

Semi-preparative purification of native and chemically modified oligodeoxyribo-nucleotide probes by reversed-phase HPLC with ESI MS detection

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Abstract

We have developed an ion-pair reversed-phase HPLC method for purification of special polymerase chain reaction (PCR) probes such as fluorescent labeled oligonucleotides, molecular beacons, and TaqMan™ probes. Purity of these oligonucleotides is crucial for quantitative PCR or genotyping reactions. Separation was performed on an XTerra™ MS C18 column using a shallow gradient of acetonitrile or methanol. Ion pairing buffers were optimized for compatibility with mass spectrometry detection. Molecular mass of target oligonucleotide was used as a criterion for automated selection of the fraction collection window. XTerra™ MS C18, 75 x 4.6 mm, 2.5 μm column is capable of purification of 0.1mmol of oligonucleotide in a single injection.

Background

Two major methods are used for purification of DNA oligonucleotides:

HPLC: Offers good separation up to ~30 mer
Convenient fraction collection.
Easily scaleable from analytical to semi-preparative scale.

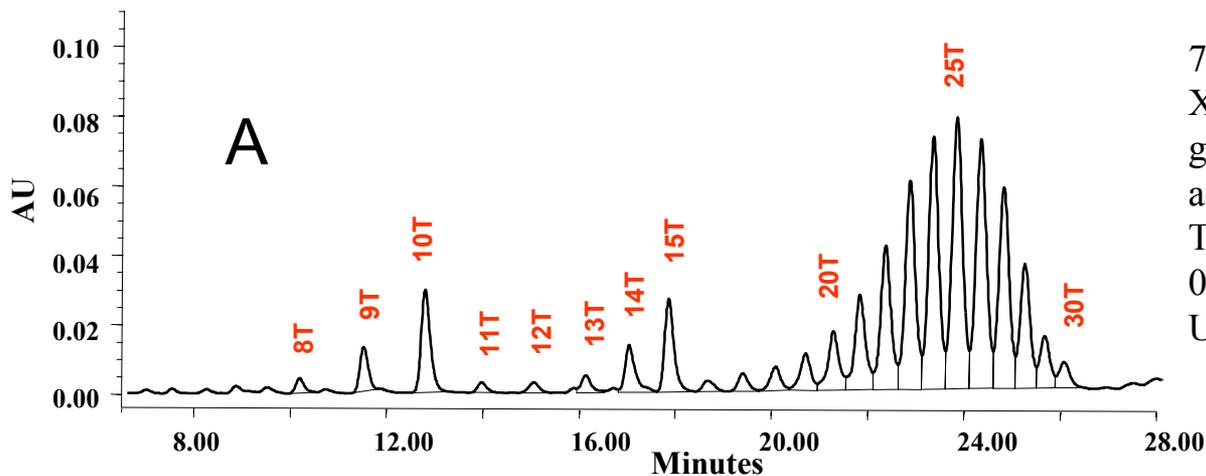
Slab gel electrophoresis: Superior separation power up to ~100 mer
Difficult band visualization and fraction collection.
The technique is not easily scaleable and automatable.

Background (Cont.)

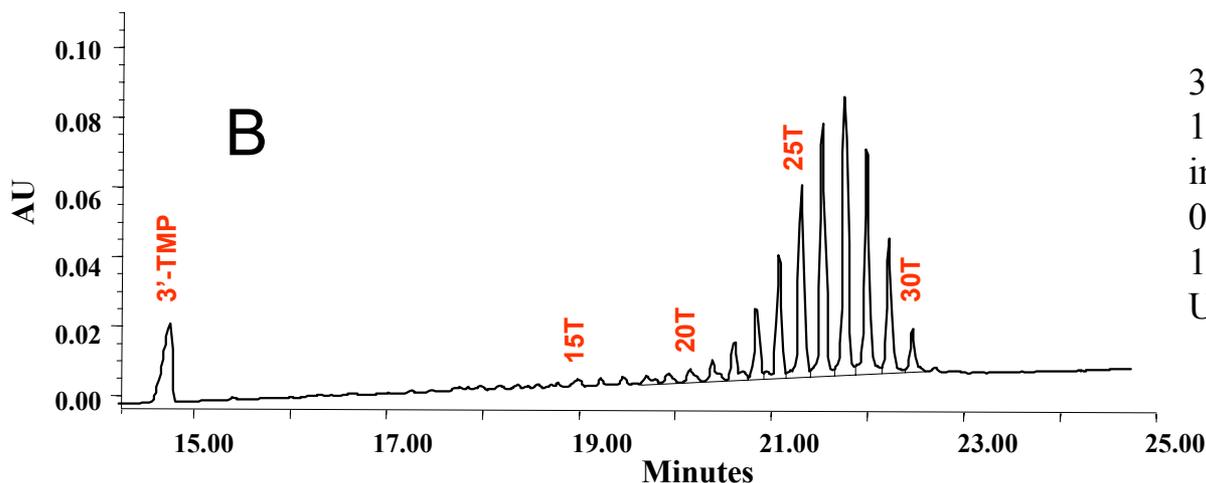
Practical aspects of DNA separation by HPLC :

- Both ion-exchange and ion-pair reversed phase HPLC offer similar separation performance.
- Separation selectivity decreases with oligonucleotide length (see panel 5A).
- Ion-pair RP-HPLC uses volatile buffers. No subsequent desalting of the sample is required. Ion-pair RP-HPLC is more compatible with MS detection.
- Generic shallow gradient is used; initial gradient strength is optimized.
- The use of elevated temperature, slow mobile phase flow rate, and small particle size sorbent (non-porous particles) improve separation performance.
- Porous 2.5 μm particles offer a good compromise between column loading capacity and chromatographic efficiency. Semi-preparative purification is possible using analytical columns (panel 8).

Comparison of separation of DNA homooligonucleotides by: (A) - RP-HPLC; (dT)₈₋₃₀, (B) - CGE; (dT)₁₅₋₃₀



75 x 4.6 mm, 2.5 μ m
XTerra™ MS C18, 36 min
gradient from 10 to 14.5 %
acetonitrile in 0.1 M
TEAAc, pH 7,
0.5 ml/min, 51.5°C,
UV 255 nm

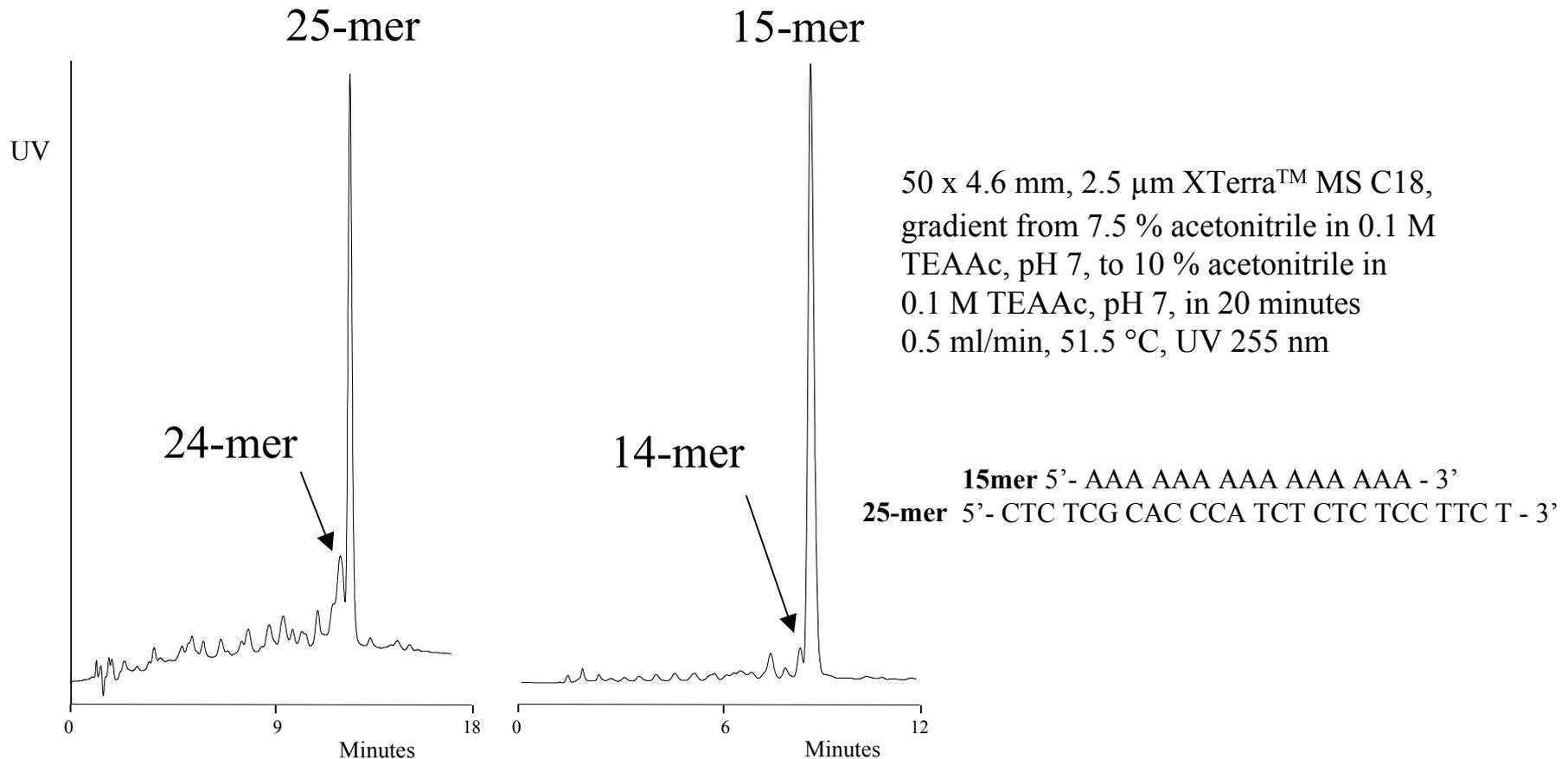


31/24 cm, 75 μ m capillary
13 % linear polyacrylamide
in 7M urea, 15% formamide
0.1 M Tris-TAPS, pH 8.3,
15 kV run, 30°C
UV 270 nm

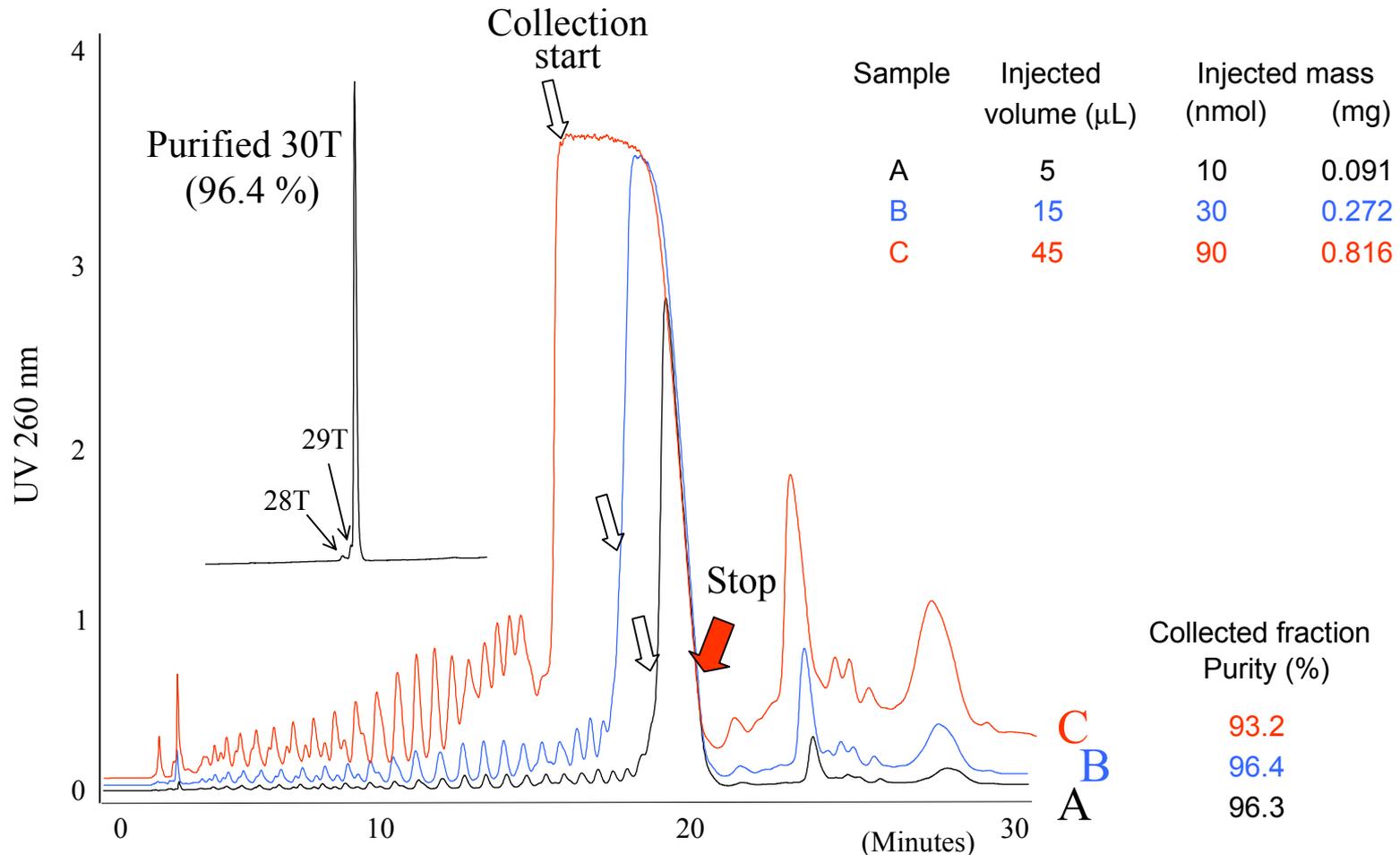
Application of XTerra™ MS C18 column for separation of DNA oligonucleotides

- 1) Fast control of synthetic oligonucleotides purity (panel 7).
- 2) Semi-preparative purification of detritylated oligonucleotides (panel 8).
- 3) High fidelity purification of special oligonucleotides, e.g. fluorescently labeled primers (panel 15), and TaqMan™ probes (panel 16).
- 4) ESI-MS identification of oligonucleotide impurities (panels 13, 14, 15, and 16).

Fast monitoring of synthetic DNA oligonucleotide purity.



Semi-preparative ion-pair RP- HPLC purification of 30-mer oligoT



HPLC conditions: 75 x 4.6 mm, 2.5 μm XTerra™ MS C18, A: 0.1 M TEAAc, pH 7; B: 25 % ACN in 0.1 M TEAAc, pH 7; gradient from 40 % B to 55 % B in 30 min, 0.5 ml/min, 50.5°C, UV 260 nm

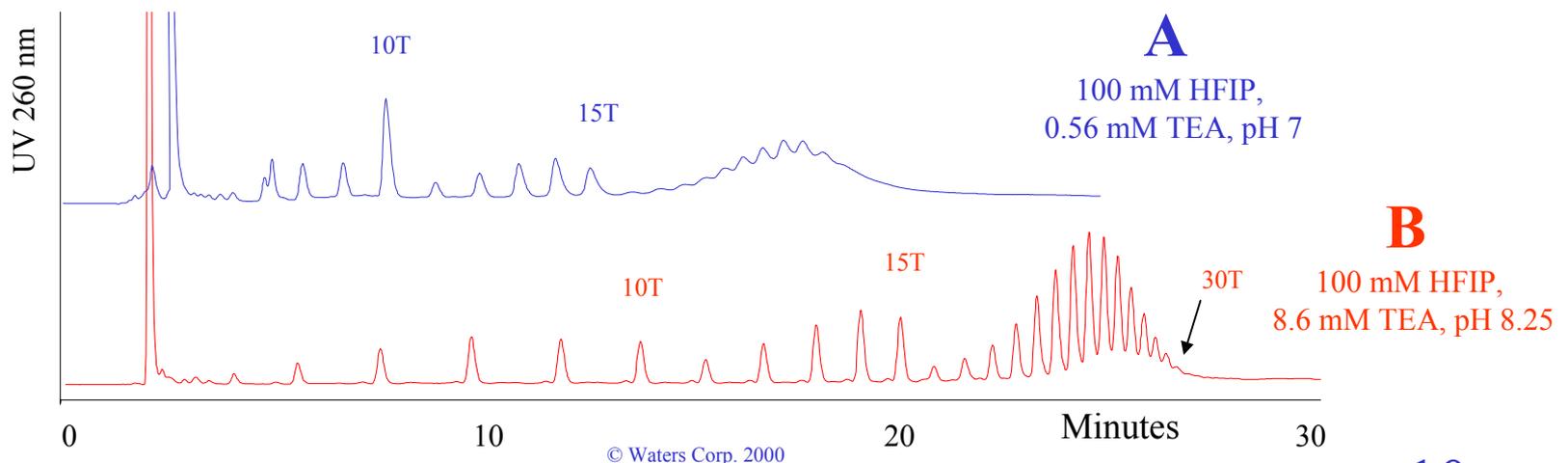
HPLC method development for semi-preparative purification of DNA oligonucleotides based on ESI-MS detection

The retention time of DNA fragment depends on its length and sequence, therefore a manual fraction collection is usually performed.

- Molecular mass is an unambiguous criterion for collection of desired fragment. We use MS detection as a triggering event for fraction collection.
- The 0.1 M concentration of triethylamine acetate (TEAAc) ion-pair buffer gives us a good RP-HPLC separation of oligonucleotides, but suppresses the sensitivity of ESI-MS (panel 11). Lower concentration of TEAAc compromises the HPLC separation (Apffel A. et al., *J. Chromatogr. A*, 1997, 777, 3; Huber C.G., Krajete A., *Anal.Chem.* 1999, 71, 3730).
- Hexafluoroisopropanol (HFIP) - triethylamine buffer was proposed instead of triethylamine acetate (Apffel A. et al., *Anal. Chem.* 1997, 69, 1320). Separation performance of HFIP-TEA buffer does not fully match the performance of TEAAc.
- We optimized the ion-pair buffer to improve the separation of oligonucleotides while keeping mobile phase directly compatible with ESI-MS detection.

Optimization of TEA-HFIP buffer

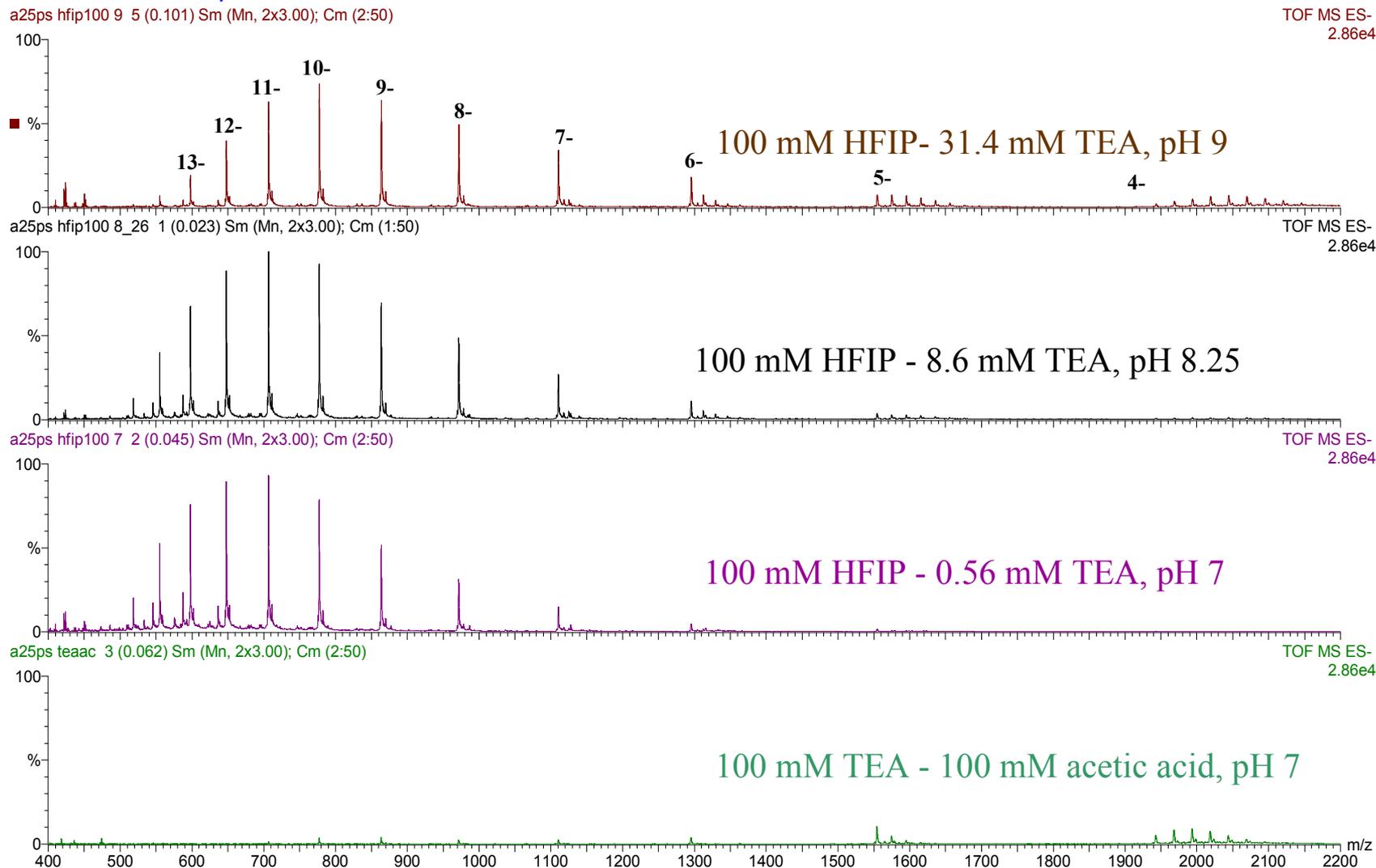
- 400 mM HFIP-2.25 mM TEA buffer, pH 7 (Apffel et al., Anal. Chem. 1997, 69, 1320) performs poorly for long oligonucleotides (> 30 mer). More pronounced peak broadening was observed with mobile phase containing 100 mM HFIP, 0.56 mM TEA, pH 7 buffer (chromatogram A, separation of oligodeoxythymidine oligonucleotide ladder 8-30mer T, XTerra™ MS C18 75x4.6mm, 2.5 μm, gradient starts from 10 % MeOH, slope 0.4 % MeOH per minute, 0.5 ml/min, column temperature 50°C, 260 nm).
- Concentration of TEA in buffer is crucial for chromatographic performance. We investigated the effect of TEA concentration (0.56-31.4 mM; pH 7-9) keeping the HFIP concentration constant (100 mM). The 100 mM HFIP-8.6 mM TEA buffer, pH 8.25 gave us an optimal separation (chromatogram B, column and HPLC conditions the same as A).
- XTerra™ MS C18 column (hybrid particle technology) tolerates the elevated pH without noticeable deterioration of column performance over several hundred injections.



Effect of buffer on ESI-MS signal of 25-mer DNA

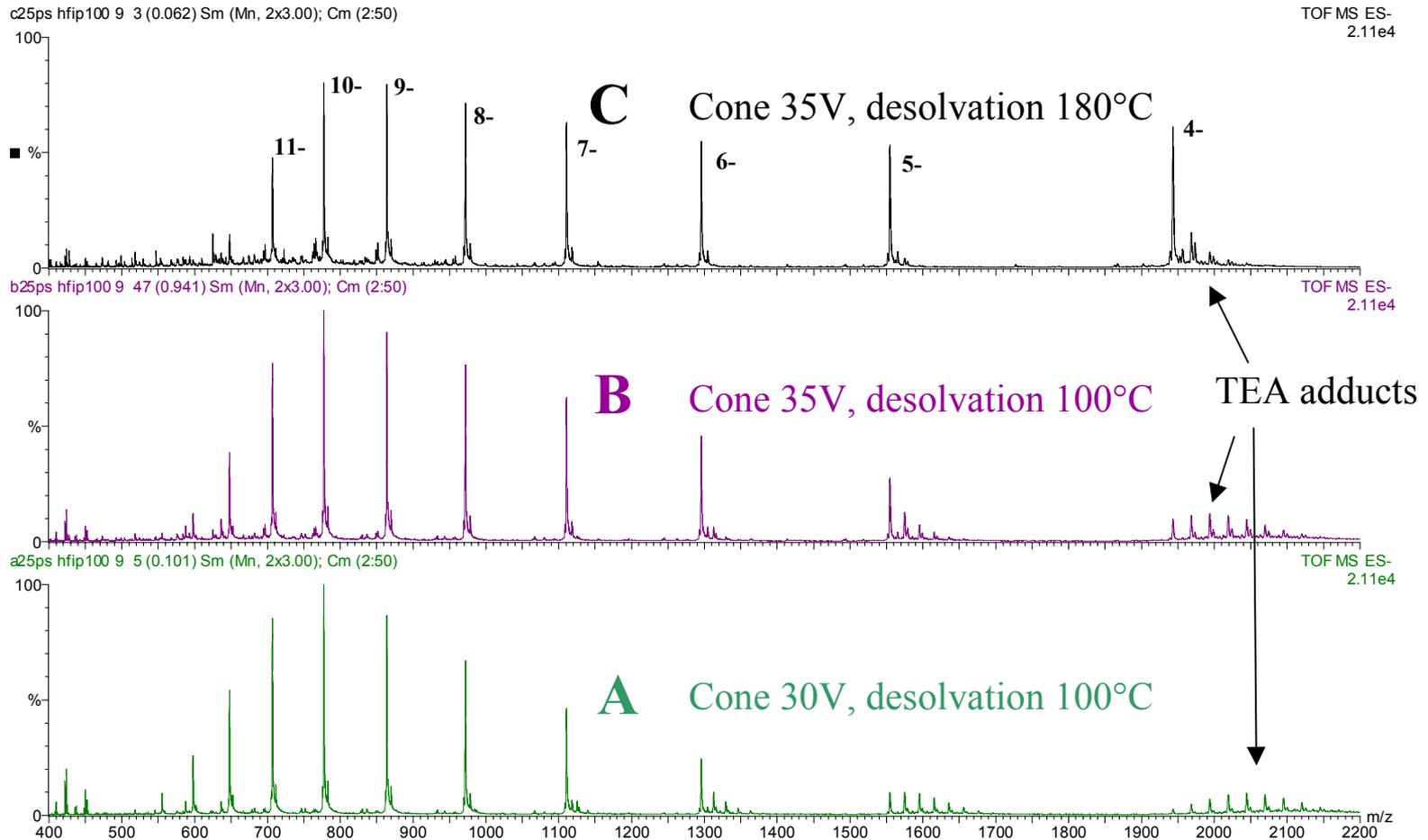
LCT, Micromass, direct infusion in 50 % ACN-buffer

electrospray capillary 2.5 kV, sample cone 30 V, 100°C desolv., 80°C source, desolvation gas 480 l/hour



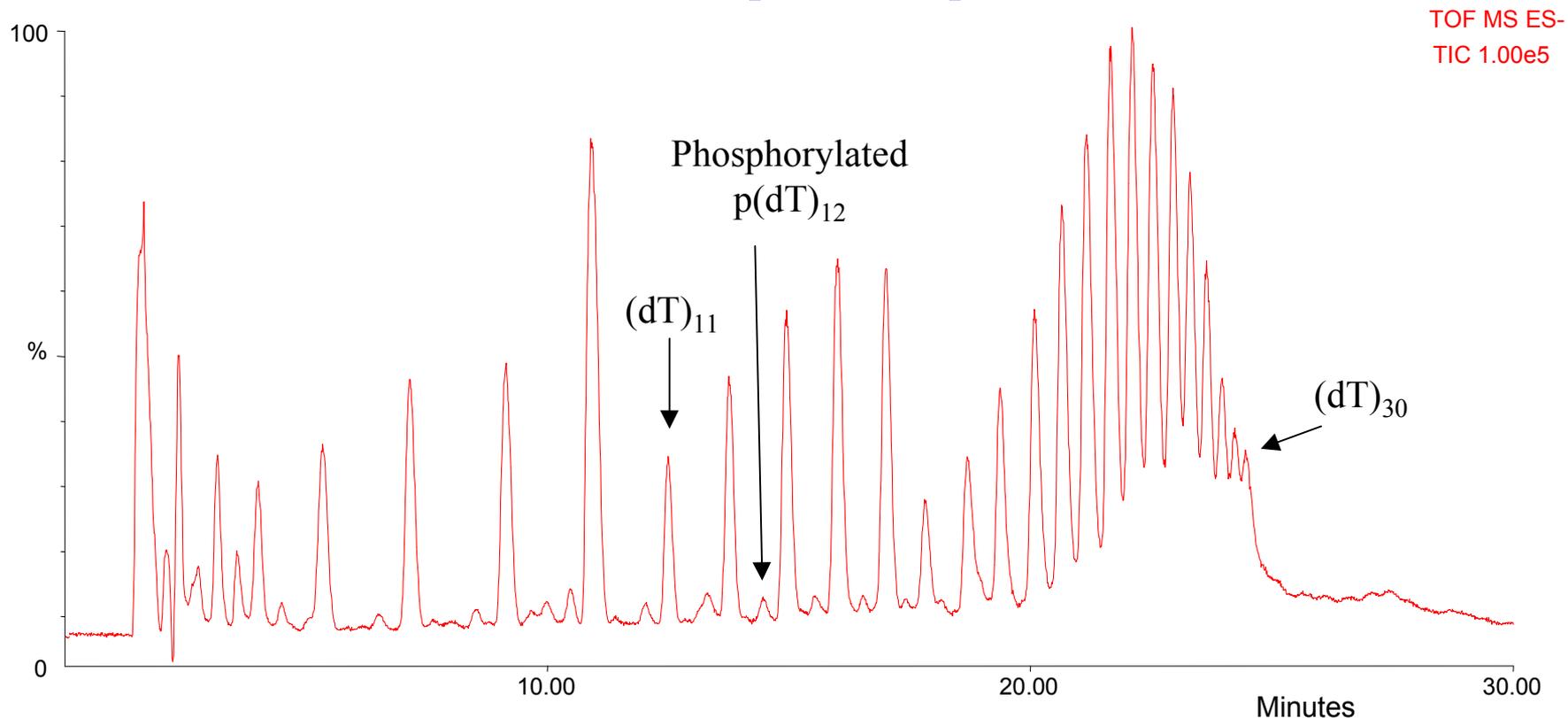
Effect of ESI-MS conditions on abundance of 25-mer DNA - TEA adducts

LCT, Micromass, direct infusion in 50 % ACN-buffer, 100 mM HFIP-31.4 mM TEA, pH 9
capillary 2.5 kV, source 80°C, desolvation gas 480 l/hour, for sample cone voltage and desolvation temperature see annotation



ESI MS chromatogram of homooligo-deoxythymidine ladder separation

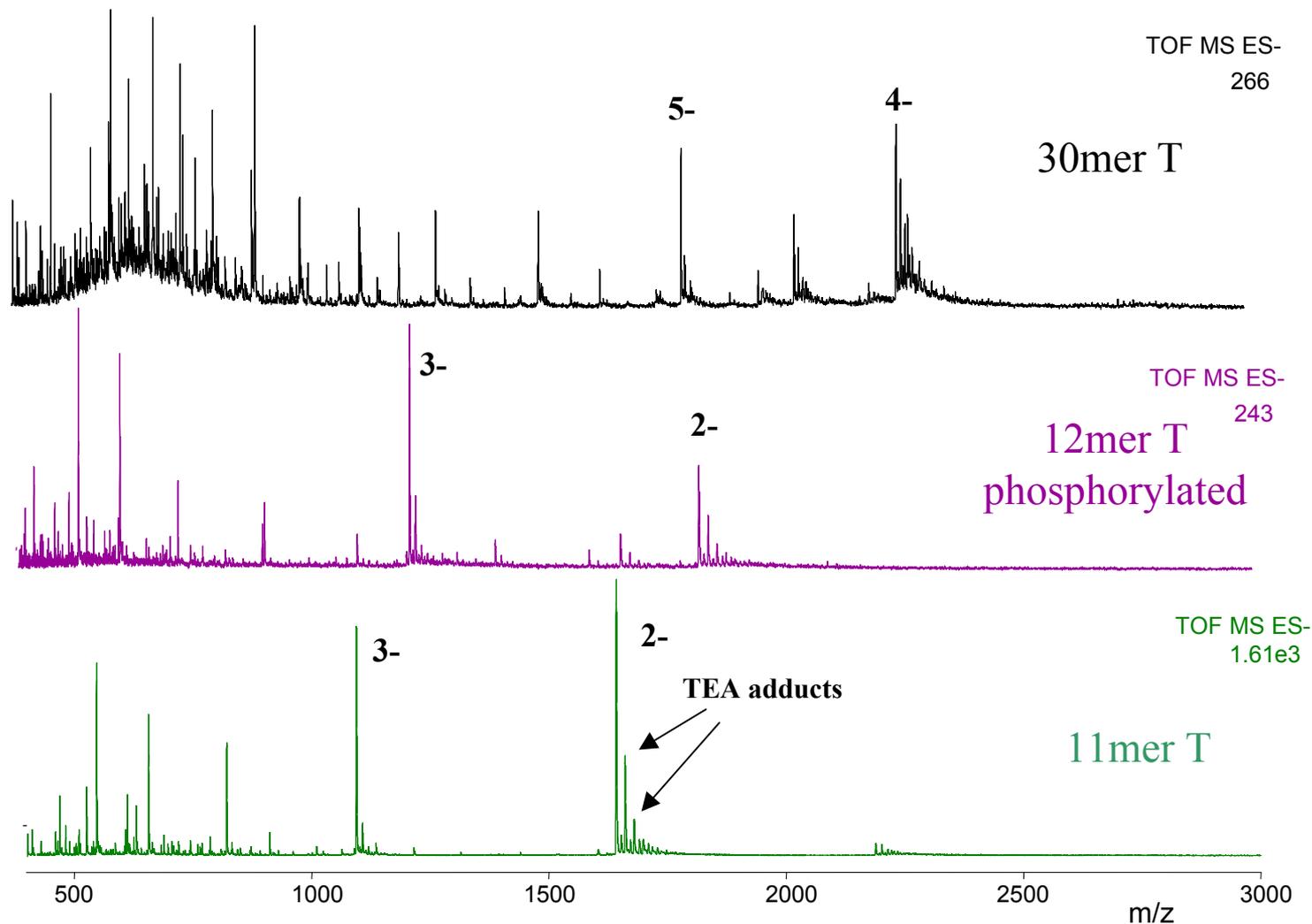
Selected ESI-MS spectra see panel 14



Capillary LC-LCT system, column 50x1 mm, XTerra™ MS C18, 2.5 μ m; mobile phase A: 100 mM- 8.6 mM TEA, pH 8.25, B: methanol; gradient starts at 10 % B, 0.4 % B per minute, flow rate 23.6 μ l/min, detection PDA and ESI-MS, 50°C, electrospray conditions see [panel 12 C](#), MS spectra see [panel 14](#)

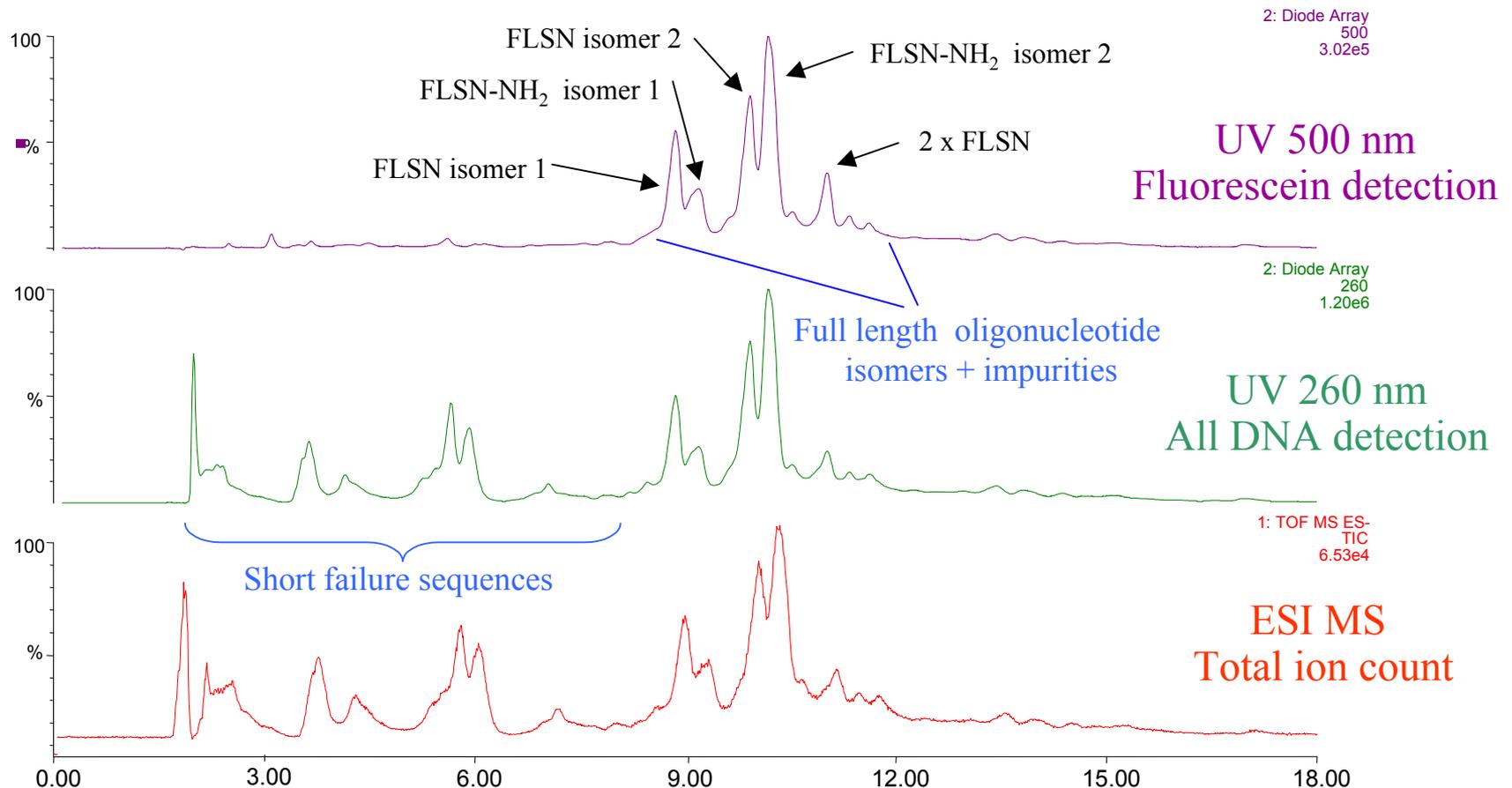
Identification of peaks from HPLC

For chromatogram see [panel 13](#), ESI-MS conditions are the same as on [panel 12C](#)



Impurities identification in 25mer fluorescein labeled probe by LC-UV-MS

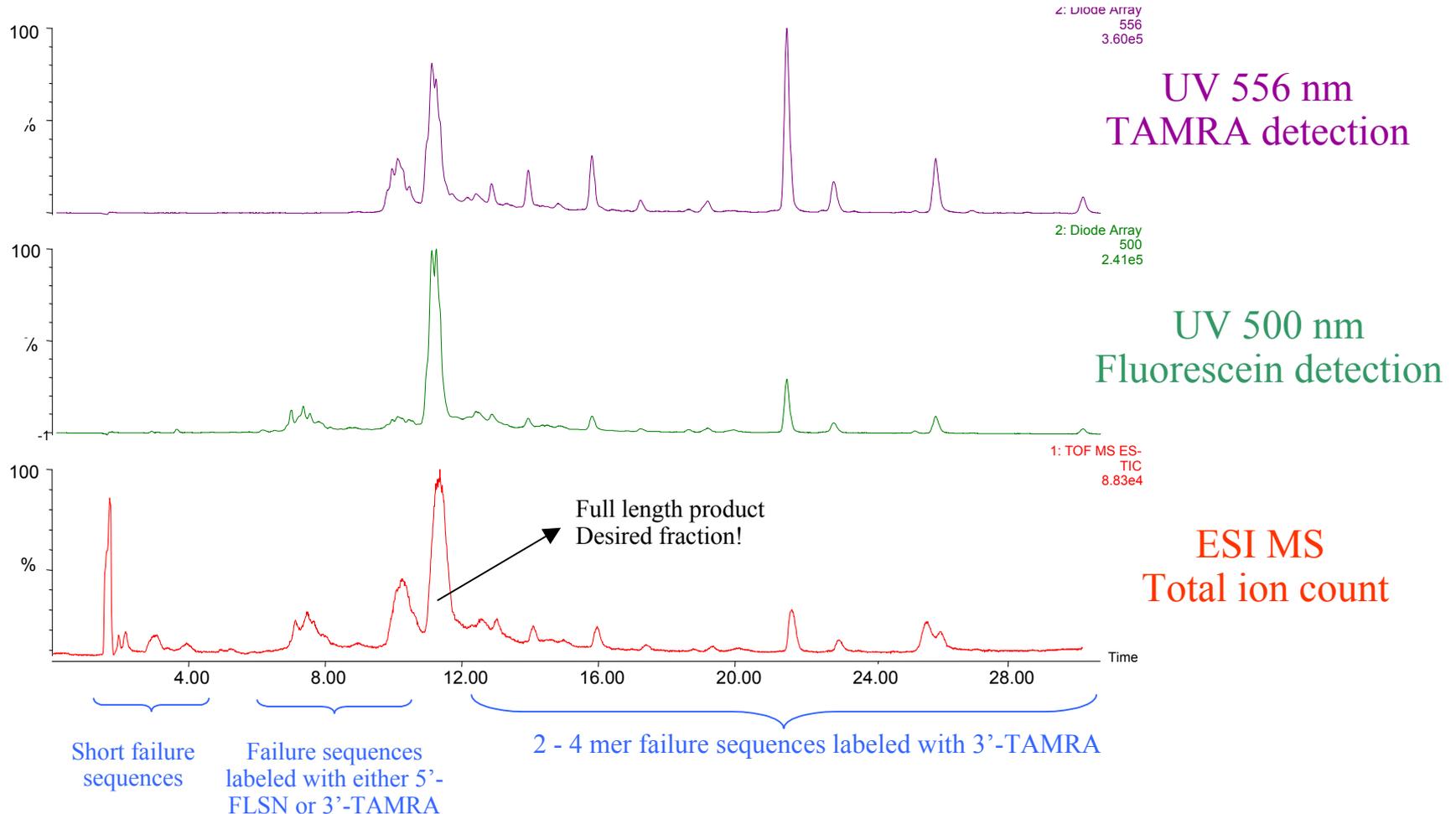
50x1mm XTerra™ MS C18, 2.5 μm, gradient starts from 15 % MeOH, slope 0.5 % MeOH per minute, 23.6 μl/min, column temperature 50°C, UV and ESI MS detection, MS condition see panel 12 C
Sample: 5' - (FLSN) GAC TTA GAC TTA GAC TTA G - 3'



Impurities identification in TaqMan 21-mer probe by LC-UV-MS of

50x1mm XTerra MS C18, 2.5 μm , gradient starts from 15 % MeOH, slope 1 % MeOH per minute, 23.6 ml/min, column temperature 50°C, UV and ESI MS detection, MS condition see panel 12 C

Sample: 5' - (FLSN) - CACCTCCAGTGGAAATCAAGT - (TAMRA) - 3'



Conclusion

- 1) XTerra™ MS C18 column is useful for DNA oligonucleotide quality control.**
- 2) Semi-preparative DNA purification was demonstrated.**
- 3) Optimized LC mobile phase offers both good HPLC separation of oligonucleotides and compatibility with ESI MS detection.**
- 4) Automated HPLC fraction collection was designed based on MS detection of selected molecular mass of desired DNA fragment.**