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MASS-DIRECTED PURIFICATION OF LONG-CHAIN SYNTHETIC OLIGONUCLEOTIDES AT µMOLE SCALE

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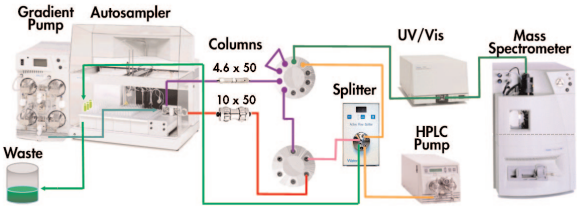
Introduction

Synthetic oligonucleotides have a wide application in molecular biology. They are used in polymerase chain reaction (PCR), RNA interference, DNA sequencing, mutagenesis and antisense drugs, etc.[1] However, purification of synthetic oligonucleotides, especially at a large-scale, can be time-consuming, expensive, and ineffective. Polyacrylamide gel electrophoresis (PAGE) and cartridges are popular purification methods for synthetic oligonucleotides.[2] Both methods become more difficult and less efficient as the oligomer chain length and synthesis scale increases. High performance liquid chromatography (HPLC) is capable of the separation; however, at the preparative scale, the UV detector becomes saturated. Using a mass spectrometer allows for a resolved target peak to be identified under the unresolved UV peak. However, the typical ion-pairing mobile phase buffer used for oligonucleotide separation by HPLC is triethylammonium acetate (TEAA), which is not compatible with MS detection. To overcome this, the separation was done using a hexaflouroisopropanol – triethylamine (HFIP-TEA) buffer.[3] We will present how existing LC/MS methodology was optimized for preparative scale mass-directed purification of long-chain oligonucleotides.



Schematic Flow Diagram of the Waters AutoPurification™ System

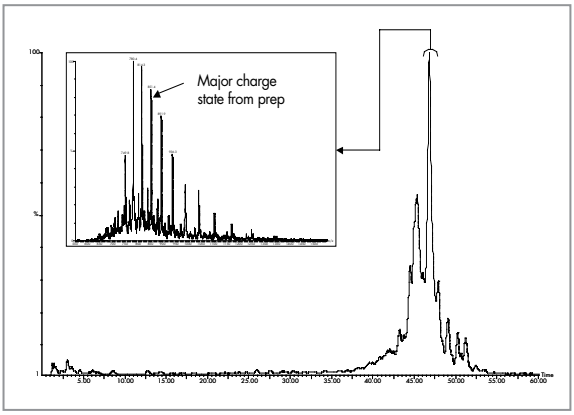
Experimental

- System
  - Waters® AutoPurification™ System
    - 2525 Binary Gradient Module
    - 2767 Sample Manager
    - Column Fluidics Organizer
    - 2996 Photodiode Array Detector
    - Micromass® ZQ™ Mass Spectrometer
    - 515 HPLC Pump
    - Active Flow Splitter (AFS)
  - Waters® XTerra® C_{18} 2.5 µm Column
    - Analytical: 4.6 x 50 mm
    - Analytical Overload: 4.6 x 50 mm
    - Preparative: 10 x 50 mm
- Samples
  - The sample shown in this experiment is a 60-mer synthesized by an in-house proprietary method at Bayer HealthCare Diagnostics Division, Walpole, MA.
- Gradient Conditions[4]
  - A: Aqueous buffer of 16.3 mM TEA: 400 mM Hexafluoroisopropanol
  - B: Methanol
  - Slope: 0.25% B/minute starting from 19% B
  - Column Temperature: Ambient
  - Flow Rate: 4.6 mm column = 0.5 mL/min, 10 mm column = 2.4 mL/min
  - Split Ratio: Analytical Overload = 1:12, Preparative = 1:60
  - Makeup solvent: 75:25 Solvent A:B @ 50 µL/min
- MS Conditions
  - ES- ionization, 550 – 1550 amu continuum scan in 0.5 seconds with a 0.1 delay.
    - Voltages: Capillary = 2.8 kV Cone: -28 V
    - Temperature: Source = 120 °C, Desolvation = 250 °C
    - Gas Flow: Desolvation = 400 L/hr, Cone = 50 L/hr

Identifying the Target Mass

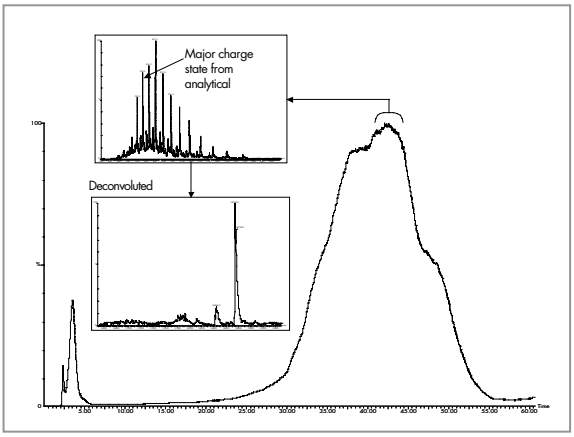
- The typical approach to identifying the target mass is to do a small scale (1 nmol) analytical injection. The trigger for the large scale preparative injection is based on those results.

Analytical Scale



1 nmol on the 4.6 mm column.

Preparative Scale



1µmole on the 10 mm column.

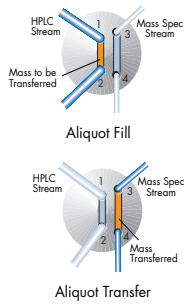
- Both the chromatography and the spectrum have changed.
- The major charge state in the analytical (1 nmol) injection was the 24^- charge (m/z = 780.4), but in the preparative injection, the 22^- charge (m/z = 851.4) was the major charge state.
- The difference between the analytical and preparative spectra increases as the difference in the mobile phase and make-up solvent composition increases.

Identifying the Target Mass without Doing a Preparative Injection

Scaling Up and Down

- By applying the correct scale factor (r^2anal/r^2prep) to the prep scaling sample load, the preparative chromatography can be observed without doing the preparative injection.
- A problem arises, however, with mass-directed fractionation. With the 1 nmol injection, the entire flow and sample enters the MS and the ionization solvent is the gradient elution solvent. With the preparative scale injection, the column was overloaded and only a portion of the column eluent is split off to the MS and then diluted with a makeup solvent.
- Because of this difference, the major charge state shifts when scaling up from the analytical to a preparative separation. Therefore, to obtain analytical data which predicts the preparative results, the concentration and solvent composition must be equivalent for both scales. A simulated preparative run, with similar overloading of the sample, on analytical column (4.6 mm) is necessary.

Scaling the Split Ratio



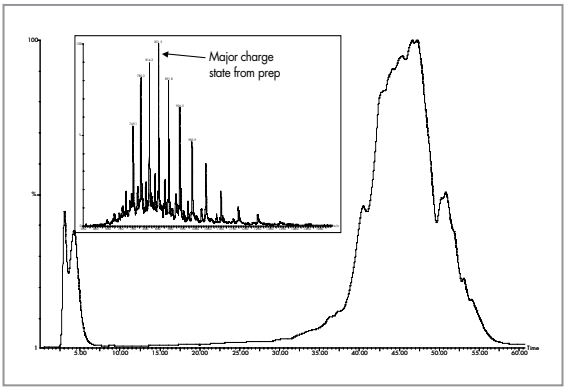
Schematic of the Waters AFS

- This exact change in split ratio was accomplished using the Waters AFS set to the same setting (Split Value #3) for both the injections.
- This is possible because the split ratio is based on a volume taken from the main stream per unit time.
- The change in flow rate is inversely proportional to the change in the split ratio.
- Flow rate to the MS is constant.

  QT = QMu + Vs/t

  Where QT is the total flow, QMu is the makeup flow and Vs/t is the split volume per unit time
- The sample concentration entering the MS is equal for both scales. At the analytical scale, 1/4.7 (0.21) of the sample is injected, but 4.7 times more is split from the main stream.

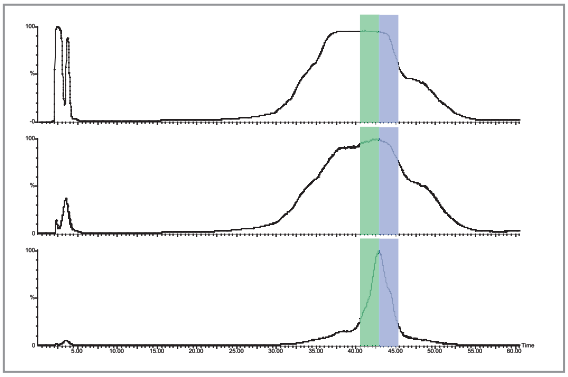
Simulated Preparative Scale (Analytical Column Overload)



213 nanomoles on the 4.6 mm column with a 1:12 mass split and 1:1 dilution with the makeup pump.

- The chromatography and spectra are now comparable between both scales
- The target mass for mass-triggered fractionation is identified as the 22^- charge state (m/z = 851.4)

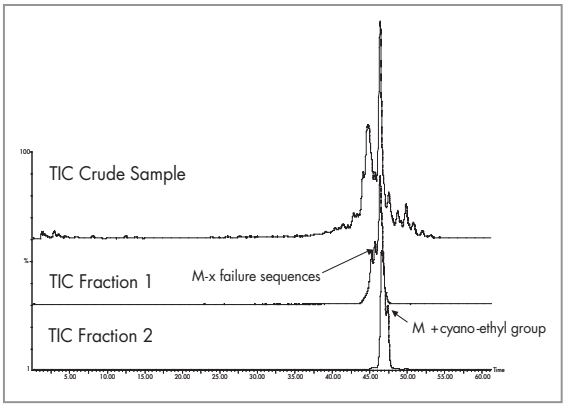
Mass-Directed Purification Results



Preparative injection of a 1 µmole 60-mer oligonucleotide synthesis on the 10 mm column.

- The m/z chromatogram for the 22^- charge state (m/z = 851.4) shows a resolved target peak to be identified under the unresolved UV peak
- Fractionation is triggered from the m/z chromatogram
- The target is collected over 2 fractions based on volume
- The UV peak is still unresolved, even after splitting and diluting with the makeup pump

Fraction Reanalysis



Overlay of the crude sample TIC and the TICs of the 2 fractions. 20 µL of each fraction injected from collection vessel onto the 4.6 mm column.

Options for Improving Fraction Purity

- Increase collection threshold
  - Cost = Decrease recovery
- Decrease the gradient slope (i.e. 0.1% MeOH increase/minute) for a better separation
  - Cost = increased run time, decreased throughput, and greater solvent

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Summary

- Purification of long-chain synthetic oligonucleotides is possible using mass triggered fractionation.
- Chromatographic separation is maintained with column overloaded with sample.
- Advantages over current methodology
  - Up to a 2-fold increase in overall purification yield, as compared to using PAGE, while still meeting the purity requirement
- Previously acquired data from a 59 mer, with an average of 38 nmol recovery by PAGE, generated 65 nmol recovery with LC/MS. Also, a 49-mer with 50 nmol recovery by PAGE gave 137 nmol recovery with LC/MS.
  - Overall time required for purification is about 5 hours with HPLC, as compared to the few days required for PAGE
- Simulating the preparative injection on an analytical column with the Waters AFS, provides a comparable chromatogram and spectrum to be expected for the actual preparative injection.
- This is especially critical for determining the target for mass-triggered fractionation, when the distribution of charge state changes.

References

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3. A. Apffel, J.A. Chakel, S. Fischer, K. Lichtenwalter, W.S. Hancock, Anal. Chem. 69 (1997) 1320.
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