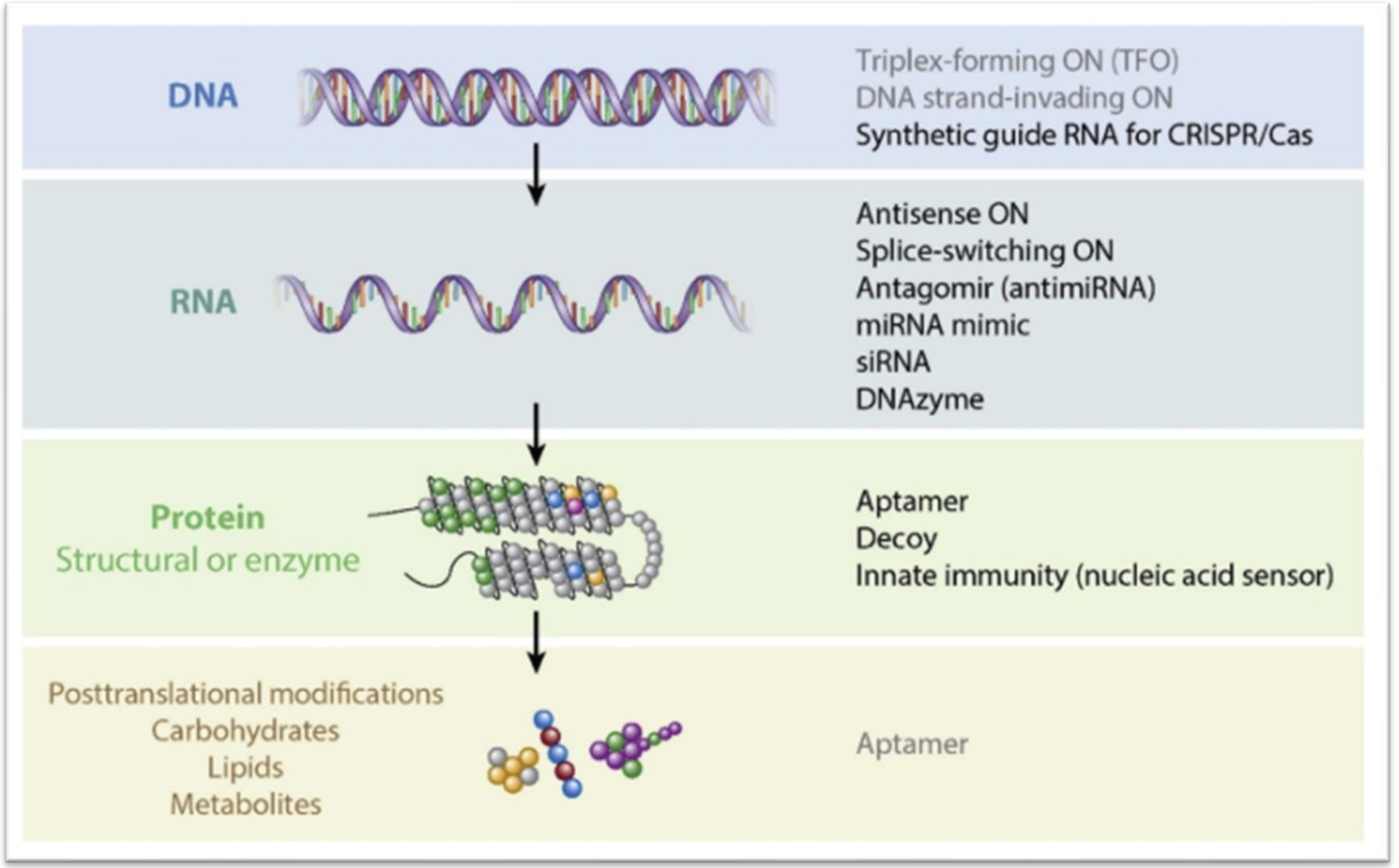


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Gel-Free Capillary Zone Electrophoresis at Acidic pH for Oligonucleotide Analysis – Challenging Old Assumptions

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Therapeutic oligonucleotides

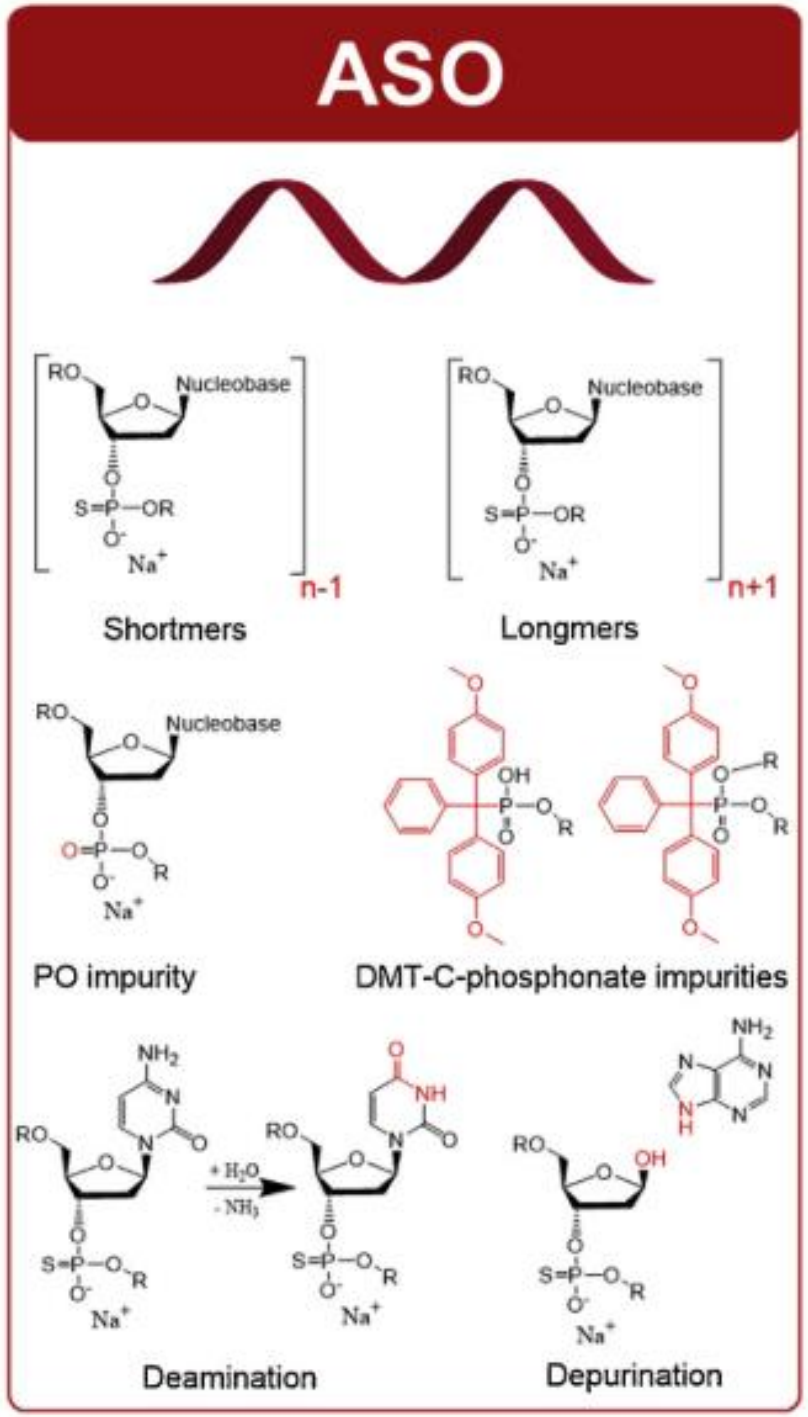


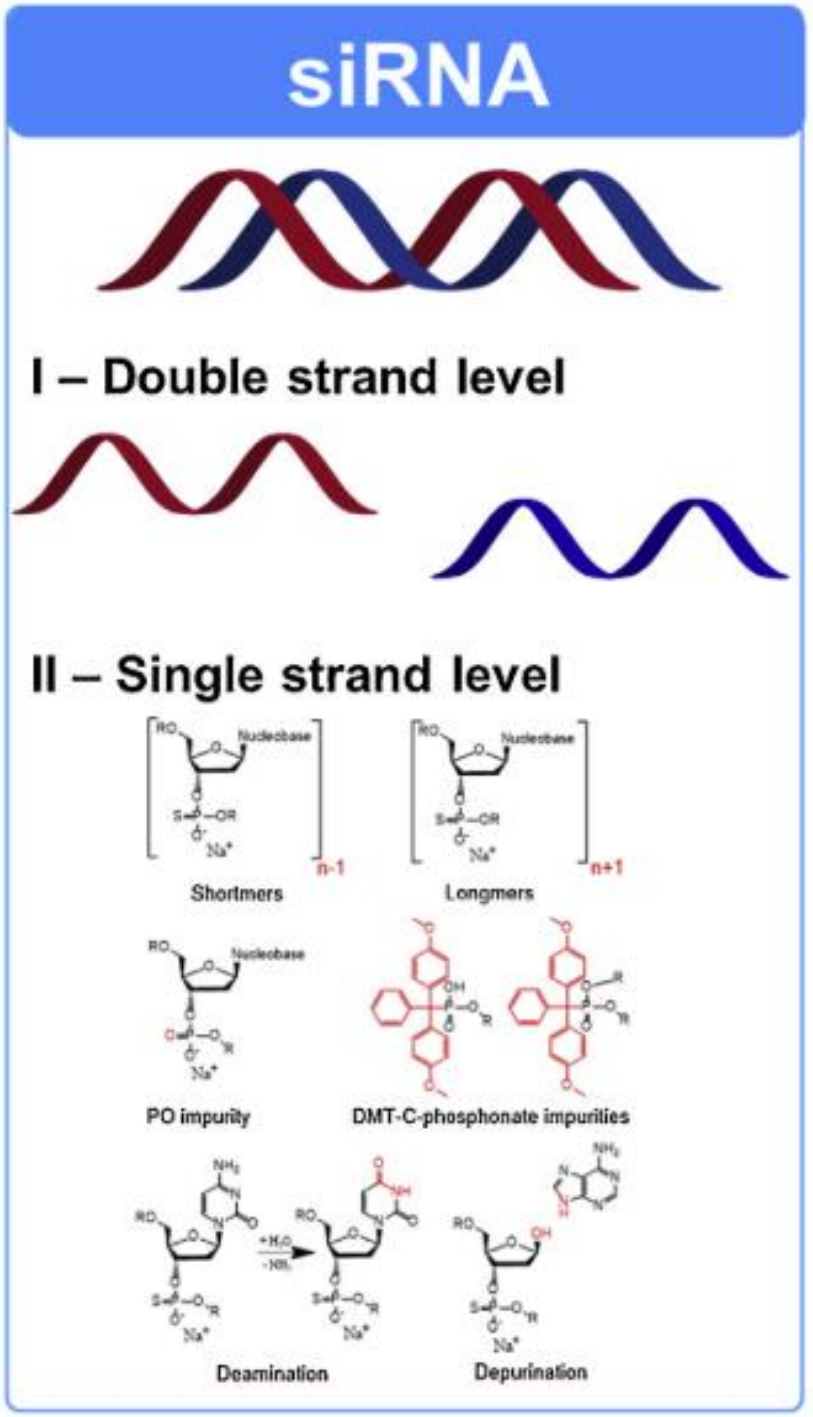
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Goyon et al, JPBA 182 (2020) 113105

Therapeutic oligonucleotides

- Impurities





Goyon et al, JPBA 182 (2020) 113105

Therapeutic oligonucleotide analysis

→ Mainly liquid chromatography:

- **IP-RP**
  Standard method for impurity analysis, detection using UV or MS.
  Separation based on structure.

- **AEX**
  Standard method for large scale production.
  Separation based on size.

- **HILIC**
  Getting increasing attention, especially due to MS-compatibility
  Separation based on structure

- MMC
- SEC
- 2D-LC



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Therapeutic oligonucleotide analysis

What about CE?

- **Capillary gel electrophoresis, CGE**
  Separations based on size

- **CZE-MS**
  Impurity charge variants
  MS to compensate for low CZE resolution at high pH



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Old assumptions – CZE of limited use

CZE separations can be achieved under native conditions for the separation of conformational variants and free drug from encapsulated drug. In addition, the CZE mode could separate charge variants such as impurities caused by deamination and depurination of the parent oligonucleotide.

Wei et al 2022

Separation technique	n/n-1 Resolution	Direct MS Compatibility	Additional Comments
CZE	<20 nt	No	Rarely used
Slab GE	~ 10-300 nt	No	High resolution; laborious for oligo purification
CGE	~ 10-600 nt	No	High resolution; Not for preparative scale
AEC	<100 nt	No	Preparative scale; Requires fraction desalting
RP LC	<20 nt	Yes	Purification of labeled ONs
IP-RPLC	<100 nt	Yes, HFIP m.p.	Most flexible; Primary LC-MS method
HILIC	<40 nt	Yes	Ion-pairing free; Lower MS sensitivity
SEC	<15 nt	No	Size-based separation
HIC	<15 nt	No	Rarely used
Affinity LC	-	No	mRNA purification based on polyA tail

Capillary Zone Electrophoresis (CZE), Slab Gel Electrophoresis (SGE), and Capillary Gel Electrophoresis (CGE)

Gilar and Stoll 2025

CE methods are seldom used in therapeutic ON analysis, but remain amongst the most established analytical techniques for characterization of biopharmaceuticals.

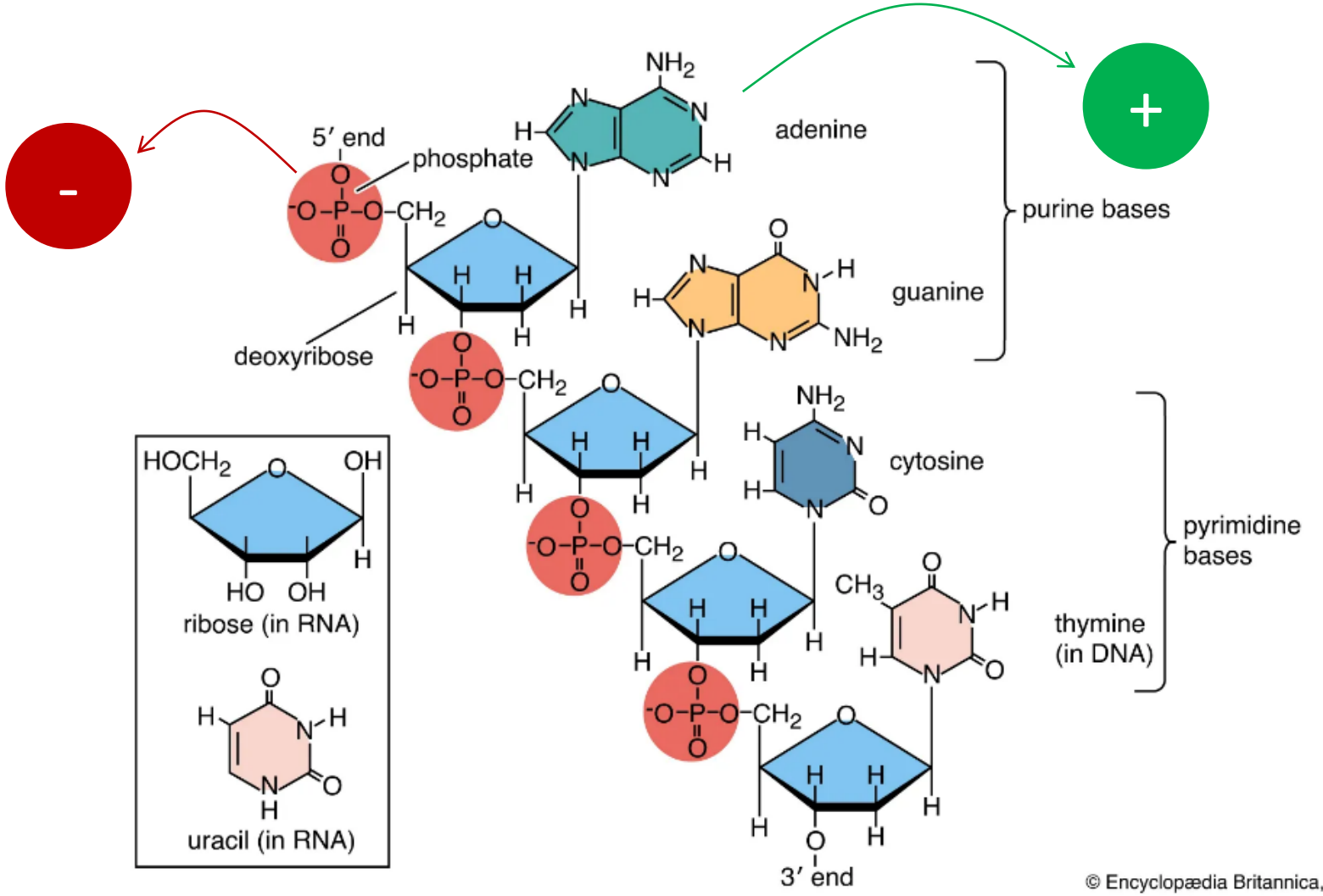
Dererova et al 2025

Historically, AEX and capillary gel electrophoresis (CGE) have been widely used for oligonucleotide analysis due to their high resolving power.

Al Bardawil et al 2026

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CZE analysis of oligonucleotides

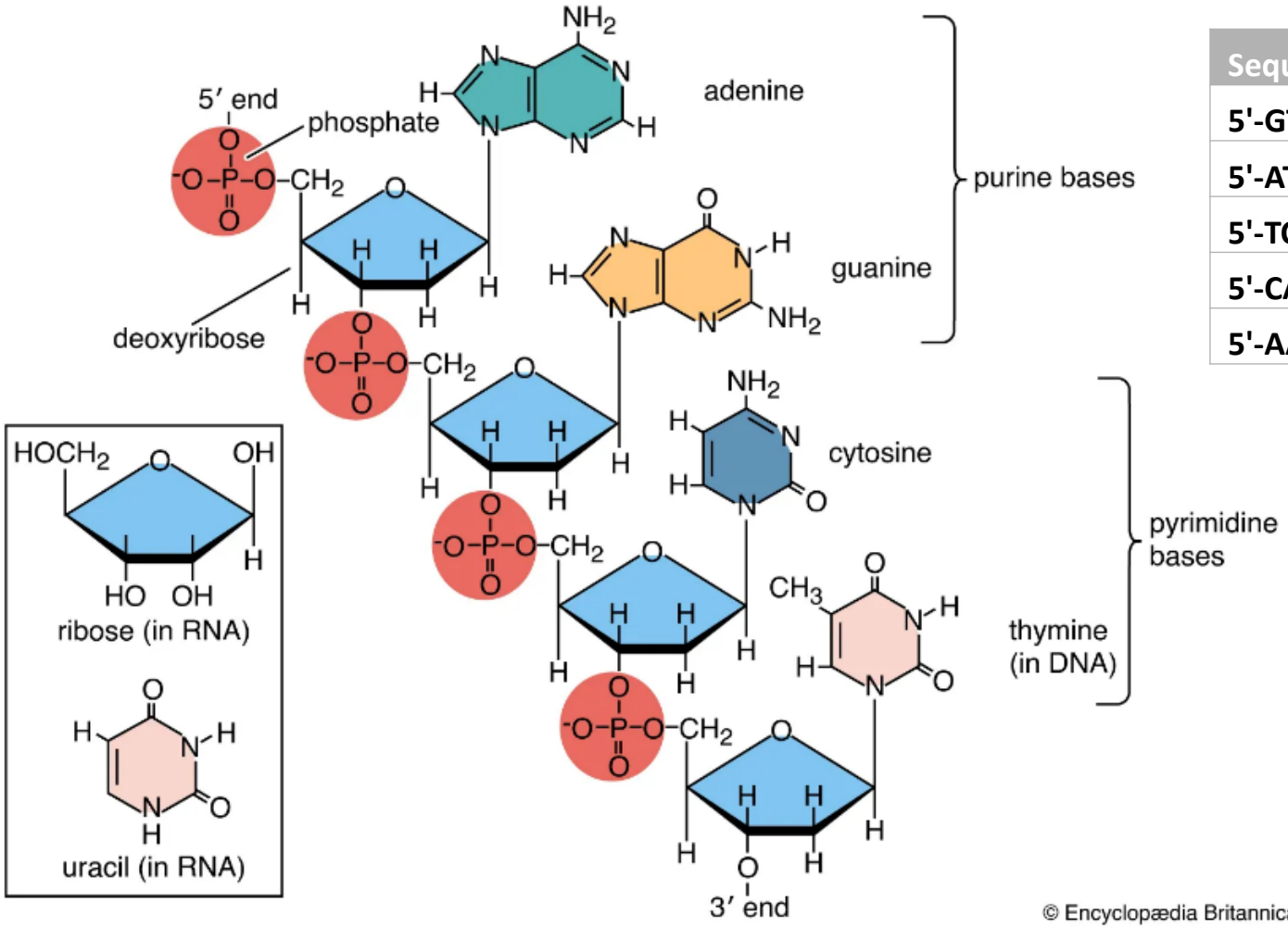


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pKa	
Adenine	3.8
Cytosine	4.5
Guanine	2.4
Thymine/	-
Uracil	-
Phosphate	0.7

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CZE analysis of oligonucleotides at acidic pH

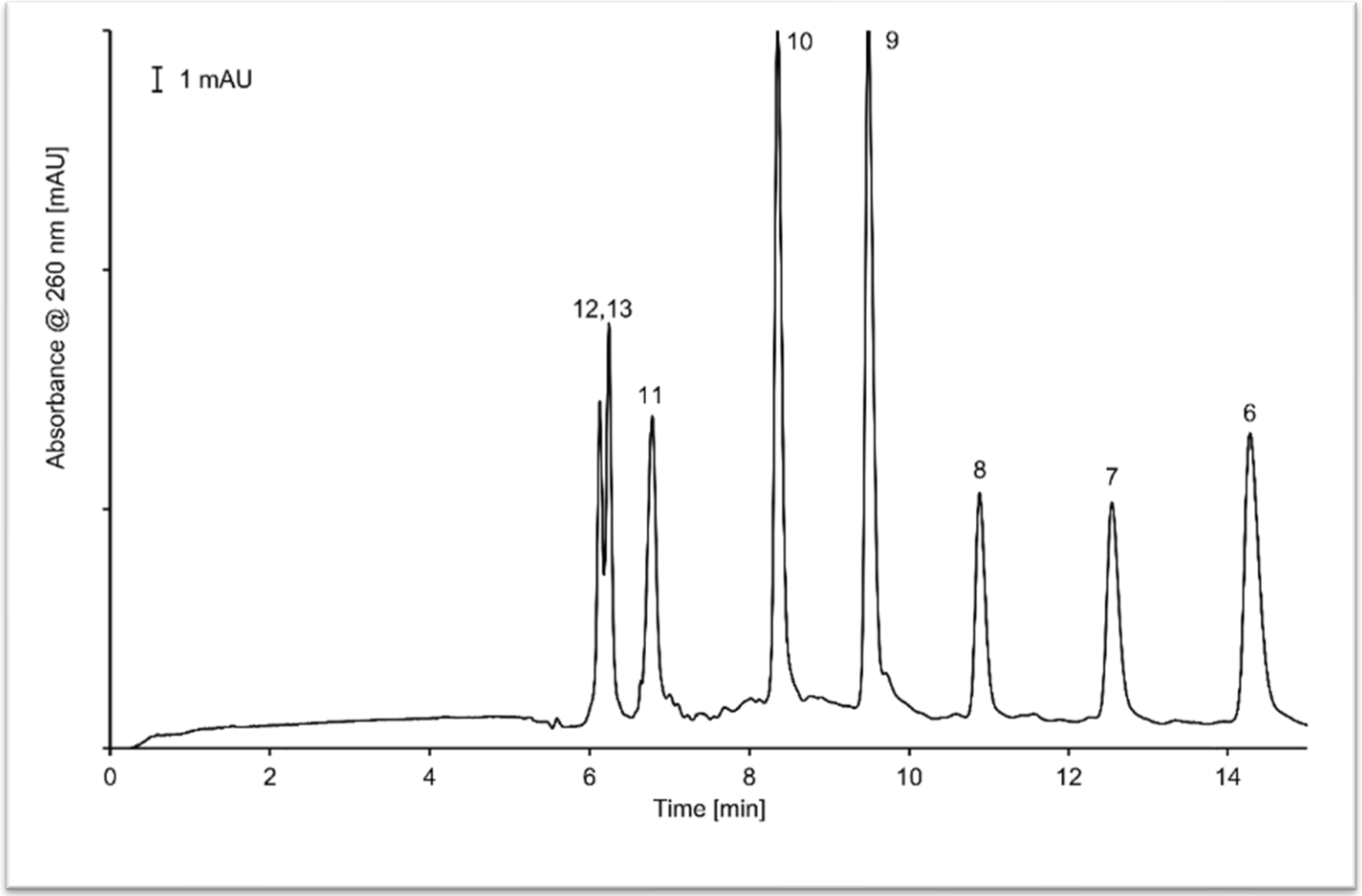


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Sequence	N	AC%	Charge
5'-GTT GTG TGT GTT GGT GTT GG-3'	20	0 %	-17.1
5'-ATG GTT GTC CGA GCT ATG GC-3'	20	35 %	-11.3
5'-TCG TGG ACC AGT CGA ATT CA-3'	20	50 %	-9.1
5'-CAA CAG TAG GCC TAA CCT TC-3'	20	65 %	-6.8
5'-AAG CCC TAG ACT ACC ACC AA-3'	20	80 %	-4.3

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Proof of concept



32.5 cm PVA capillary
100 mM H_3PO_4
80 mM NaOH
pH 2.8
20 °C
-10 kV
UV @260 nm

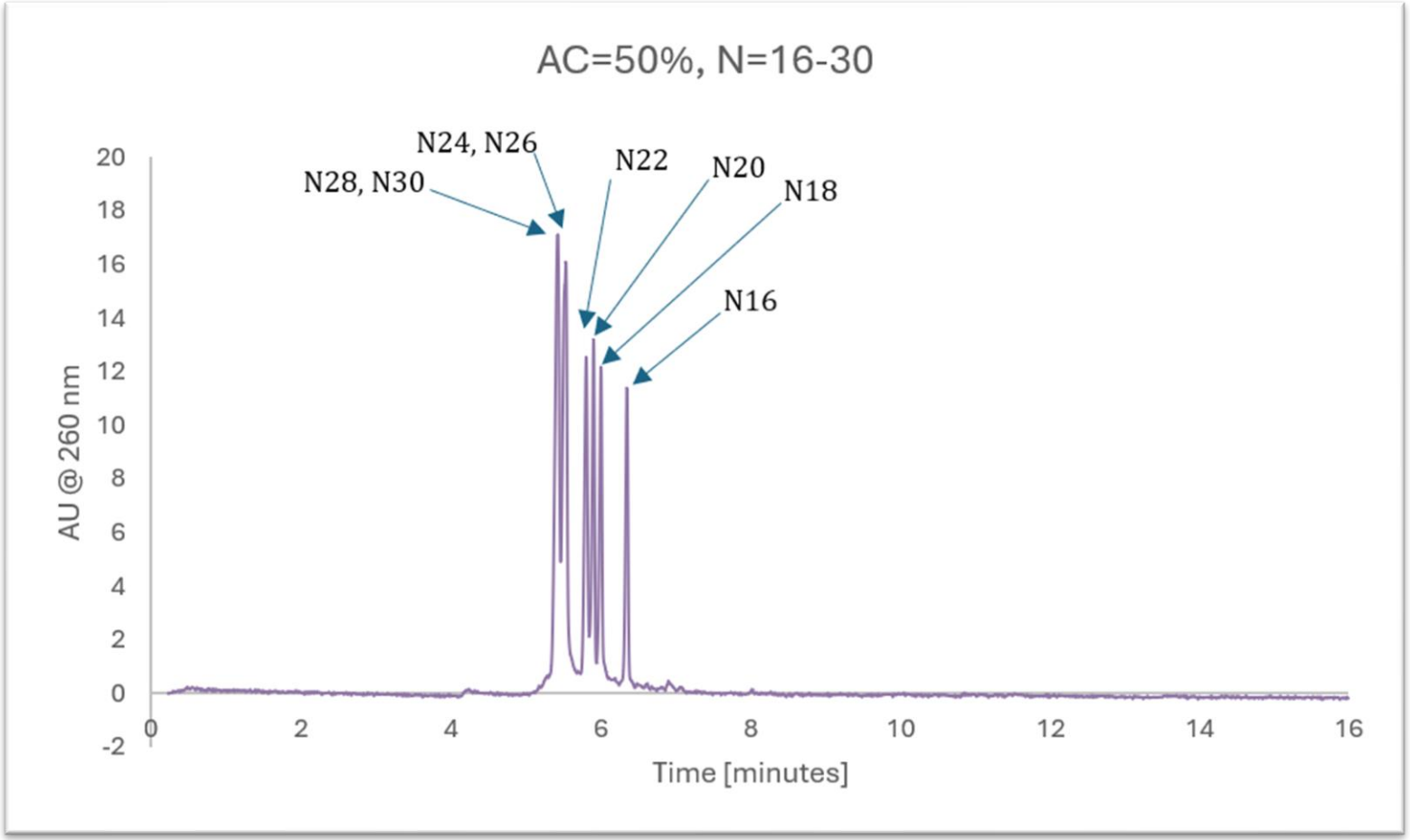
<u>8 ssDNA sequences</u>

18 – 25 nt
8 – 78 % AC

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Haglöf et al, Talanta 297 (2026) 128610

Proof of concept



32.5 cm PVA capillary
100 mM Malonic acid
50 mM NaOH
pH 2.8
20 °C
-10 kV
UV @260 nm

<u>8 ssDNA sequences</u>

16 – 30 nt
50 % AC

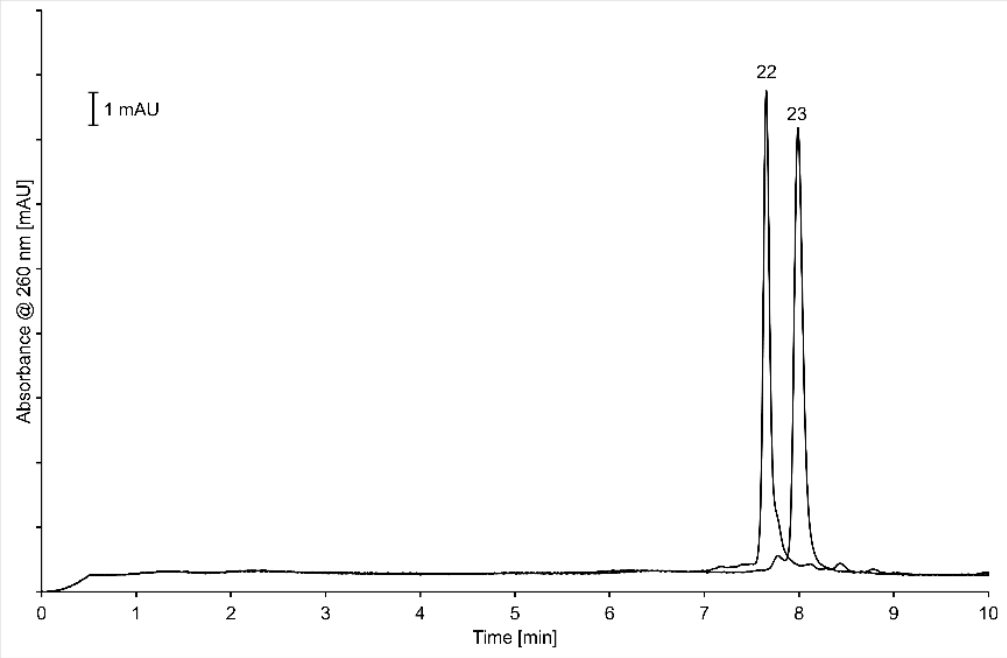
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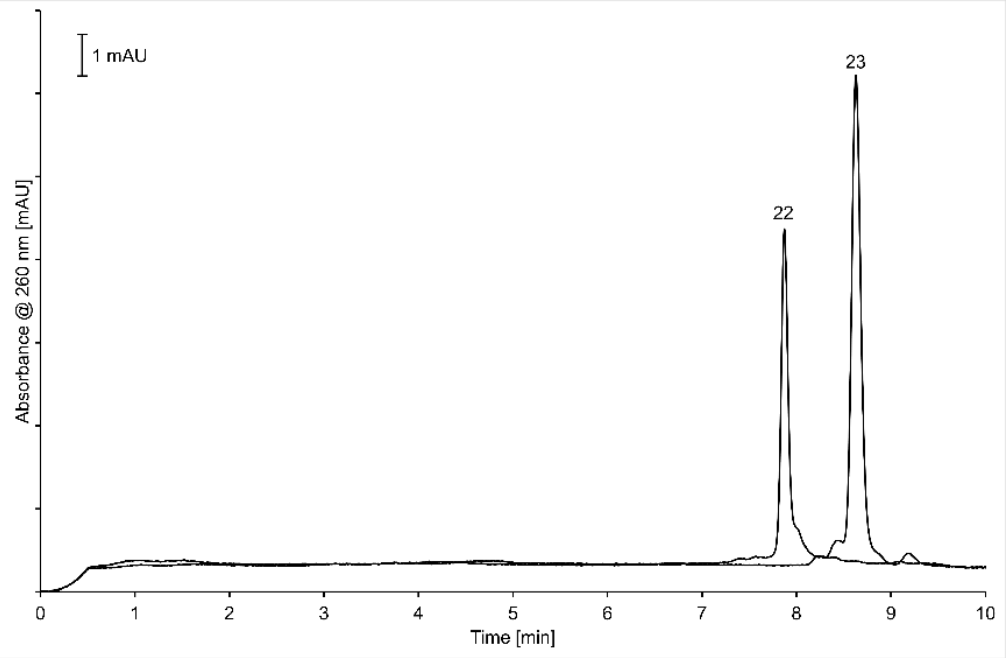
Paraschou, MSc thesis, 2026

Proof of concept

Works for both DNA and RNA

→ Can use DNA as a model

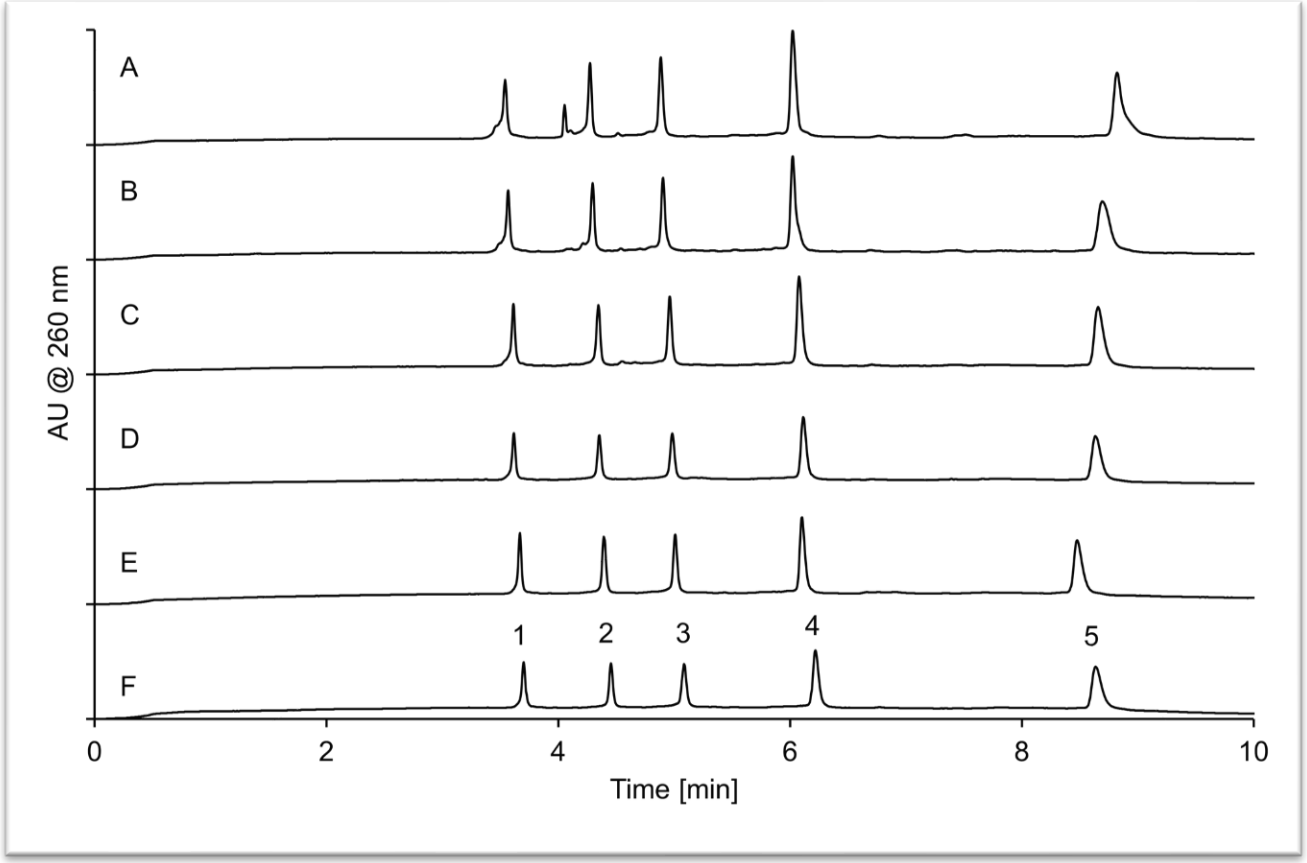




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Haglöf et al, Talanta 297 (2026) 128610

Knowledge building



32.5 cm PVA capillary
0 – 100 mM Malonic acid
100 – 0 mM Phosphoric acid
48 – 82 mM NaOH
pH 2.8
20 °C
-10 kV
UV @260 nm

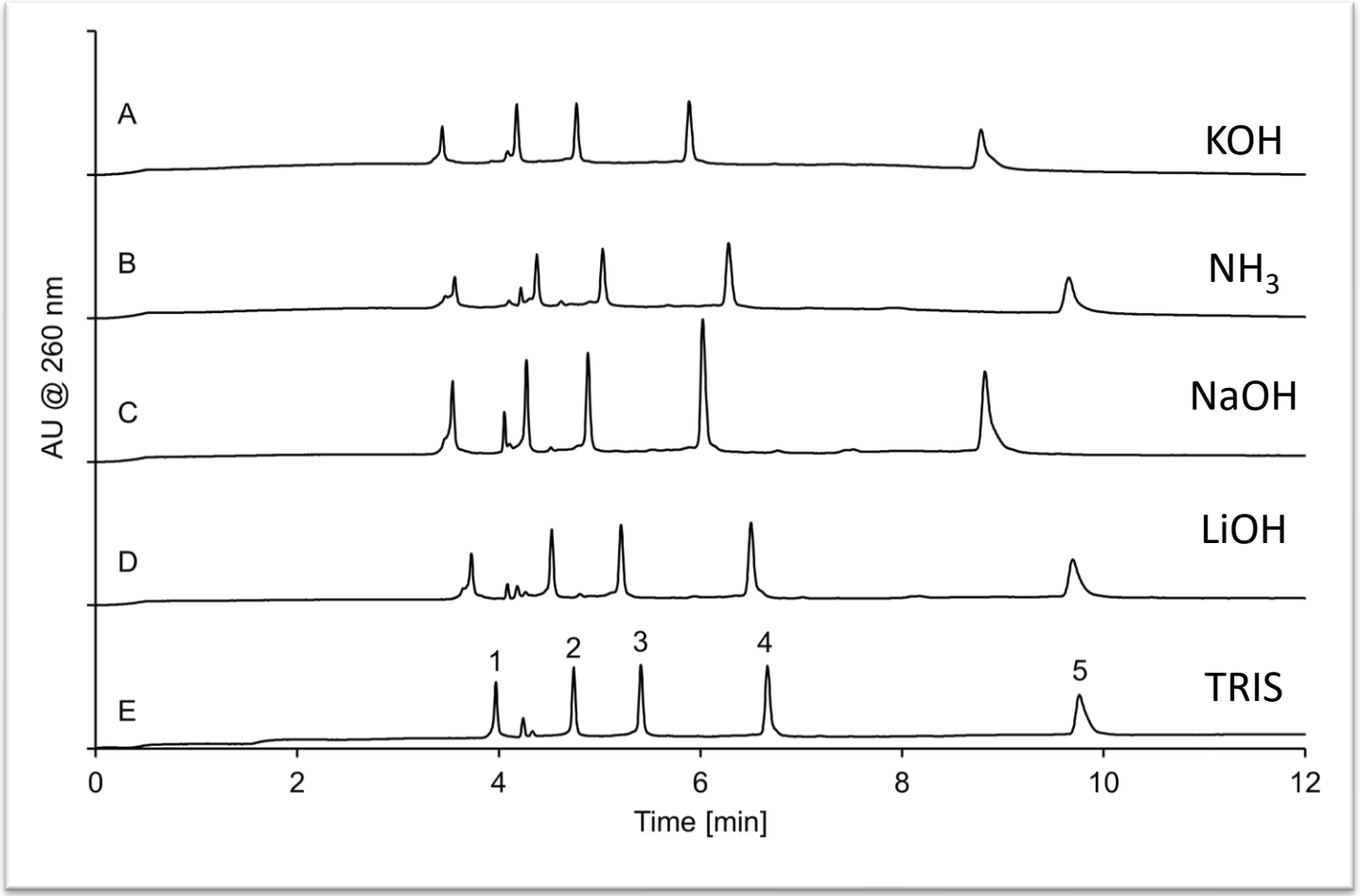
5 ssDNA sequences

20 nt
0 – 80 % AC

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Knowledge building



32.5 cm PVA capillary
100 mM Malonic acid
50 mM base
pH 2.8
20 °C
-10 kV
UV @260 nm

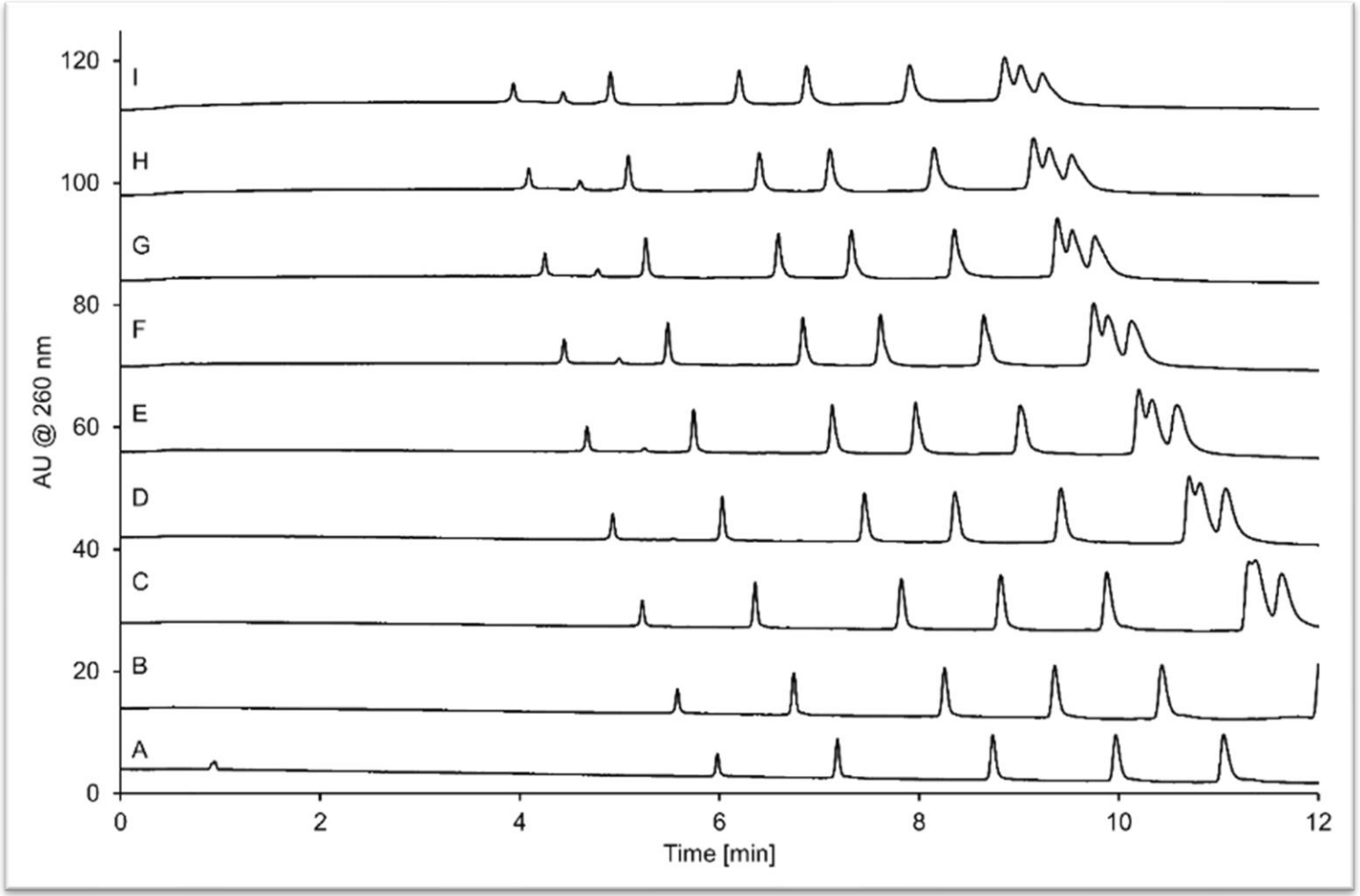
5 ssDNA sequences

20 nt
0 – 80 % AC

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Knowledge building



32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
20 – 60 °C
-10 kV
UV @260 nm

8 ssDNA sequences

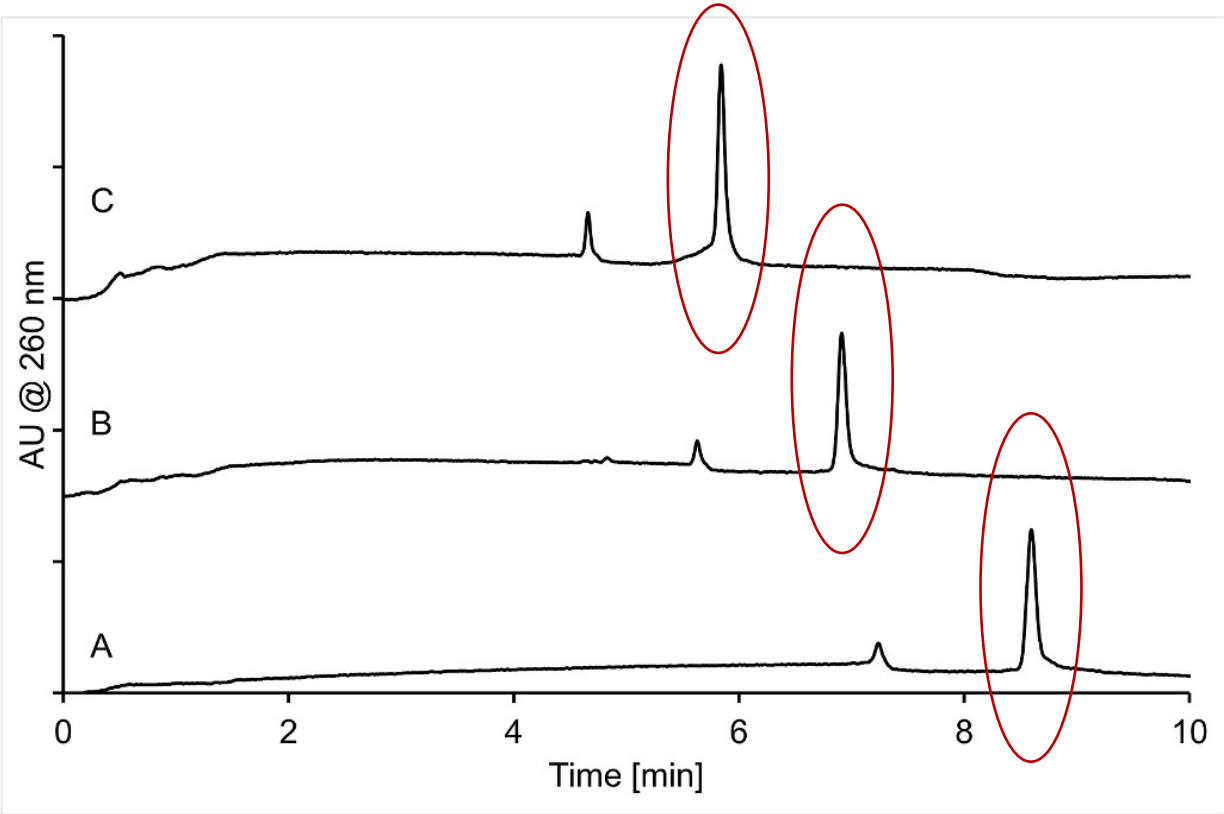
18 – 25 nt
5 – 80 % AC

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Secondary structure investigation

Hypothesis: no secondary structure formation at acidic pH and elevated temperature.



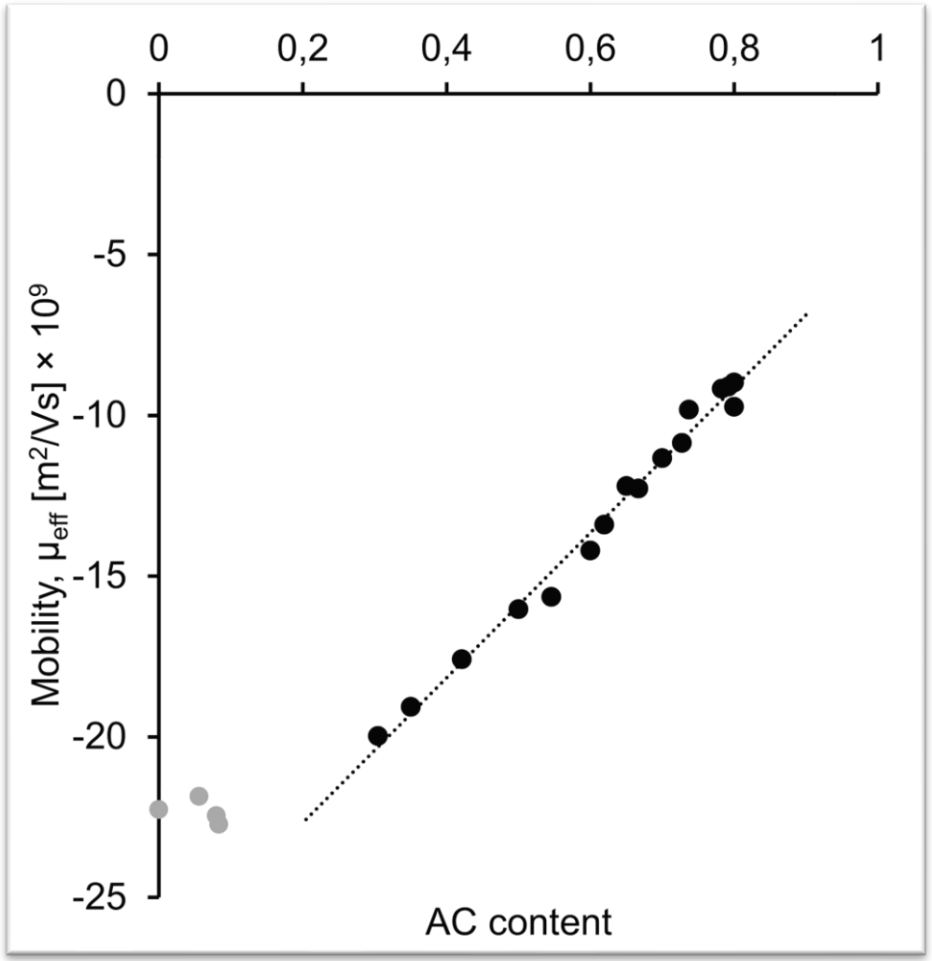
32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
20, 40, 60 °C
-10 kV
UV @260 nm

<u>3 ssDNA sequences</u>
20 nt
50 % AC
Different duplex or hair-pin formation

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Mobility dependance on AC content



Modelable!
$R^2 > 0.96$

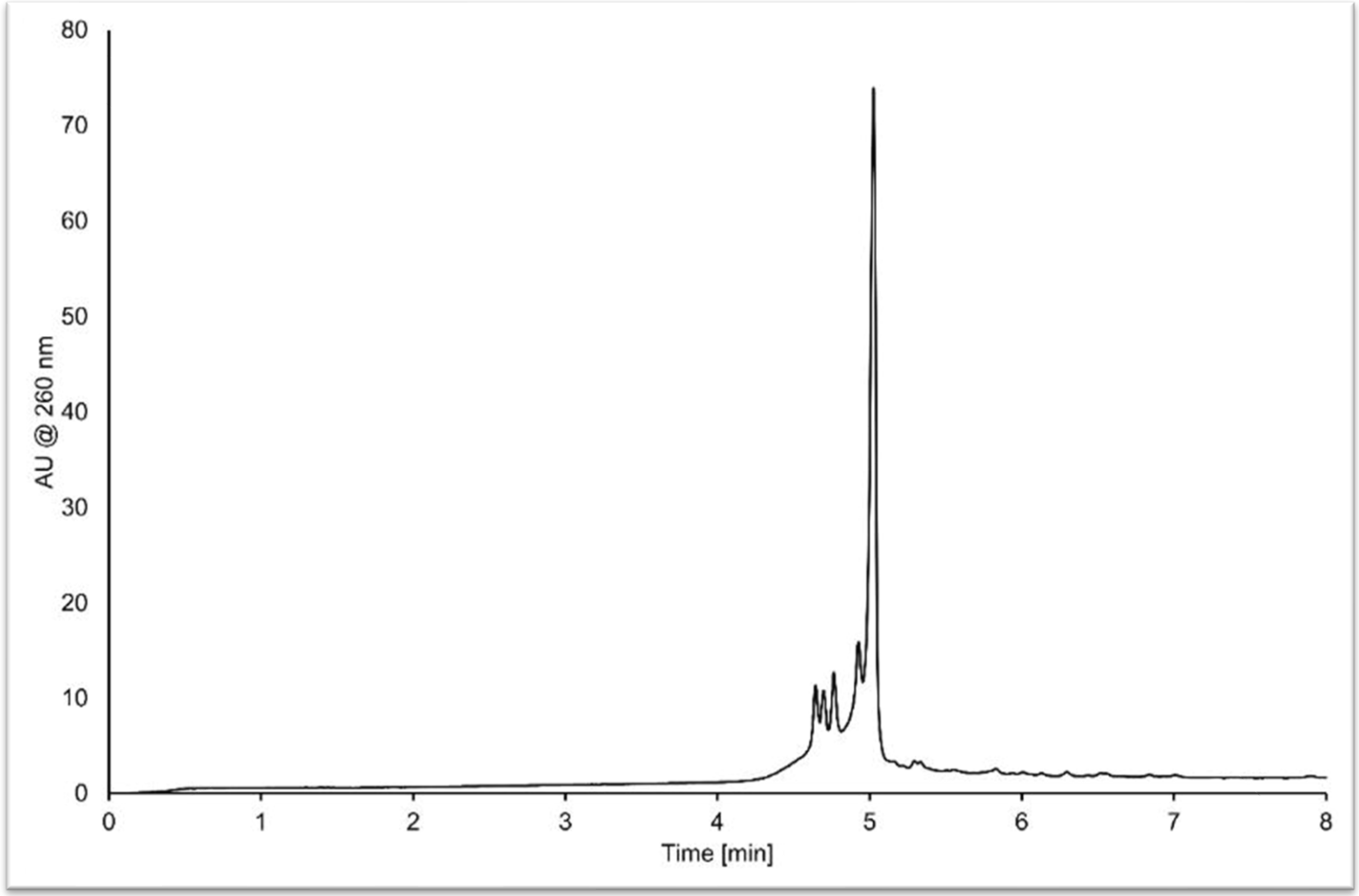
<u>20+ ssDNA sequences</u>

18 – 25 nt
5 – 80 % AC

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Oligonucleotide impurity profiling



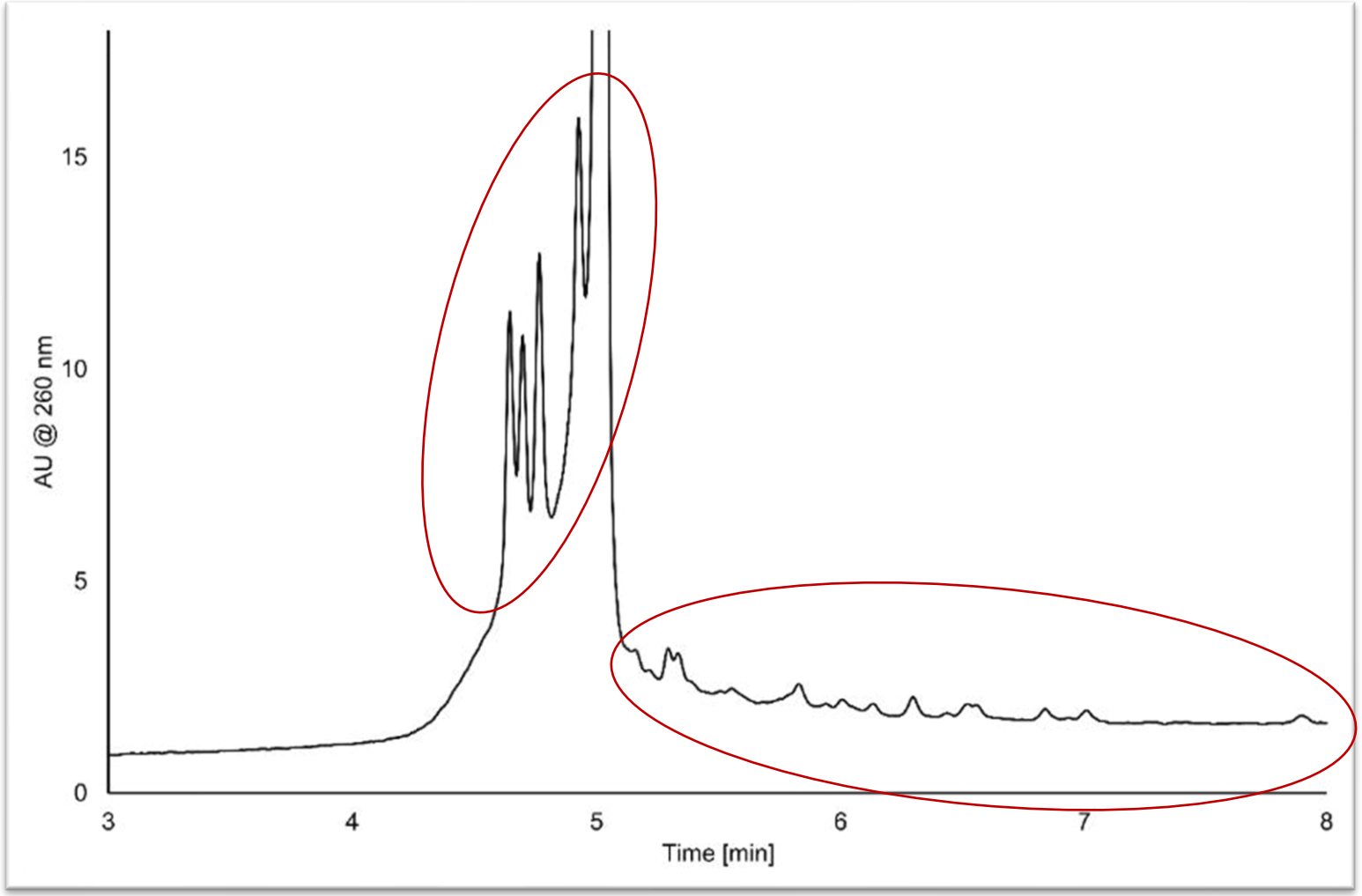
32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
60 °C
-10 kV
UV @260 nm

<u>Synthetic 20 nt oligo</u>
+ 4 impurities:
shortmers
deaminations

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Oligonucleotide impurity profiling



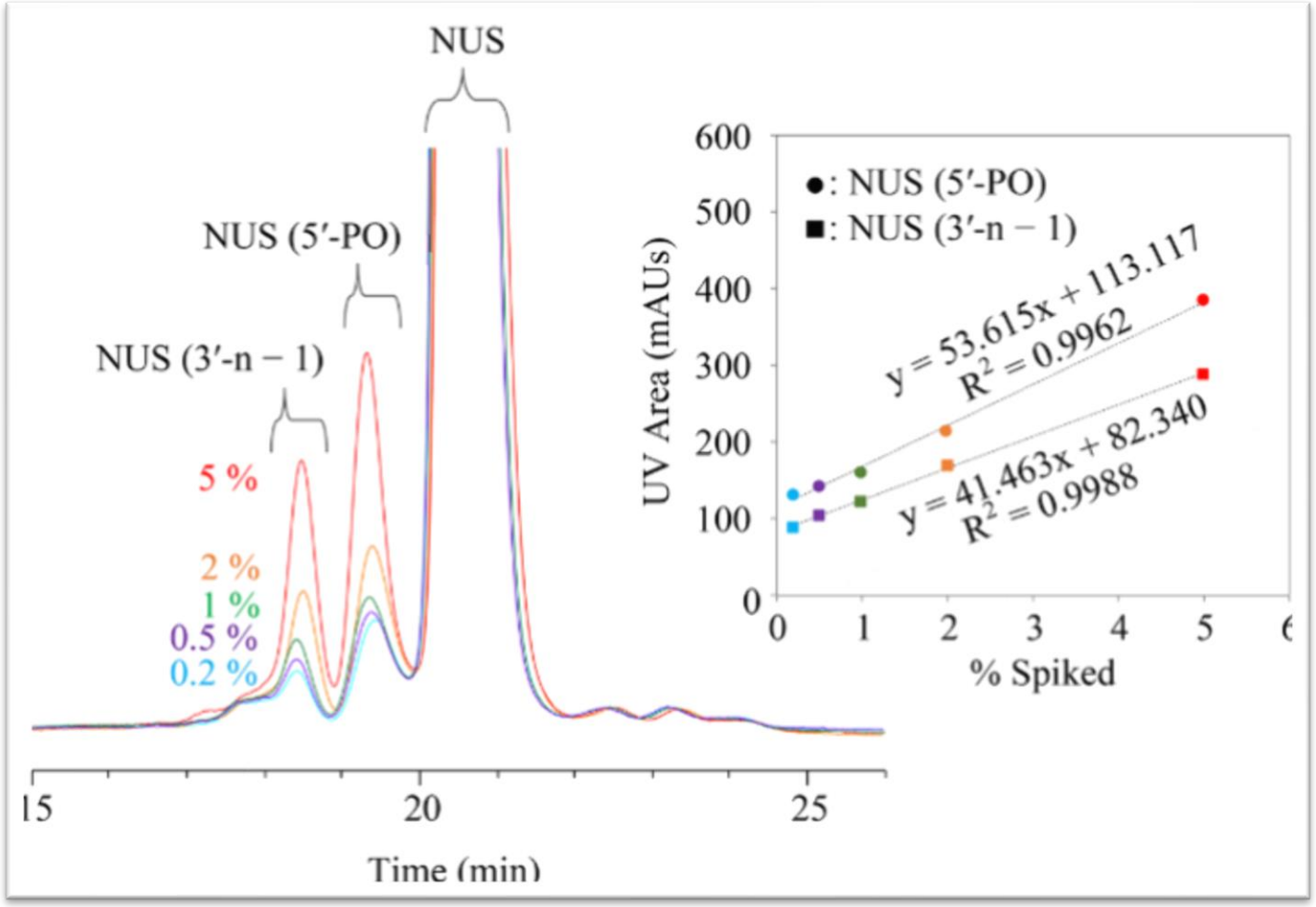
32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
60 °C
-10 kV
UV @260 nm

<u>Synthetic 20 nt oligo</u>
+ 4 impurities:
shortmers
deaminations

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Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Oligonucleotide impurity profiling

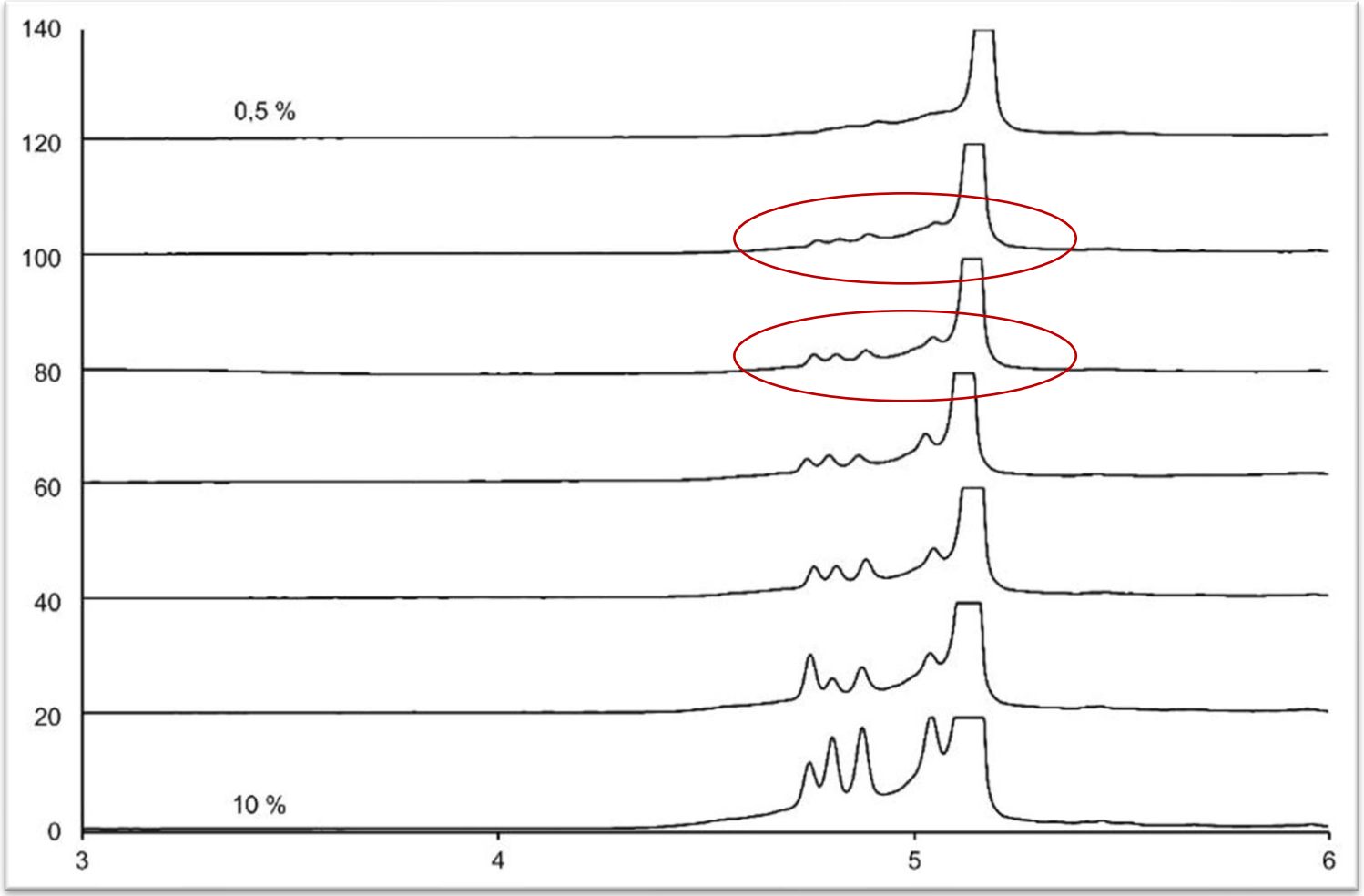


The recently published EMA guideline on oligonucleotide development and manufacture indicates that identification and qualification thresholds of 1.0% and 1.5%, respectively, are generally acceptable.

Al Bardawil et al, Anal Chem 98 (2026) 10895

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Oligonucleotide impurity profiling



32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
60 °C
-10 kV
UV @260 nm

<u>Synthetic 20 nt oligo</u>
+ 4 impurities:
shortmers
deaminations

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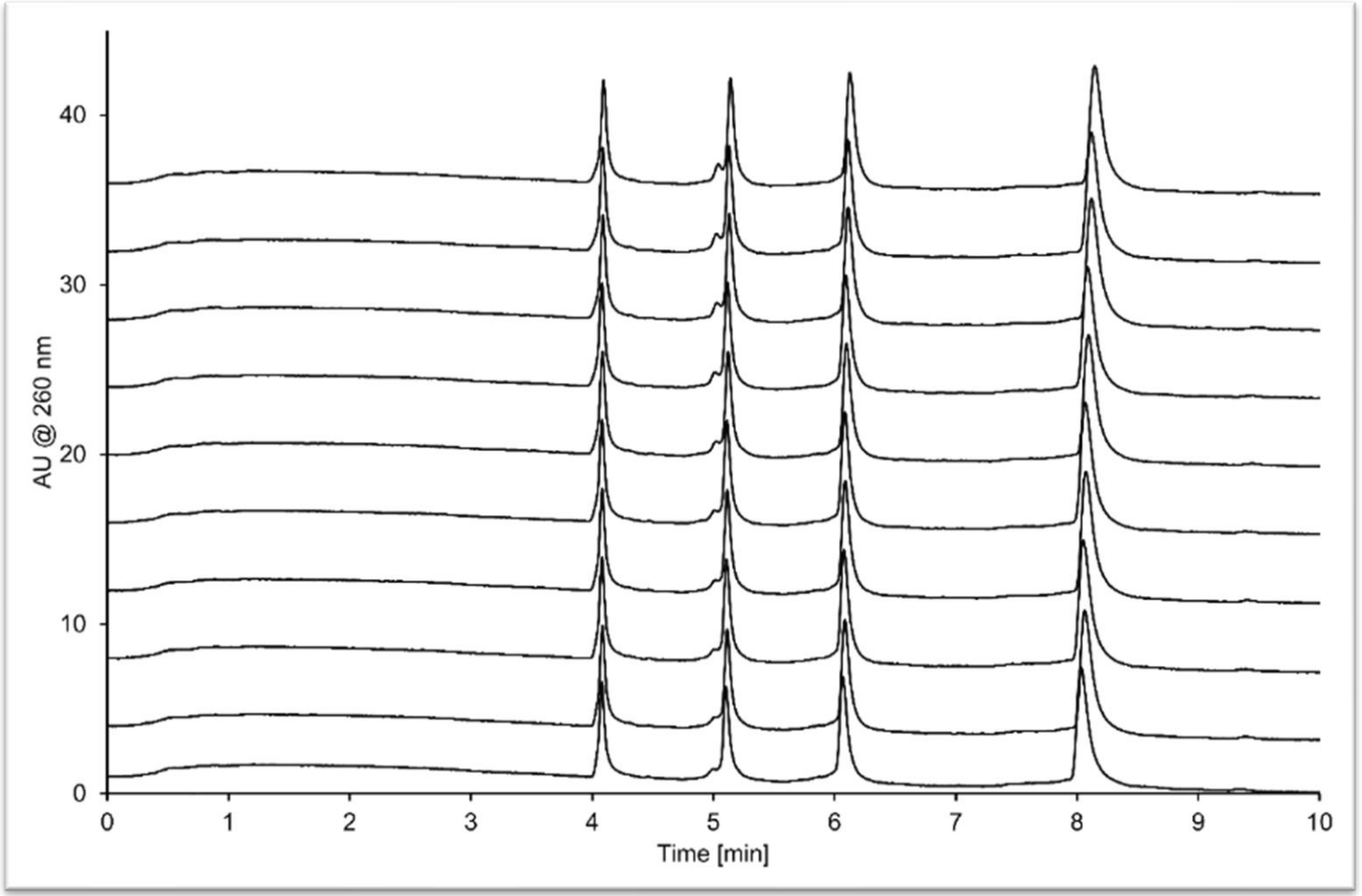
Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Oligonucleotide impurity profiling

Repeatability

32.5 cm PVA capillary
75 mM Malonic acid
38 mM NH_3
pH 2.8
60 °C
-10 kV
UV @260 nm

<u>Precision</u>
Mobility: < 0.5 % CV
Resolution: < 1.6 % CV



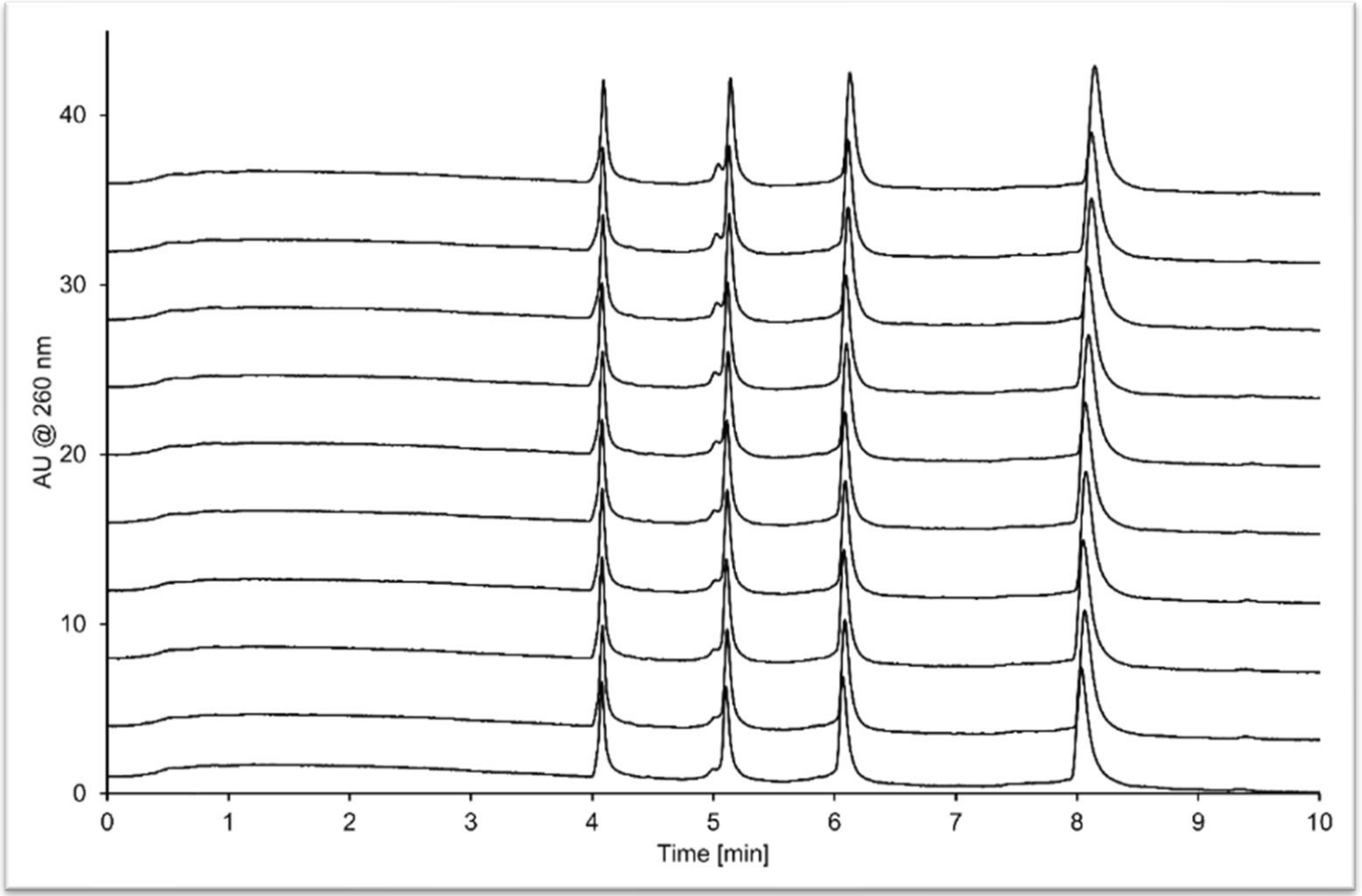
Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Oligonucleotide impurity profiling

Repeatability

Key parameters

- Sample cooling
- Buffered samples
- Capillary washing (10 mM H_3PO_4)



Precision

Mobility: < 0.5 % CV

Resolution: < 1.6 % CV

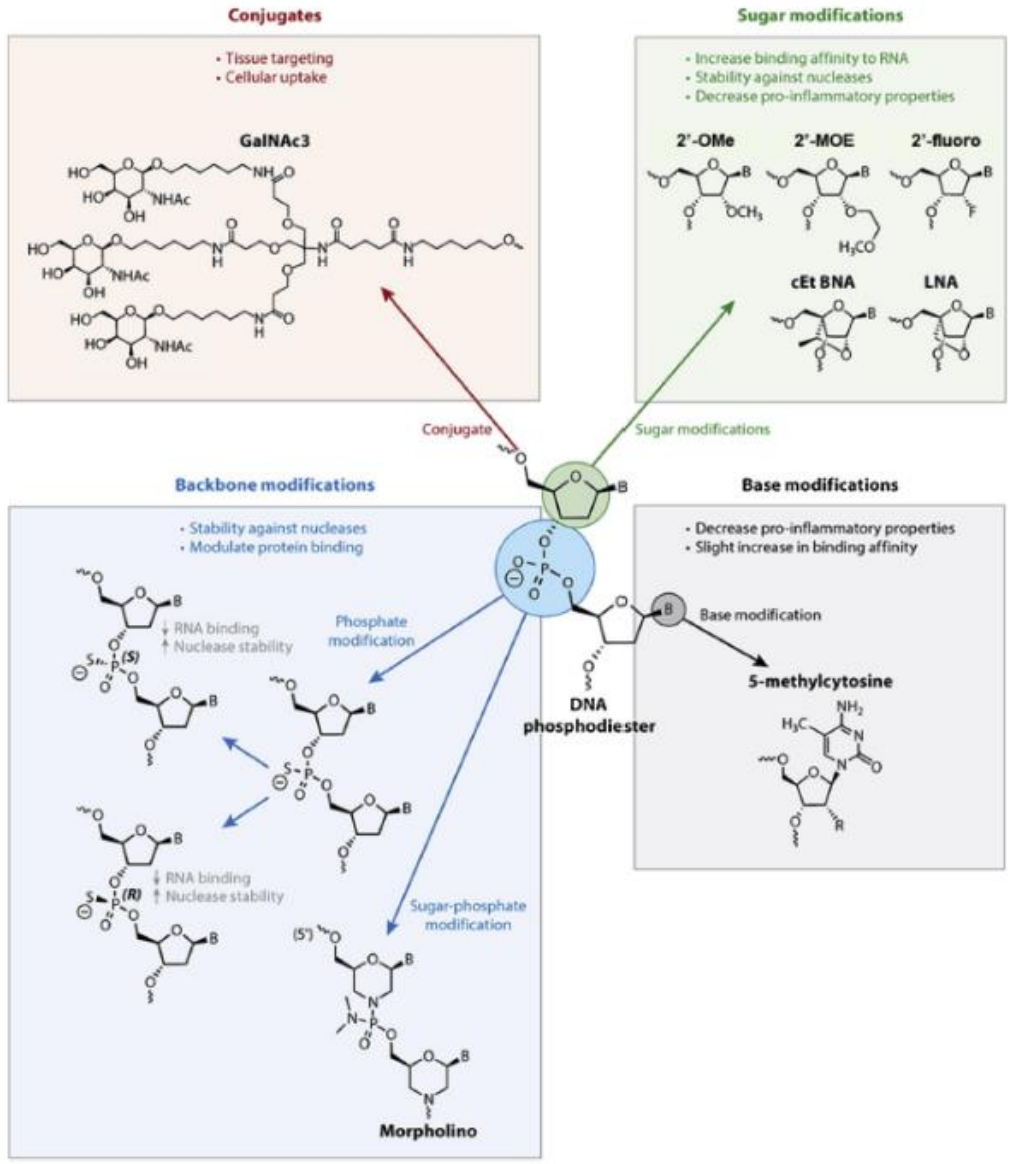
Haglöf et al, Analytica Chimica Acta 1402 (2026) 345395

Ongoing work

- MS detection
- ON modifications
- Bioanalysis (miRNA)

Conjugates

Backbone



Sugar

Base

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Goyon et al, JPBA 182 (2020) 113105

Acknowledgements

- Professor Cari Sänger-van de Griend
- Julia Regnell, PhD student
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