Ion-Pairing Reversed Phase Chromatography for LC MS and MS/MS Characterization of RNAi

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Sean M. McCarthy, Vera Ivleva, Martin Gilar, Ying-Qing Yu, John C. Gebler
Waters Corporation, 34 Maple Street, Milford, MA 01757

OVERVIEW

- **There is an increased desire for MS compatible mobile phases for single stranded and duplex oligonucleotide separations.**
- **A systematic comparison of the resolving power and MS compatibility of mobile phase modifiers for oligonucleotide separations is presented.**
- **Choice of mobile phase modifier for ion-paring (IP) reversed phase liquid chromatography requires consideration of many factors including IP ability, ease of removal following separation , cost of purchase and disposal, and toxicity.**
- **We studied various mobile phase compositions for their ability to separate homo and heteromeric oligonucleotides.**
- **Successful candidates should yield predictable elution order based on oligo length and adequate compatibility with ESI-MS. detection.**

HOMOMERIC OLIGONUCLEOTIDE SEPARATIONS

UV 260nm DETECTION

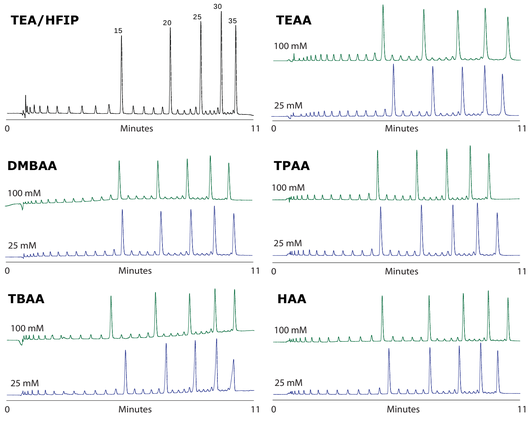
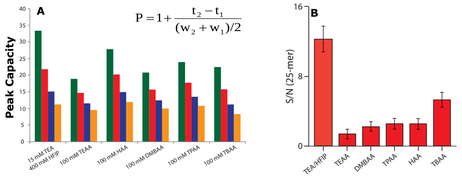


Figure 1: Separations were accomplished with a Waters ACQUITY UPLC® System using a Waters Oligonucleotide Separations Technology column (ACQUITY UPLC® OST C18, 1.7µm, 2.1x50) maintained at 60 °C. On column loading was 20 pmol/oligo of Waters MassPREP™ OST standard.

SINGLE QUADRUPOLE MS DETECTION

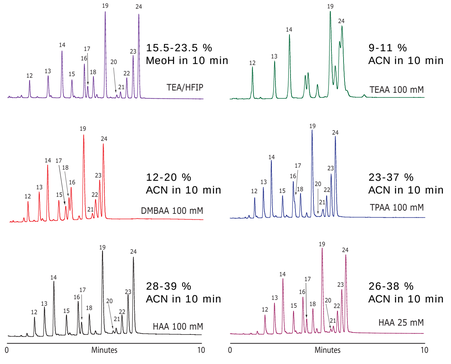


$$P = 1 + \frac{t_2 - t_1}{(w_2 + w_1)/2}$$

Figure 2: (A) Peak capacities for mobile phases at 100 mM for data shown in Figure 1. (B) Signal to Noise determined from three separate analysis of 25-mer oligo dT via single quadrupole MS.

- **Elution conditions (ACN gradient) were adjusted so 15-mer eluted at ≈ 5 minutes and 35-mer eluted at ≈ 10 min. Separations were run in triplicate with exceptional reproducibility.**
- **Generally, the more hydrophobic IP agents (longer alkyl chain) required higher ACN content for oligo elution.**
- **Mobile phases were prepared by the addition of equimolar ratios of acetic acid and appropriate base. The pH was adjusted to ≈ 7.0 by the addition of either acetic acid or amine as needed.**

HETEROMERIC OLIGONUCLEOTIDE SEPARATIONS



- **Figure 3: (A) Separation of a digested mixture of 24-mer, 19-mer and 14-mers (5'- CCC CTT GGT TAA CCA AGG TTC CAA-3') Gradients and conditions are the same as those in Figure 1.**
- **HAA and TPAA give predictable retention of heteromeric oligonucleotides, but HAA provides better resolution.**
- **Increased IP efficiency makes the retention pattern of heteromeric oligos more regular.**

RNAi CHARACTERIZATION WITH LC-MS

- **Use LC-MS compatible mobile phases, such as 15mM TEA, 400 mM HFIP ion-pairing buffer.**
- **Sum mass spectra under the peaks of interest, deconvolute mass with MaxEnt1, calculate Δ mass between adjacent peaks, assign failed sequences based on Δ mass (Figure 4).**

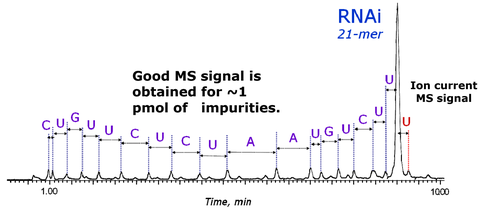


Figure 4: UPLC-MS analysis of crude 21nt RNAi, UUC UGU AAU CUC UUG UCU ATT (5'-3'), ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm column, 60 °C, UV 260 nm. m.p. A: 15mM TEA, 400 mM HFIP, pH 7.9, B: 50% m.p. A, 50% MeOH. Gradient 20-40 % B in 10 minutes, 0.2 mL/min.

RNA DUPLEX ANALYSIS

- **LC analysis of siRNA can be difficult due to on-column melting and incomplete resolution of full length from mis-matched duplexes and single stranded impurities.**
- **OST column technology coupled with HPLC and UPLC, using duplex compatible mobile phases, solves these problems by giving predictable retention and non-denaturing separation conditions.**
- **Use of IP RP HPLC and UPLC analysis of duplexes can be interfaced with MS to provide mass data not available with other methods such as PAGE and AX-HPLC**

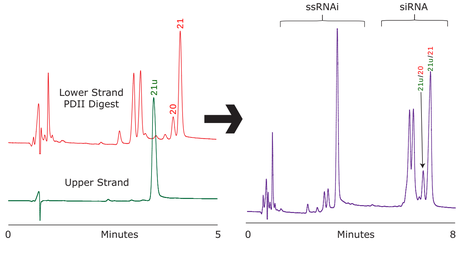


Figure 5. Separation of mis-matched duplexes via UPLC with HAA mobile phase. ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm column, 60 °C, UV 260 nm.

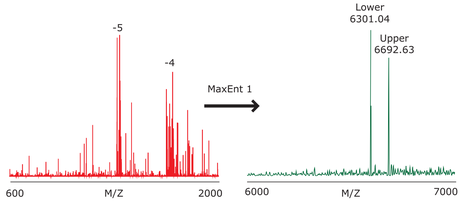


Figure 6: Raw and MaxEnt 1 deconvoluted spectra collected with single quadrupole detector from truncated siRNA separation shown in Figure 5. Data shown is for full length upper strand hybridized with N-1 lower strand.

RNA DUPLEX PURIFICATION

- **Purification of siRNA is necessary for therapeutics to ensure specificity and unwanted gene silencing**
- **Purification accomplished by *on-column annealing* of siRNA**
  - **Inject first strand under initial gradient conditions immediately followed by injection of second complementary strand**
  - **After injection of second strand, gradient started to elute products**
- **Purification is scalable and siRNA is formed quantitatively**
- **siRNA is easily collected by proper hearth cutting of peak**
- **Analysis of collected peak confirms that collected fraction contains desired full length duplex composed of equimolar ratio of each complementary strand**

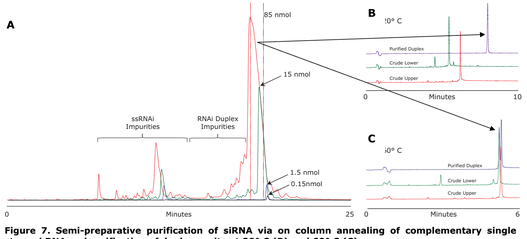


Figure 7. Semi-preparative purification of siRNA via on column annealing of complementary single straned RNA and verification of duplex purity at 20° C (B) and 60° C (C).

CONCLUSIONS

- **HAA and TPAA mobile phases provide exceptional resolution and peak capacities for homo and hetero-oligonucleotide analysis**
- **IP RP LC allows for MS analysis and sequencing of RNA**
- **HAA is siRNA and MS compatible and resolves partial duplexes**
- **IP RP LC combined with non-denaturing column conditions allows for scalable on column annealing and purification of siRNA**

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