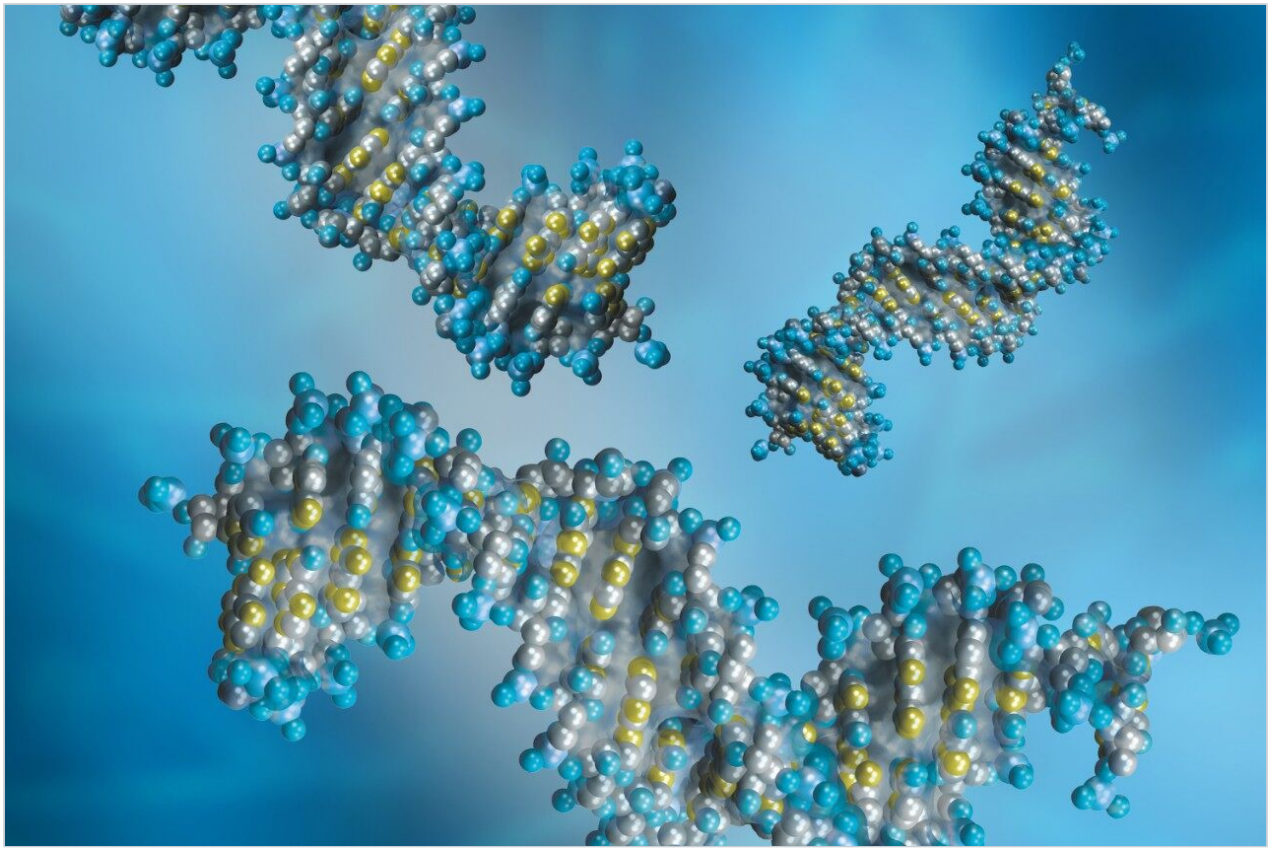
Waters™

Application Note

# LC-MS Analysis of Synthetic Oligonucleotides

Waters Corporation



## Abstract

This application note demonstrates LC-MS analysis of synthetic oligonucleotides.

## Introduction

### LC-MS – The method of choice for characterization of oligonucleotides

Quality control and characterization is an important requirement for therapeutic oligonucleotides. Liquid chromatography with mass spectrometry detection (LC-MS) is the most suitable method for this analysis. Liquid chromatography using XTerra MS $C_{18}$ Columns provides good oligonucleotide resolution up to 60mer using a mobile phase compatible with electrospray mass spectrometry (ESI-MS). The methods were developed for sensitive LC-MS analysis of native and modified oligonucleotides using a 1.0 x 50 mm XTerra MS $C_{18}$ Column with a Capillary HPLC System and ESI (Tof) mass spectrometer.

## Experimental

### Preparation of 16.3 mM TEA - 400 mM HFIP buffer

Dissolve 41.5 mL of HFIP in ~950 mL of water. While mixing vigorously add 2.3 mL of TEA. Adjust volume to 1L with water. The pH of solution should be close to 7.9.

### Preparation of 8.6 mM TEA - 100 mM HFIP buffer

Dissolve 10.5 mL of HFIP in ~950 mL of water. While mixing vigorously add 1.2 mL of TEA. Adjust volume to 1L with water. The pH of solution should be close to 8.3.

## Results and Discussion

### XTerra Columns for Sensitive LC-MS analysis and HPLC conditions

XTerra MS $C_{18}$ Columns are packed with porous 2.5 µm hybrid particles. The sorbent has extended stability at temperatures and pH's typically used for oligonucleotide separations (50–60 °C; pH 7–9). The 1.0 x 50 mm column is operated at the mobile phase flow rate compatible with direct sensitive MS detection. Mobile phases consist from aqueous triethylamine (TEA) and hexafluoroisopropanol (HFIP) solutions (ion-pairing buffer) and methanol. Oligonucleotide resolution achieved with this system was greater than with traditional

triethylammonium acetate (TEAA) ion-pairing buffer. Contrary to TEAA based mobile phases, little or no ion suppression was observed with TEA-HFIP buffers.

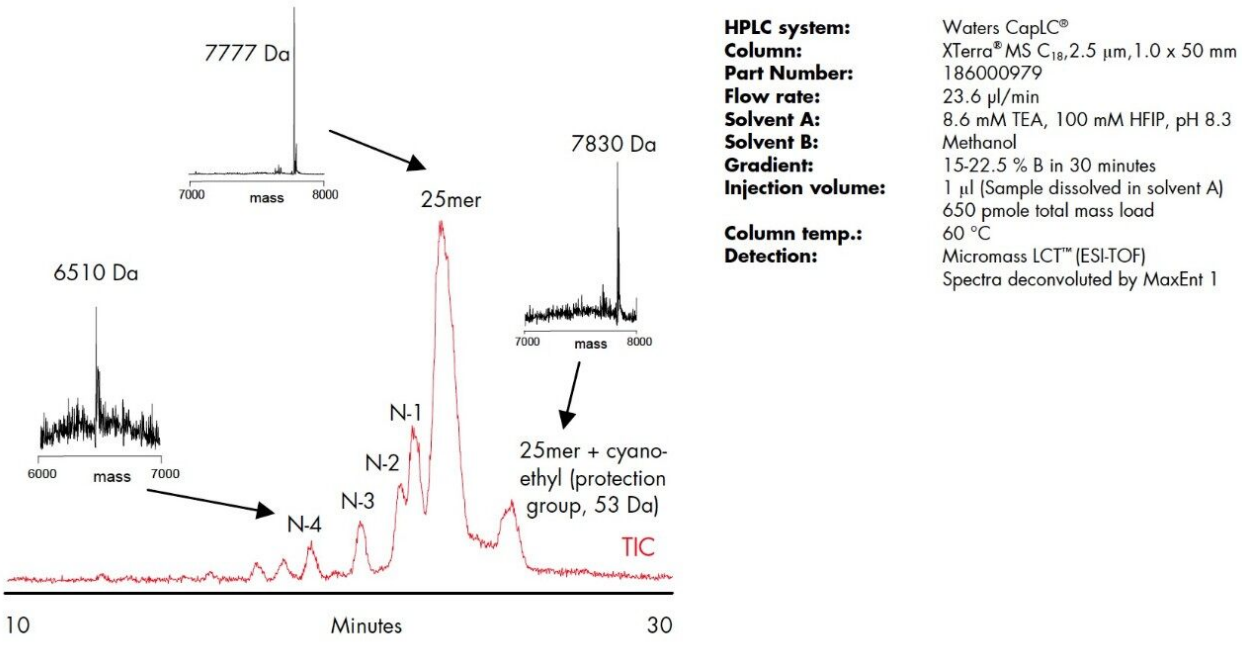
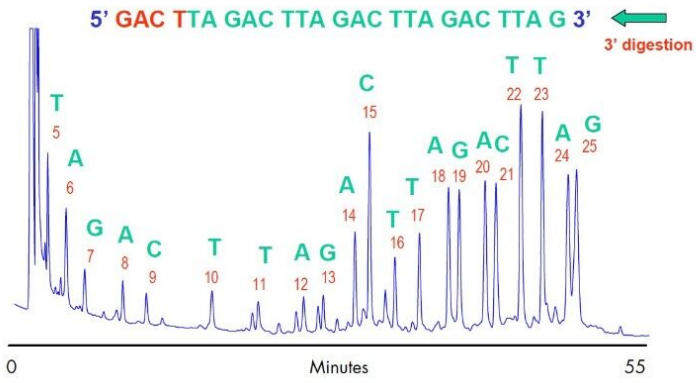



*Figure 1. LC-MS analysis of 25mer phosphorothioate oligonucleotides.*

## Principles of Oligonucleotide Identification by MS

The comparison of theoretical and measured mass was used for oligonucleotide identification. The Tof mass spectrometer routinely achieves mass accuracy ±1Da for oligonucleotides <50mer or even longer. Figure 2 shows oligonucleotides generated by digesting 25mer with 3′ exonuclease. They were positively identified by their molecular mass. The difference in mass for 24/25mer pair was 329.2 Da, indicating the loss of G mononucleotide. Similarly, the difference in mass for 23/24, 22/23, and 20/21 shows a loss of A, T and C mononucleotide, respectively (313.2, 304.2 304.2, and 289.2 Da). This method can be used for sequence verification and failure products identification of therapeutic and diagnostic oligonucleotides (Gilar, *Anal. Biochem*. 298 (2001) 196–206). Figure 3 shows analysis of TaqMan oligonucleotide. Accurate mass measurement was used for identification of singly-labeled failure products from “one-pot” synthesis. First elute nonlabeled oligonucleotides, followed with 5′FAM products, 3′TAMRA labeled oligonucleotides and the dually-labeled target product. Later eluting peaks are 1-4mer TAMRA labeled products and un-conjugated dye.

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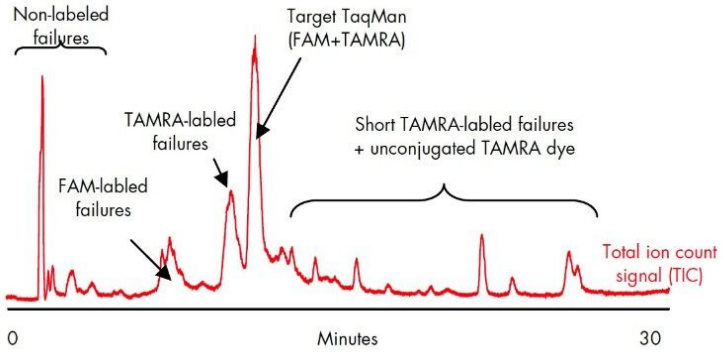
|---|---|
| **HPLC system:** | Waters CapLC® |
| **Column:** | XTerra® MS $C_{18}$, 2.5 µm,1.0 x 50 mm |
| **Part Number:** | 186000979 |
| **Flow rate** | 23.6 µl/min |
| **Solvent A** | 16.3 mM TEA, 400 mM HFIP, pH 7.9 |
| **Solvent B** | Methanol |
| **Gradient:** | 11-20 % B in 30 minutes, 20-24.5 % B from 30 to 60 min |
| **Injection volume:** | 1 µl (sample dissolved in solvent A) |
| **Column temp.:** | 50 °C |
| **Detection:** | PDA and MS. Micromass™ LCT (ESI-TOF) Spectra were deconvoluted by MaxEnt 1. |

*Figure 2. LC-MS identification of oligonucleotides.*




| | |
|---|---|
| **HPLC system:** | Waters CapLC® |
| **Column:** | XTerra® MS $C_{18}$, 2.5 µm, 1.0 x 50 mm |
| **Part Number:** | 186000386 |
| **Flow rate:** | 23.6 µl/min |
| **Solvent A** | 8.6 mM TEA, 100 mM HFIP, pH 8.3 |
| **Solvent B:** | Methanol |
| **Gradient:** | 15-22.5 % B in 30 minutes |
| **Injection volume:** | 1 µl (Sample dissolved in solvent A), 650 pmole total mass load |
| **Column temp.:** | 60 °C |
| **Detection:** | Micromass LCT (ESI-TOF) Spectra deconvoluted by MaxEnt 1 |

*Figure 3. LC-MS identification of 21mer TaqMan and failure by-products generated by one-pot Probe synthesis.*

WA20773, June 2003