UPLC separation of oligonucleotides: Method development

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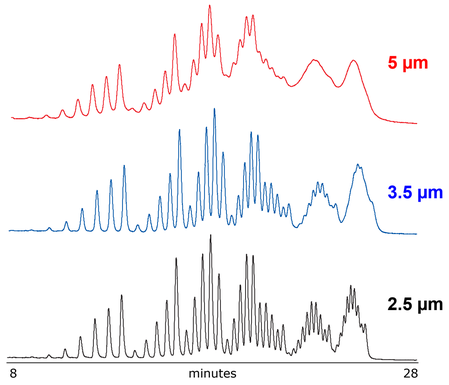
OVERVIEW

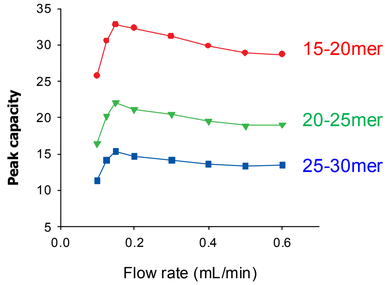
- **UPLC system and columns packed with sub 2 µm sorbents offer better resolution oligonucleotides than conventional HPLC.**
- **Suitable flow rates for 2.1 mm i.d. UPLC columns are between 0.1 and 0.4 mL/min (1.7 µm sorbent, 60 ° C).**
- **Shallow gradients are recommended.**
- **Analysis time (gradient time) can be reduced proportionally with increase of flow rate (gradient volume is constant).**
- **Separation of short oligonucleotides is achievable within minutes.**
- **Most useful oligonucleotide separation technology (OST) column dimensions are 50 x 2.1 mm.**
- **Demanding separations may require longer (100 mm) OST column. However, gradient time should be increased proportionally. For example, 30 minutes gradient operated with 50 mm column should be extended to 60 minutes for 100 mm column.**

IMPACT OF PARTICLE SIZE ON OLIGONUCLEOTIDE SEPARATION



BEH OST C18, 50 x 2.1 mm column, 15-60T ladder, 0.1 M TEAA system, gradient 8-12.5% acetonitrile in 30 minutes, 60 °C, 0.2 mL/minute, **Alliance Bio, 2796**, PDA detector, 260 nm.

CHOICE OF FLOW RATE TO ACHIEVE BEST SEPARATION PERFORMANCE IN UPLC



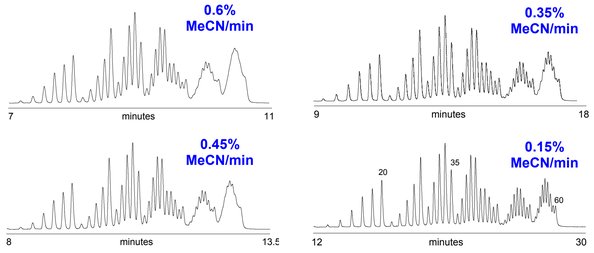
BEH OST C18, 50 x 2.1 mm, 1.7µm, 15, 20, 25, 30, 35 mer oligodeoxythymidines, TEA-HFIP ion pairing system (15mM, 400 mM), gradient 15-27% methanol in 26.7 30 minutes, 60 °C, 0.2 mL/minute, **ACQUITY UPLC**, PDA detector, 260 nm.

- **Description of experiment above: Best peak capacity for 50 x 2.1 mm column packed with 1.7µm sorbent was found for flow rate 0.15-0.2 mL per minute.**
- **Gradient time was kept constant; flow rate varied. The gradient slope is effectively shallower at higher flow rates (in terms of column volumes used for oligouncleotide elution).**
- **Peak capacity *P* was calculated from equation below.**

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

- **t_1 and t_2 represent retention times of two selected peaks, e.g. 15 and 20 mer. w_1 and w_2 are peak widths of peaks measured at 13.4% peak height.**
- **The peak capacity value indicates how many peaks can be fitted between two selected mers. For example if peak capacity is greater than 6 for 25-30mers, one expects to achieve baseline separation for all 25-30mer peaks.**
- **Due to separation selectivity, separation of shorter oligonucleotides is easier than resolution of longer ones.**

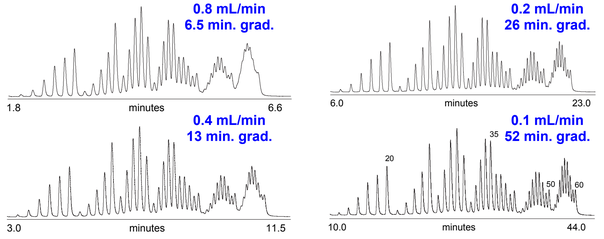
IMPACT OF GRADIENT SLOPE



BEH OST C18, 50 x 2.1 mm, 1.7µm, 15-60T ladder, 0.1M TEAA ion pairing system, gradient starts at 8% MeCN, variable slope, 60 °C, 0.2 mL/minute, **ACQUITY UPLC**, PDA 260 nm.

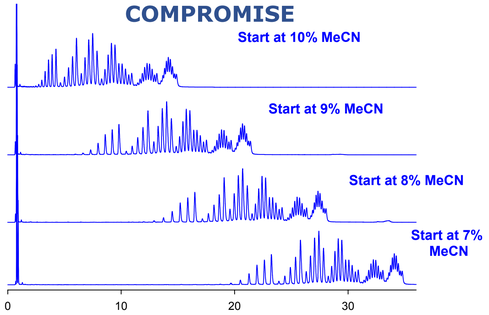
- **Shallow (long) gradients are more favorable for resolution.**
- **Best strategy to reduce the analysis time while maintaining shallow gradient is to scale the flow rate proportionally with gradient time. Gradient volume stays constant. See figure below.**

CONSTANT GRADIENT VOLUME: CHANGING GRADIENT TIME AND FLOW RATE PROPORTIONALLY



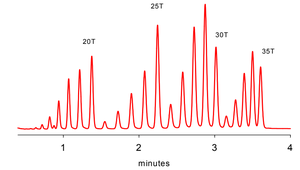
BEH OST C18, 50 x 2.1 mm, 1.7µm, 15-60T ladder, 0.1M TEAA ion pairing system, gradient starts at 9% MeCN, constant gradient volume (time:flow rate ratio), 60 °C, 0.2 mL/minute, **ACQUITY UPLC**, PDA 260 nm.

OPTIMIZING INITIAL GRADIENT STRENGTH: FASTER SEPARATION WITHOUT RESOLUTION COMPROMISE

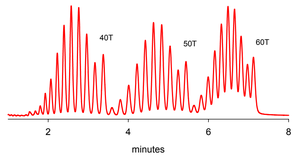


BEH OST C18, 50 x 2.1 mm, 1.7µm, 15-60T ladder, 0.1M TEAA ion pairing system, gradient starts at indicated MeCN %, constant gradient slope 0.15% MeCN/min, 60 °C, 0.2 mL/minute, **ACQUITY UPLC**, PDA 260 nm.

UPLC FOR FAST ANALYSIS OF OLIGONUCLEOTIDES



BEH OST C18, 50 x 2.1 mm, 1.7µm, 15-35T, TEA-HFIP (15mM-400mM), start 20% MeOH, 0.5% MeOH/min, 60 °C, 0.4 mL/min., **ACQUITY UPLC**, PDA 260 nm, 50 µL mixer, ~4500 psi (37 MPa).



BEH OST C18, 50 x 2.1 mm, 1.7µm, 15-35T, TEA-HFIP (15mM-400mM), start 23% MeOH, 0.15% MeOH/min, 60 °C, 0.2 mL/min., **ACQUITY UPLC**, PDA 260 nm, 50 µL mixer, ~2500 psi (17 MPa).

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