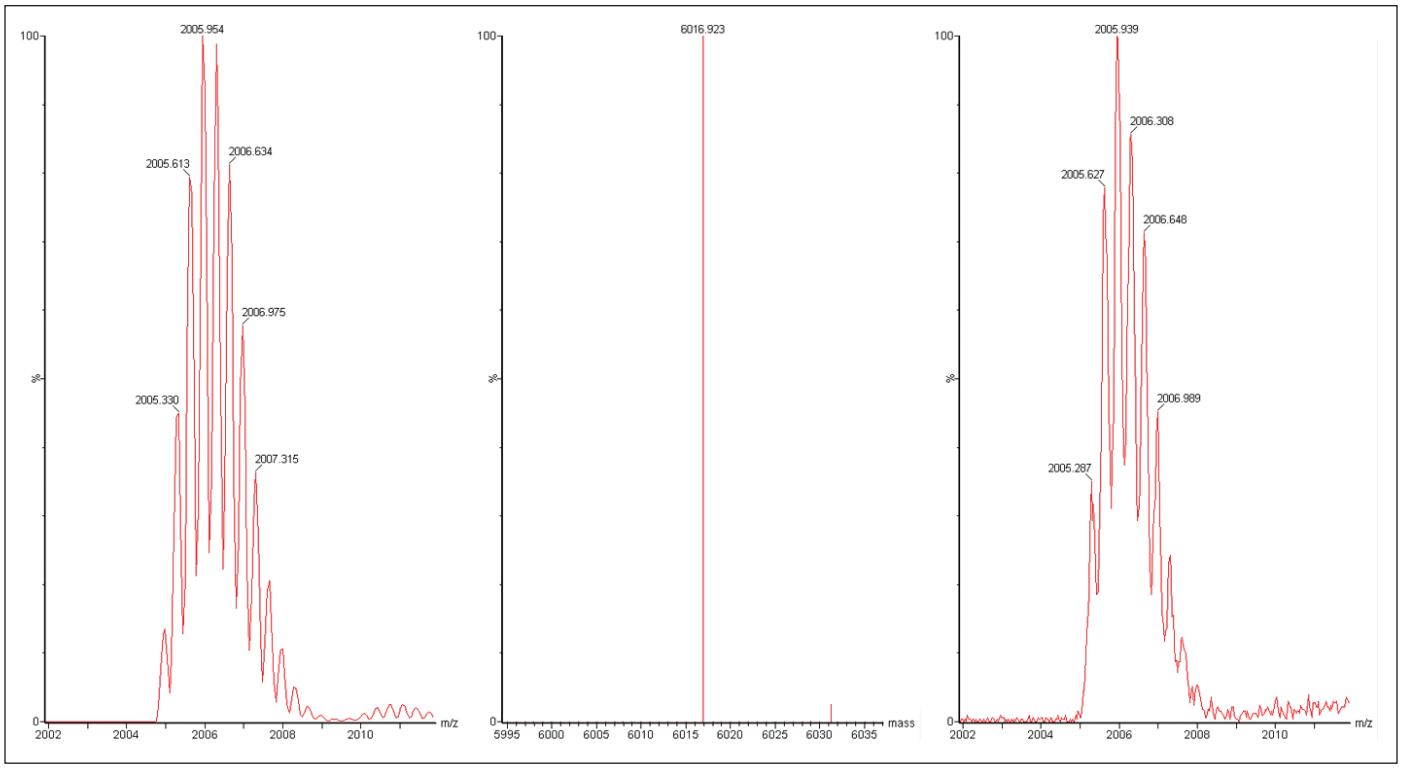

Figure 4. MaxEnt1 deconvoluted spectrum of the 20-mer oligomer at 100 pMol level to confirm the accurate mass of the analyte.

It is important to note that being able to achieve these separations relies on the chromatographic system. UPLC OST Column Technology has been specifically designed for the ACQUITY UPLC System and for these types of sample. The lack of carryover is shown in Figure 5, where a blank run and a 25 femtomolar sample are shown.

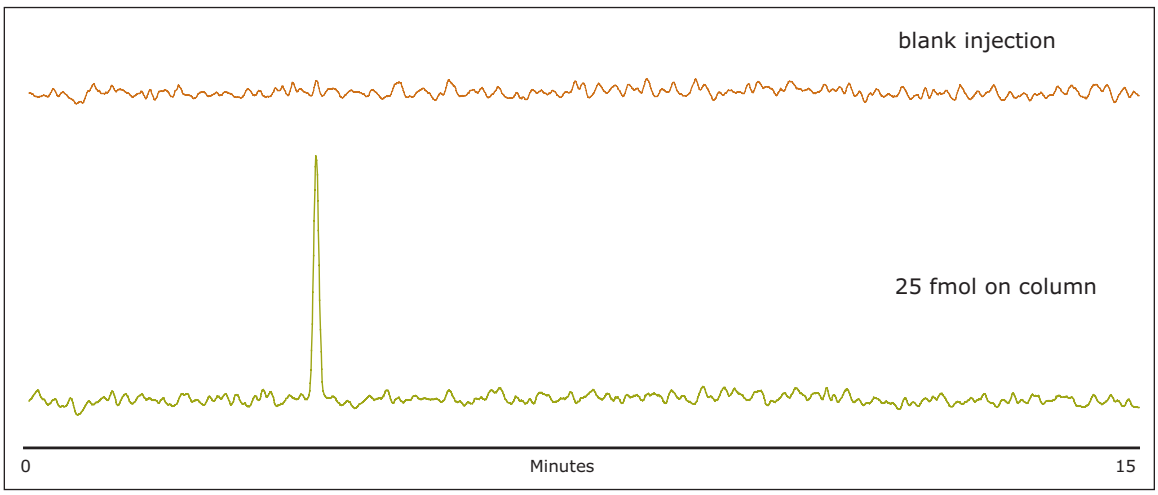

Figure 5. Extracted ion chromatogram of the 20-mer oligomer (bottom trace) showing the lack of carryover in the blank sample (top trace).

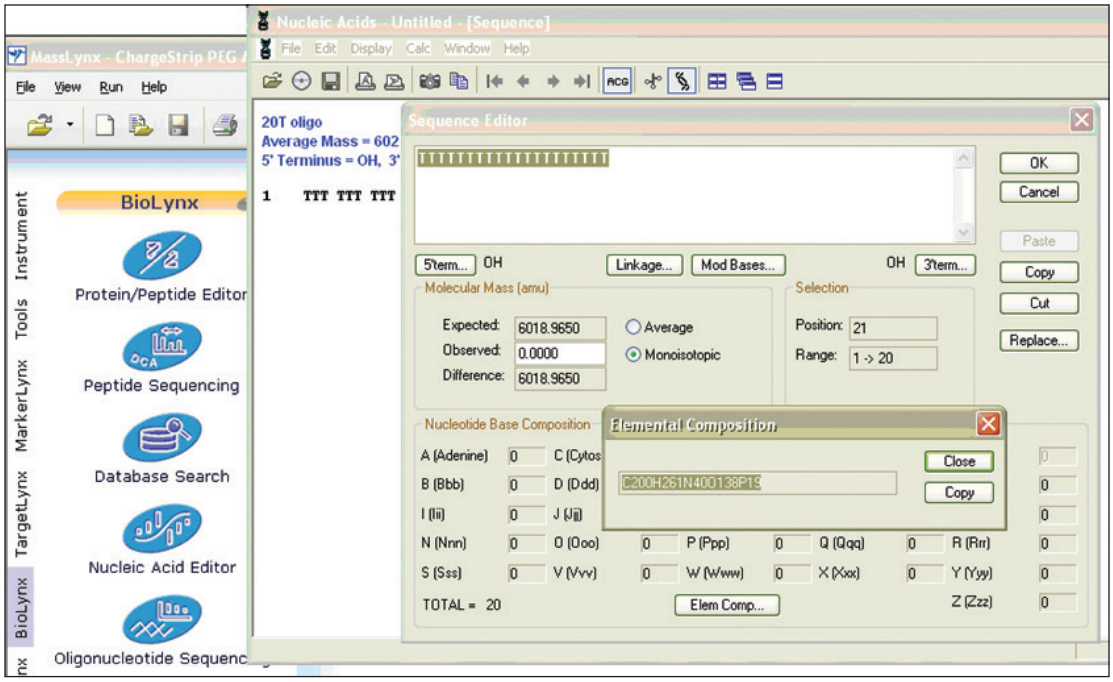

Figure 6. Mass calculations based on the sequence of the 20-mer showing the elemental composition of the oligonucleotides from which an accurate mass is derived.

CONCLUSIONS

The sensitivity demonstrated in this application note illustrates that Waters has achieved approximately three to six times higher sensitivity when compared to previous-generation MS instrumentation. This will potentially prove useful for those laboratories that wish to push the levels of sensitivity further. The ability to obtain a linear calibration curve over multiple orders of magnitude at this low level is useful for those who need to quantify oligonucleotides at low levels.

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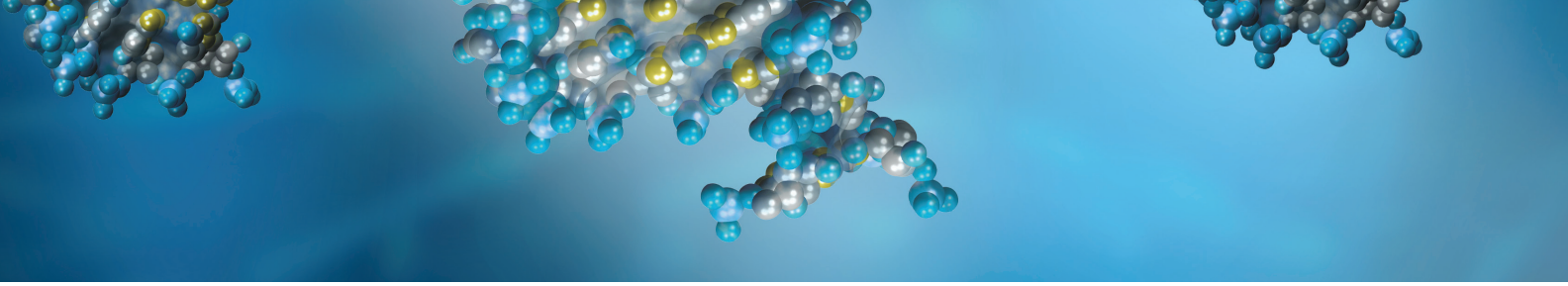

[UPLC-HDMS]

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UPLC SYNAPT MS-MS Method for Structural Characterization of siRNA Oligonucleotides

Vera B. Ivleva, Ying Qing Yu, and Martin Gilar
Waters Corporation, Milford, MA, USA

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 ACQUITY UPLC Oligonucleotide Separation Technology (OST) C_{18} Column, 1.7 µm, 2.1 x 50 mm (P/N 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:	ACQUITY UPLC
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:	SYNAPT HDMS
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 wellresolved from the original target 21 nt (Figure 1). Components were assigned based on their exact mass measurement, providing partial sequence verification.[1]

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).

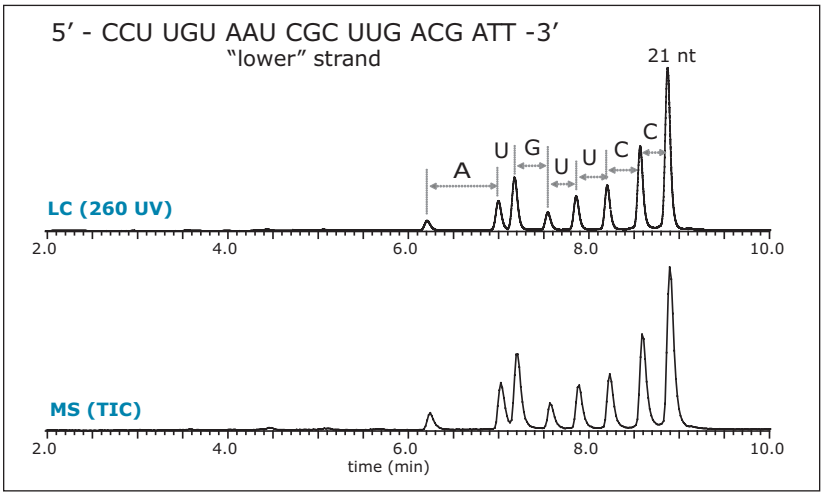

Figure 1. Fast UPLC-MS chromatograms of the lower RNA 21 nt strand.

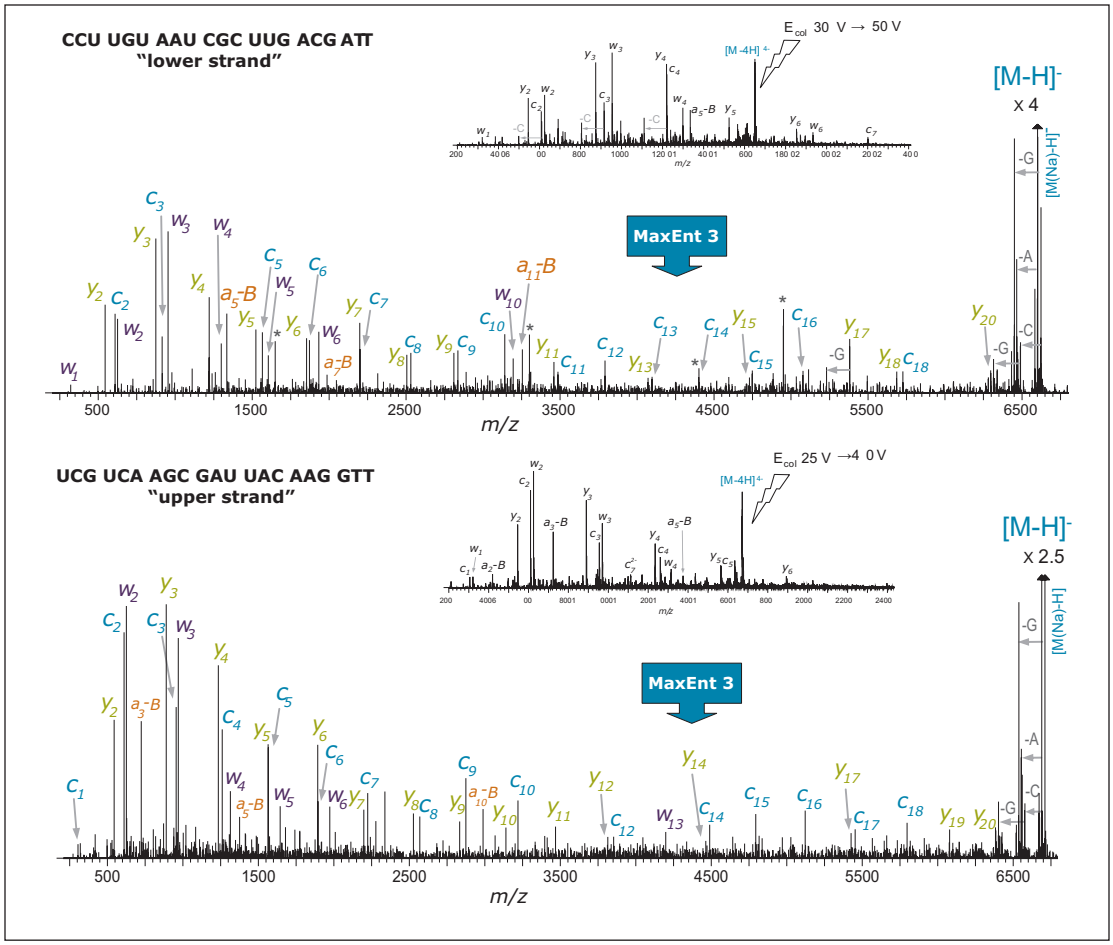

Figure 2. MaxEnt3 deconvoluted MS/MS spectra of 21 nt RNA oligonucleotides. Asterisk denotes harmonic peak (deconvolution artifacts).

Ion fragment	Theoretical *m/z*	Observed *m/z*	PPM
$[a_2\text{-}B]^-$	418.066	418.065	2.4
y_2^-	545.129	545.127	3.7
c_2^-	609.075	609.074	1.6
w_2^-	625.095	625.094	1.6
$[c_3 - Cyt]^-$	804.057	804.057	0.0
y_3^-	874.181	874.183	-2.3
c_3^-	915.100	915.102	-2.2
c_7^{2-}	1100.122	1100.114	7.3
$[c_4 - Cyt]^-$	1110.083	1110.085	-1.8
y_4^-	1219.229	1219.231	-1.6
c_8^{2-}	1264.648	1264.639	7.1
w_4^-	1299.195	1299.196	-0.8
$[a_5\text{-}B]^-$	1335.157	1335.157	0.0
c_9^{2-}	1417.660	1417.647	9.2
w_9^{2-}	1444.690	1444.683	4.8
$[c_5 - Cyt]^-$	1455.130	1455.131	-0.7

Ion fragment	Theoretical *m/z*	Observed *m/z*	PPM
c_{14}^{3-}	1467.164	1467.150	9.5
y_5^-	1524.270	1524.269	0.7
y_{10}^{2-}	1557.227	1557.200	17.3
c_5^-	1566.173	1566.174	-0.6
w_{10}^{2-}	1597.210	1597.198	7.5
c_{16}^{3-}	1691.863	1691.863	0.0
y_{11}^{2-}	1729.751	1729.747	2.3
c_{11}^{2-}	1742.705	1742.709	-2.3
w_{11}^{2-}	1769.734	1769.729	2.8
y_6^-	1853.323	1853.330	-3.8
y_{12}^{2-}	1882.271	1882.255	8.5
c_{12}^{2-}	1895.225	1895.229	-2.1
w_6^-	1933.289	1933.289	0.0
y_{13}^{2-}	2035.284	2035.298	-6.9
c_{13}^{2-}	2048.238	2048.242	-2.0
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.

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.

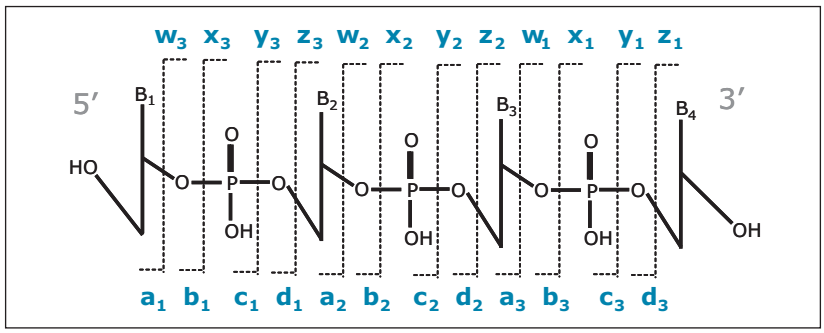

Figure 3. Nomenclature of oligonucleotide fragmentation.[3]

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.

References

1. UPLC-MS Analysis of Interfering RNA Oligonucleotides. Waters Application Note. 2008 (P/N 720002412EN)
2. Kirperkar F, Krogh TN. Rapid Commun. *Mass Spectrom.* 2001; 15(1): 8–14.
3. Adopted from McLuckey SA, et al. *J. Am. Soc. Mass Spectrom.* 1992; 3(1) 60–70.

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Characterizing Polyethylene Glycol (PEG) by SYNAPT High Definition Mass Spectrometry (HDMS)

Petra Olivova, Weibin Chen, and John C. Gebler
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Rapidly assess the general molecular weight distribution of PEG material used in biopharmaceuticals
- Achieve fast, more detailed characterizations of PEG
- Gain more confidence that PEGylated biopharmaceutical products will pass quality control tests

WATERS SOLUTIONS

SYNAPT® High Definition Mass Spectrometry® (HDMS®) System

Triwave® Technology

DriftScope™ Software

KEYWORDS

SYNAPT, HDMS, high definition, Triwave, DriftScope, PEG, IMS, ESI-Tof

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 Mass Spectrometry (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 gasphase 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 Polyethylene Glycol Standards Kit (P/N 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–water 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:	N2 gas
IMS gas pressure:	0.8 mbar
Pulse height:	Variable, 7–15 V
Ionization mode:	ESI+
Capillary voltage:	3200 V
Cone voltage:	40 V
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Source temp.:	150 °C
Acquisition range:	100–4000 *m/z*
Trap collision energies:	8 V

RESULTS AND DISCUSSION

An electrospray ionization time-of-flight (ESI-Tof) mass spectrum of PEG 4450 [0.5% (w/v) in 50:50 water–acetonitrile 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 polydiperse 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.

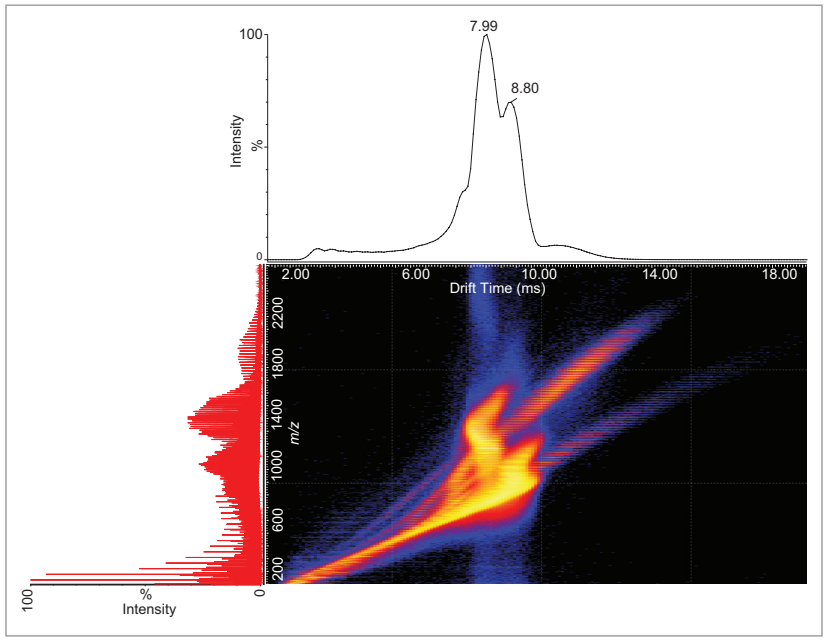

Figure 1. Data for analysis of PEG 4450 using the SYNAPT HDMS System, displayed in DriftScope Software. Each pixel represents an ion, with color representing its intensity (blue-low, to orange-high). To the left of the plot is the ESI-Tof spectrum without IMS separation. On the top of the DriftScope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

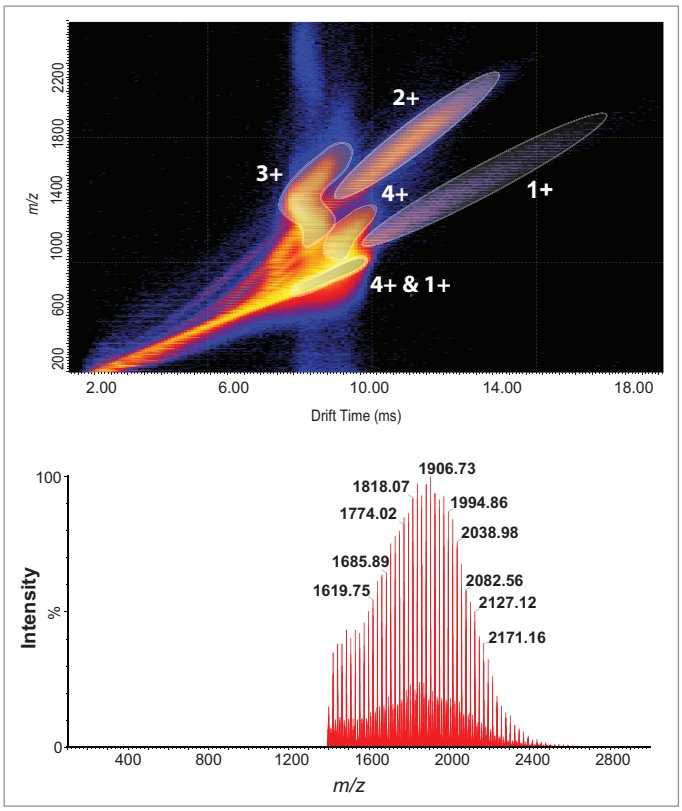

Figure 2. Analysis of PEG 4450 using HDMS. Top panel: HDMS data show the gas-phase separation power of the SYNAPT HDMS System in the analysis of PEG 4450. Components with different charge states are separated via ion mobility, thus enabling the examinations of different (minor) components in the PEG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

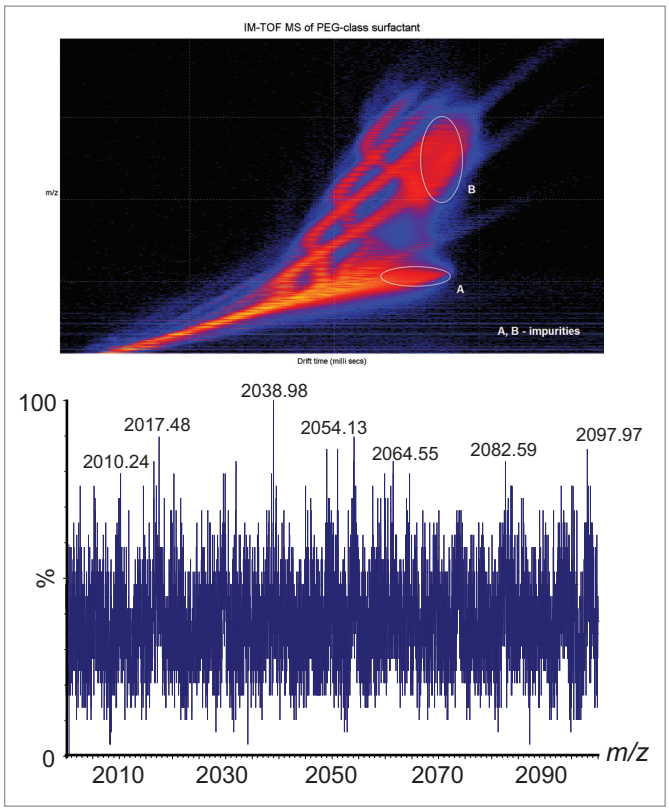

Figure 3. Analysis of PEG 20,000 using HDMS. Top panel: Using DriftScope Software, the HDMS data exhibit the separation of potential impurities (labeled as circle A and B) contained in the PEG materials from the rest of PEG components. These impurities would not be readily discovered without the gas-phase 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.

CONCLUSIONS

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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