Application NOTE

Waters

LC/MS Analysis of Synthetic Oligonucleotides

LC/MS– The method of choice for characterization of oligonucleotides

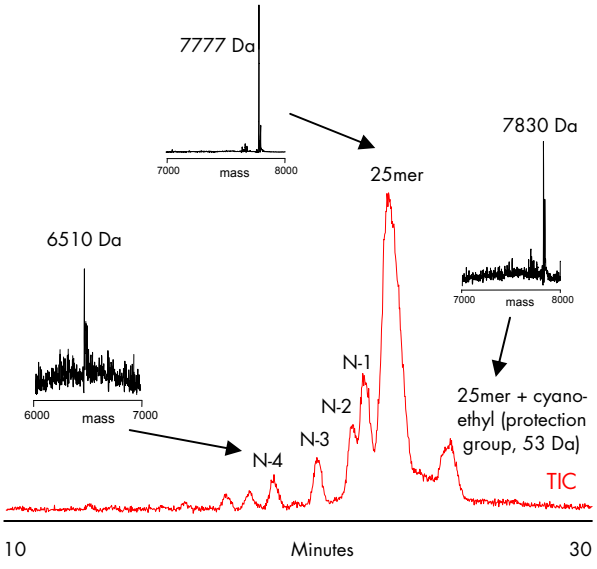
Quality control and characterization is an important requirement for therapeutic oligonucleotides. Liquid chromatography with mass spectrometry detection (LC/MS) is the most suitable method for this analysis. Liquid chromatography using XTerra® MS C_{18} columns provides good oligonucleotide resolution 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.

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.

XTerra® columns for Sensitive LC/MS analysis and HPLC conditions.

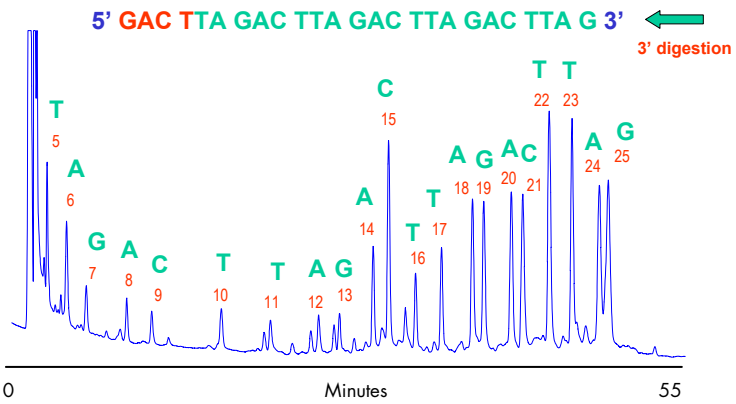
XTerra® MS C_{18} columns are packed with porous 2.5 µm hybrid particles. The sorbent has extended stability at temperatures and pH's typically used for oligonucleotide separations (50-60 °C; pH 7-9). The 1.0 x 50 mm column is operated at the mobile phase flow rate compatible with direct sensitive MS 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.

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.



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:	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 1. LC-MS analysis of 25mer phosphorothioate oligonucleotides.

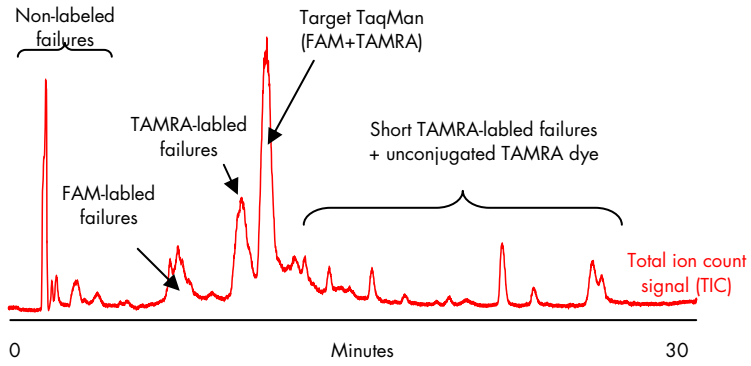


HPLC system:	Waters CapLC®
Column:	XTerra® MS C_{18}, 2.5 µm,1.0 x 50 mm
Part Number:	186000979
Flow rate	23.6 µl/min
Solvent A	16.3 mM TEA, 400 mM HFIP, pH 7.9
Solvent B	Methanol
Gradient:	11-20 % B in 30 minutes, 20-24.5 % B from 30 to 60 min
Injection volume:	1 µl (sample dissolved in solvent A)
Column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

Figure 2. LC-MS identification of oligonucleotides.

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 non-labeled 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.



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.

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