EBF Spring Focus Workshop 2023 – Matthijs Pijnappel

METHOD DEVELOPMENT FOR QUANTIFICATION OF OLIGONUCLEOTIDES BY LC-MS IN BIOLOGICAL SAMPLES SUITABLE FOR PRE-CLINICAL STUDIES

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INTRODUCTION

- Challenges in oligonucleotide analysis method development
  - Ionization
    - Signal intensity
    - Division over many charge states
    - Adduct formation
  - Chromatographic peak shape
  - Extraction efficiency from plasma

- Analyte
  - 7.5 – 11.0 kDa
  - Single stranded oligonucleotide

- Pre-validation of the method

- Peak shape issues over time

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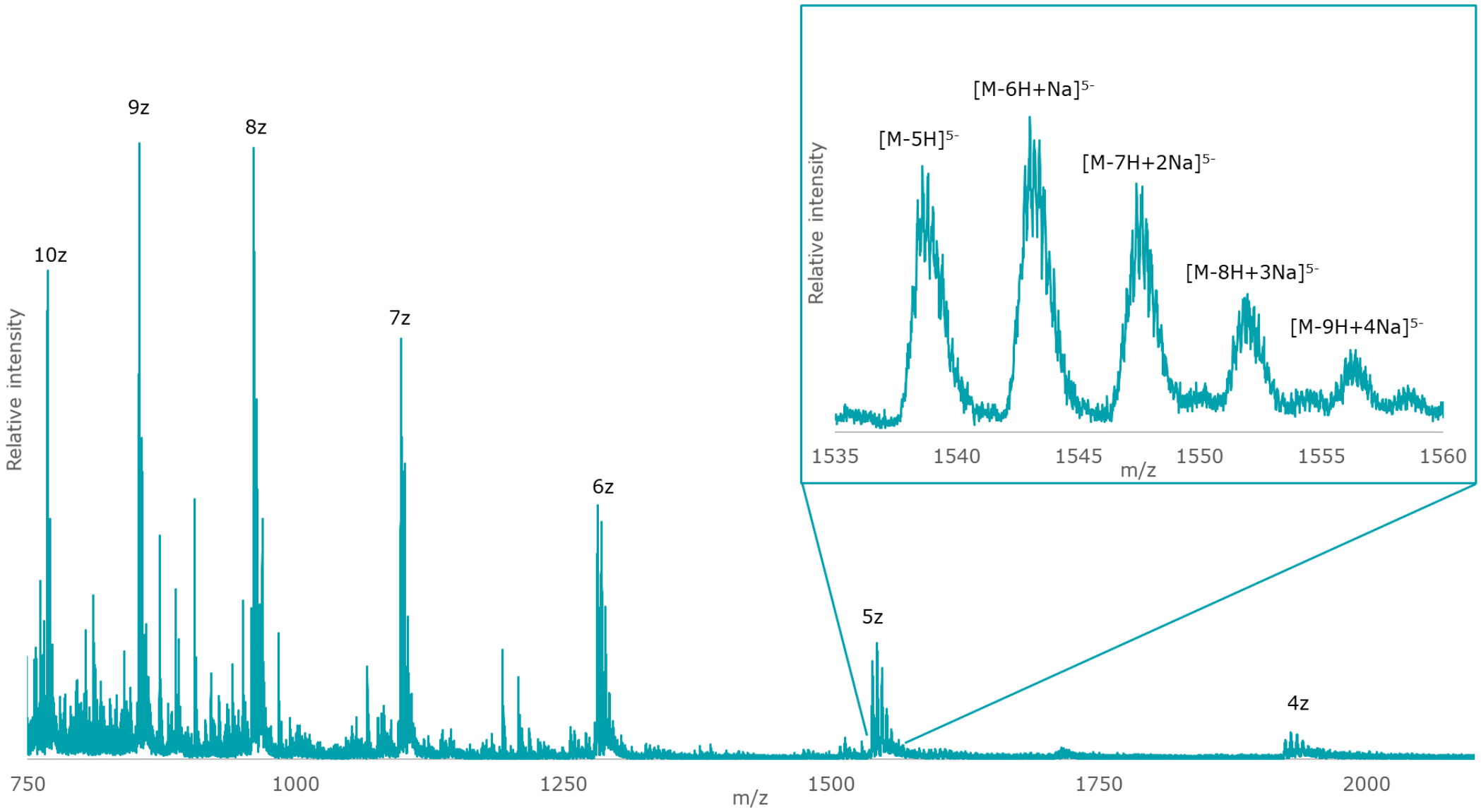
CHALLENGES IN OLIGONUCLEOTIDE MASS SPECTROMETRY



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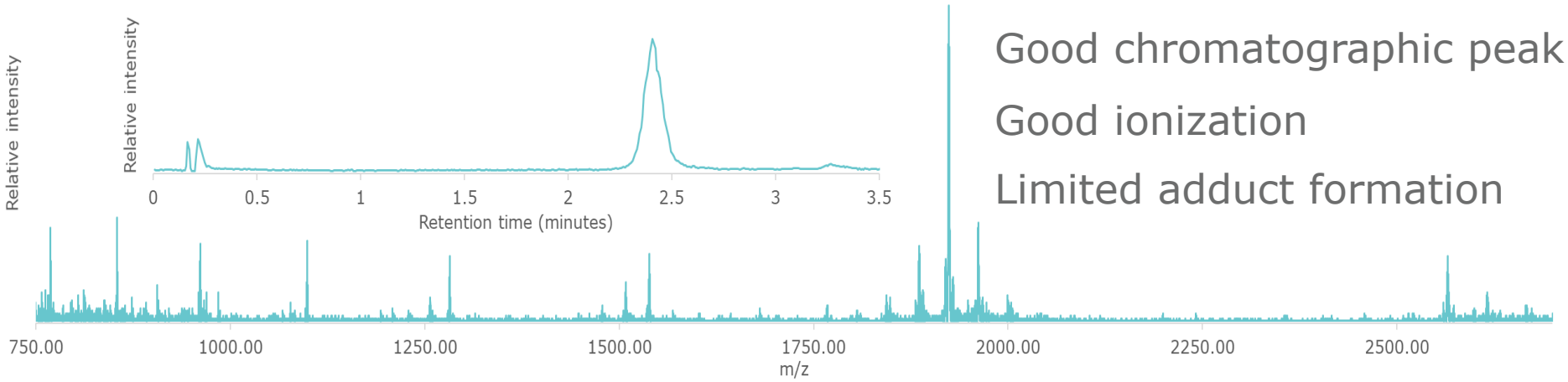
CHROMATOGRAPHIC AND MASS SPECTROMETRIC IMPROVEMENTS

- Ionization and chromatographic separation are connected by the mobile phase used
- Initially acid and base combinations were screened for ionization characteristics in a high throughput way, using flow injection, monitoring:
  - Adduct formation
  - Signal intensity
  - Most prevalent charge state
- Chromatographic performance
  - Chromatographic peak shape
  - Adduct formation
  - Signal intensity
  - Focused charge state

MOBILE PHASE SELECTION

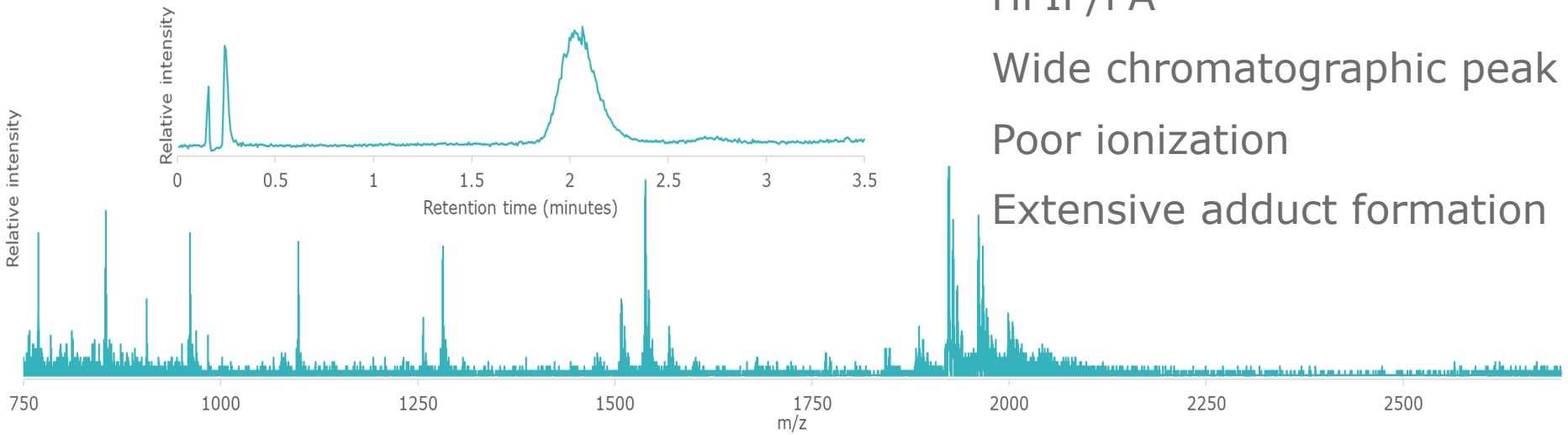


HFIP/DIPA

Good chromatographic peak

Good ionization

Limited adduct formation



HFIP/PA

Wide chromatographic peak

Poor ionization

Extensive adduct formation

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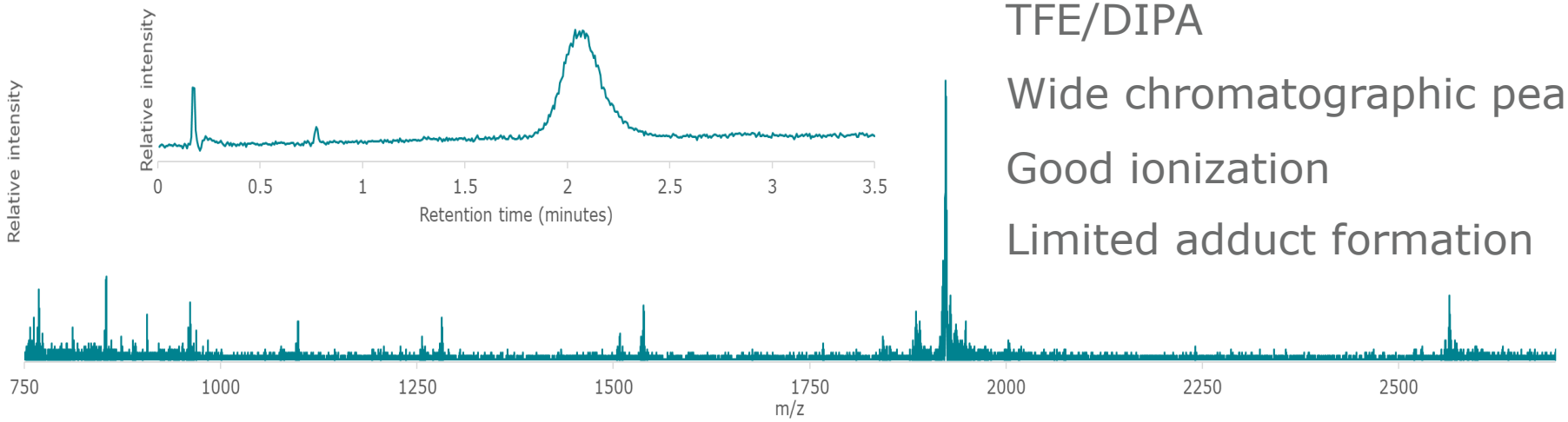
MOBILE PHASE SELECTION

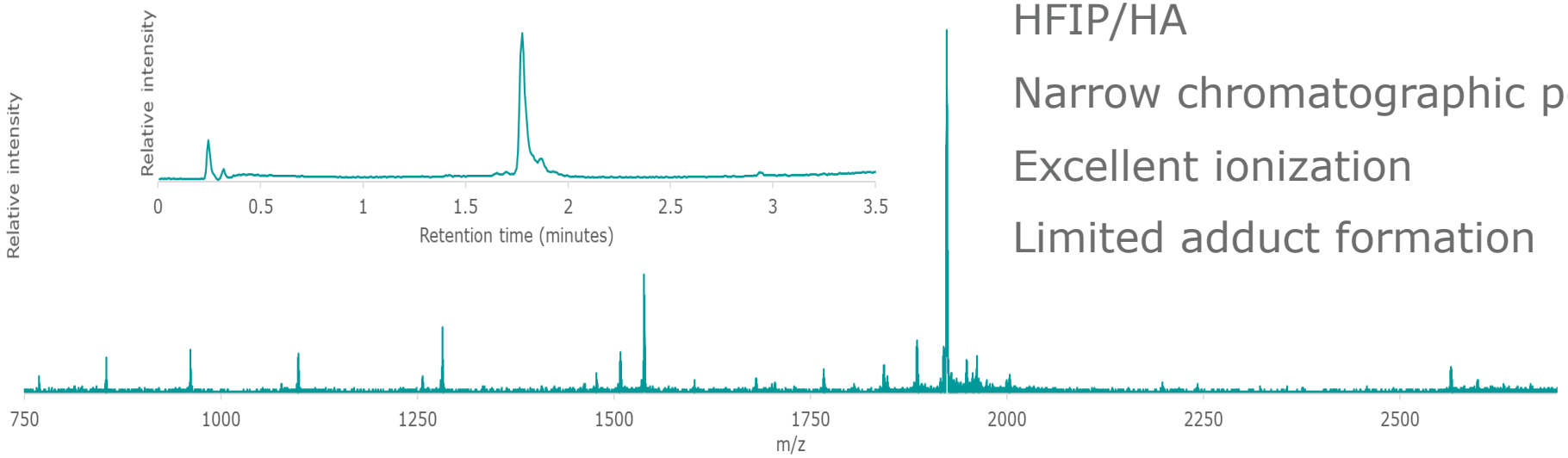


TFE/DIPA

Wide chromatographic peak

Good ionization

Limited adduct formation



HFIP/HA

Narrow chromatographic peak

Excellent ionization

Limited adduct formation

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SAMPLE PREPARATION OF PLASMA SAMPLES

- Different sample preparation techniques tested
  - Liquid-Liquid Extraction folowed by Solid Phase Extractions
  - Digestion combined with Liquid-Liquid Extraction
  - Solid Phase Extractions
  - Liquid-Liquid Extraction

- The extraction efficiency of 2 oligonucleotides from plasma was monitored using LC-MS

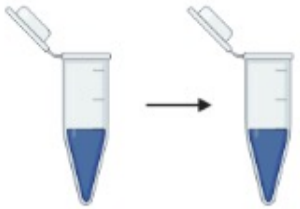
LIQUID-LIQUID EXTRACTION FOLLOWED BY SOLID PHASE EXTRACTION

LLE

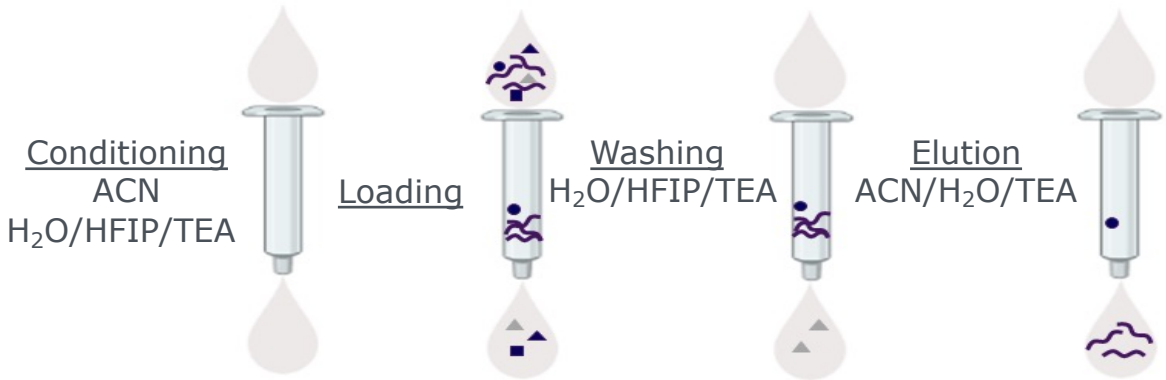
NH_4OH

Phenol/DCM

Centrifugation



SPE



- Spiked plasma
- Liquid liquid extraction
- Waters HLB
- Elution performed with ACN:H_2O with TEA

Sample preparation methods	Recoveries Oligonucleotide A	RSD	Recoveries Oligonucleotide B	RSD
LLE/SPE	49%	9%	20%	4%

Ewles, M.; Goodwin, L.; Schneider, A.; Rothhammer-Hampl, T. Quantification of Oligonucleotides by LC–MS/MS: The Challenges of Quantifying a Phosphorothioate Oligonucleotide and Multiple Metabolites. *Bioanalysis* **2014**, *6* (4), 447–464. https://doi.org/10.4155/bio.13.319.

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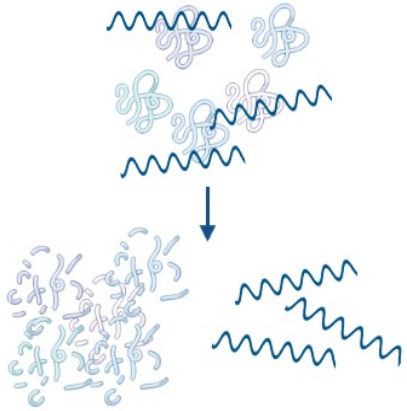
DIGESTION FOLLOWED BY LIQUID-LIQUID EXTRACTION

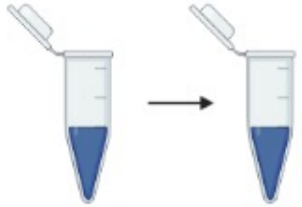
Digestion

Lysis buffer
Proteinase K
Incubation



LLE

First LLE : Phenol/DCM/H_2O
Centrifugation
Second LLE : DCM
Centrifugation



- Spiked plasma
- Digestion with Proteinase K
- Liquid liquid extraction using phenol/DCM and DCM

Sample preparation methods	Recoveries Oligonucleotide A	RSD	Recoveries Oligonucleotide B	RSD
Digestion/LLE	0%	81%	2%	62%

Bigelow, J. C.; Chrin, L. R.; Mathews, L. A.; McCormack, J. J. High-Performance Liquid Chromatographic Analysis of Phosphorothioate Analogues of Oligodeoxynucleotides in Biological Fluids. *J. Chromatogr. B. Biomed. Sci. App.* **1990**, *533*, 133–140. https://doi.org/10.1016/S0378-4347(00)82193-3.

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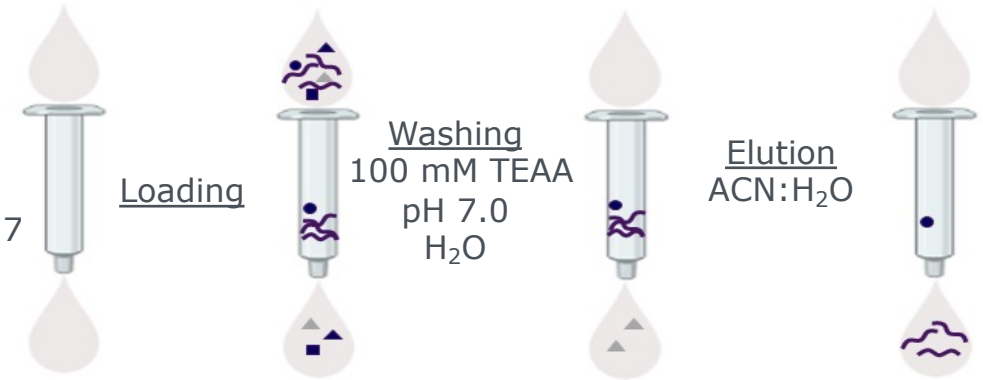
SOLID PHASE EXTRACTION

SPE

100mM
TEAA pH 7



- Spiked plasma
- Liquid liquid extraction
- Waters HLB SPE cartridges

Sample preparation methods	Recoveries Oligonucleotide A	RSD	Recoveries Oligonucleotide B	RSD
SPE	50%	81%	21%	115%

Johnson, J. L.; Guo, W.; Zang, J.; Khan, S.; Bardin, S.; Ahmad, A.; Duggan, J. X.; Ahmad, I. Quantification of Raf Antisense Oligonucleotide (RafAON) in Biological Matrices by LC-MS/MS to Support Pharmacokinetics of a Liposome-Entrapped RafAON Formulation. *Biomed. Chromatogr.* **2005**, *19* (4), 272–278. https://doi.org/10.1002/bmc.450.

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LIQUID-LIQUID EXTRACTION

- Spiked plasma sample
- LLE using phenol:DCM
- LLE using DCE

Sample preparation methods	Recoveries Oligonucleotide A	RSD	Recoveries Oligonucleotide B	RSD
LLE	84%	2%	74%	3%

- Water layer is used for analysis

Turnpenny, P.; Rawal, J.; Schardt, T.; Lamoratta, S.; Mueller, H.; Weber, M.; Brady, K. Quantitation of Locked Nucleic Acid Antisense Oligonucleotides in Mouse Tissue Using a Liquid–Liquid Extraction LC–MS/MS Analytical Approach. *Bioanalysis* **2011**, *3* (17), 1911–1921. https://doi.org/10.4155/bio.11.100.

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EXTRACTION EFFICIENCY

- 3 out of 4 literature methods gave poor results
- One sample preparation method showed promising results

Sample preparation methods	Recoveries Oligonucleotide A	RSD	Recoveries Oligonucleotide B	RSD
LLE/SPE	49%	9%	20%	4%
Digestion/LLE	0%	81%	2%	62%
SPE	50%	81%	21%	115%
LLE	84%	2%	74%	3%

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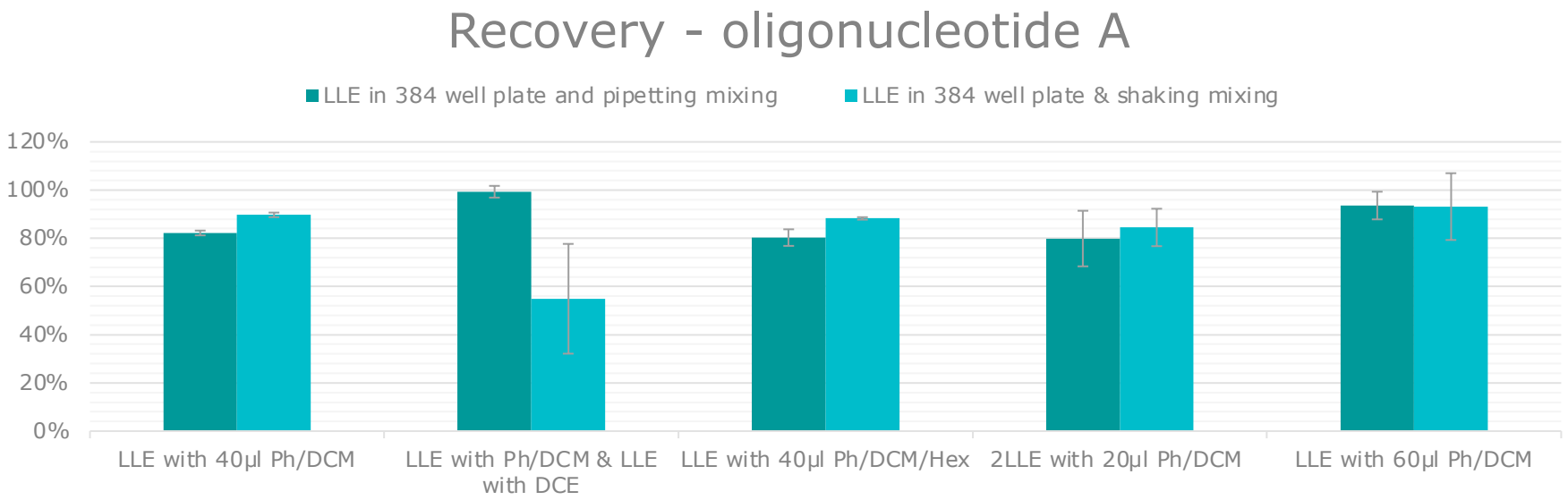
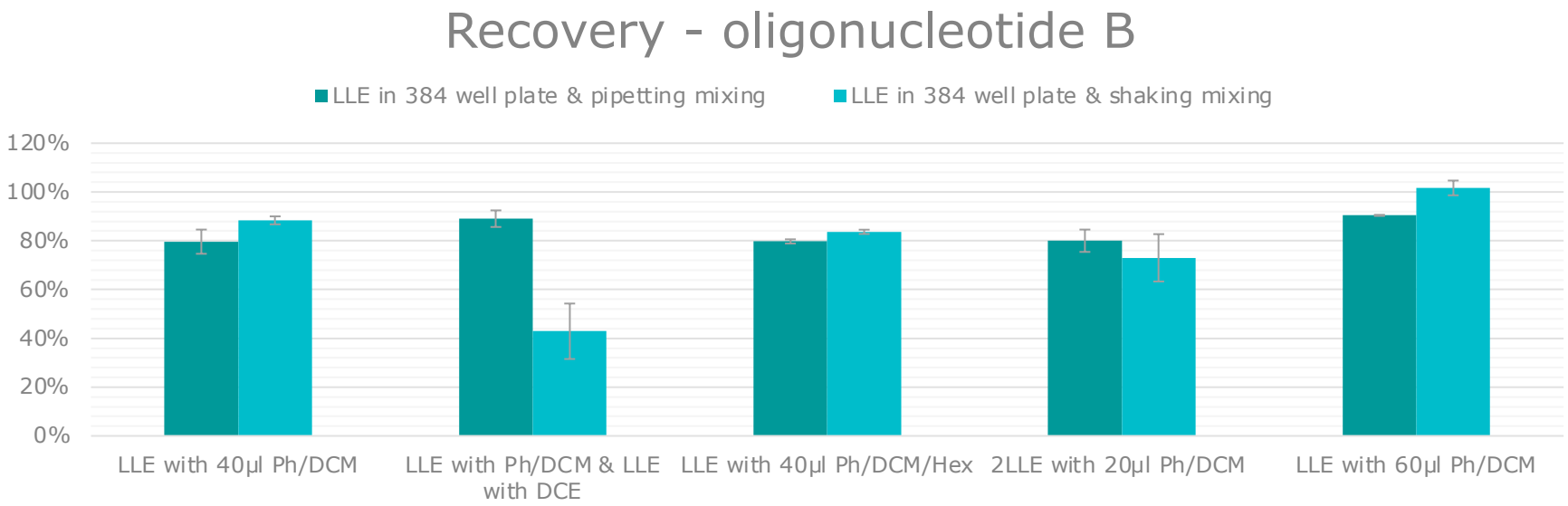
SAMPLE PREPARATION OPTIMISATION





PRE-VALIDATION

- Method was pre-validated in the range from 15.0 – 3000 ng/mL
- Control samples spiked at 4 levels (LLOQ, QC L, QC M and QC H)
- Within- and between-run accuracy and precision
- No carry-over

Oligonucleotide A - LLOQ 15 ng/ml		
Day	**Precision**	**Accuracy**
Day 1	11.7%	99.9%
Day 7	12.0%	120%
5 days auto-sampler stability	4.61%	92.7%

Oligonucleotide A - QC L 30 ng/ml		
Day	**Precision**	**Accuracy**
Day 1	5.11%	97.6%
Day 7	4.19%	103%
5 days auto-sampler stability	2.14%	99.6%

Oligonucleotide A - QC M 300 ng/ml		
Day	**Precision**	**Accuracy**
Day 1	0.96%	101%
Day 7	2.91%	105%
5 days auto-sampler stability	2.92%	99%

Oligonucleotide A - QC H 3000 ng/ml		
Day	**Precision**	**Accuracy**
Day 1	1.86%	96.8%
Day 7	4.14%	98.4%
5 days auto-sampler stability	3.98%	98.4%

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COLUMN PROBLEMS

LLE

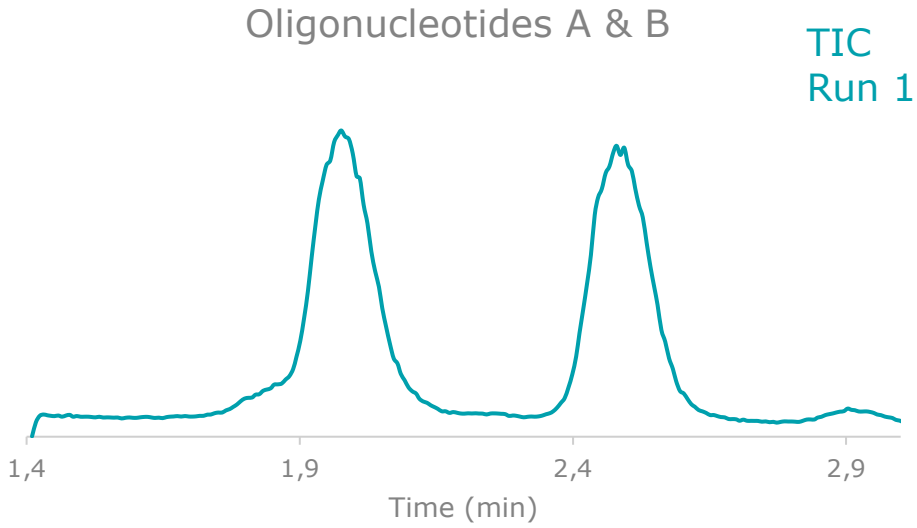
NH_4OH

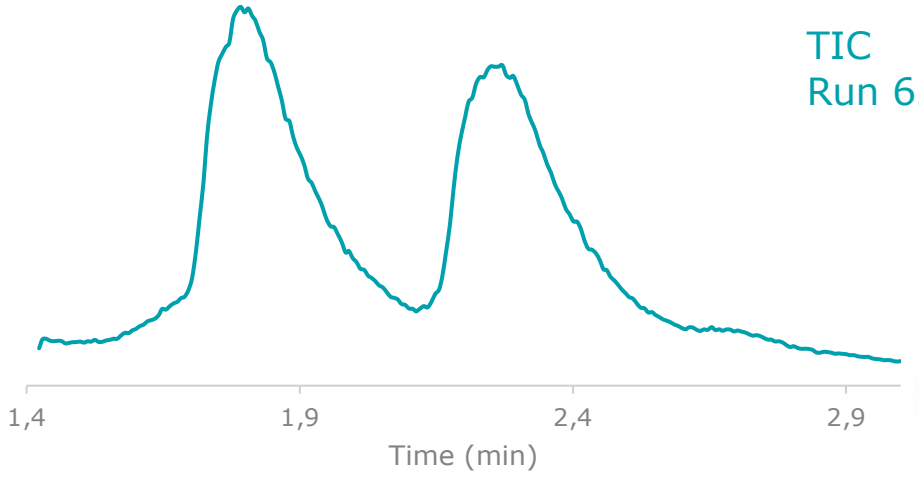
DTT

20 µL Ph/DCM

Pipette mixing

Centrifuge 20 min





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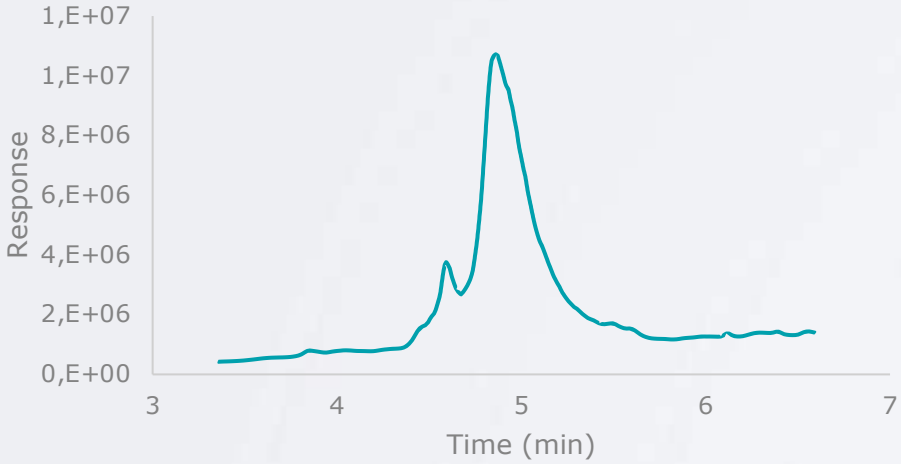
REMAINING CHALLENGE

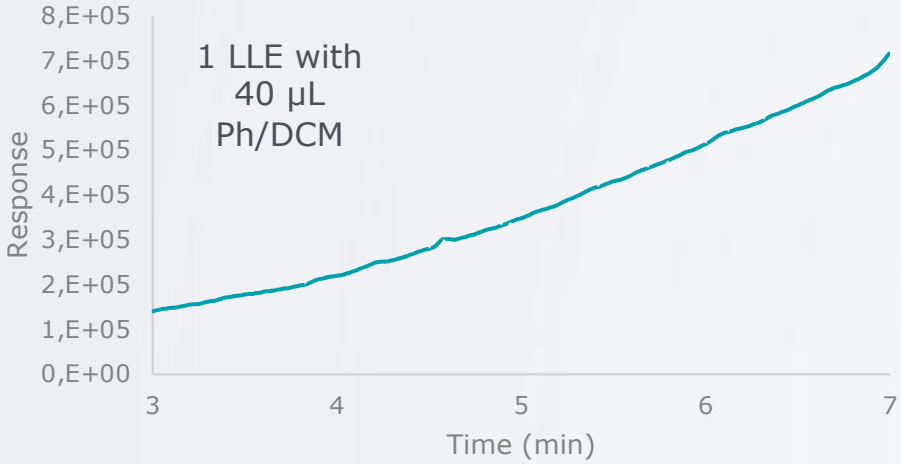
x10 diluted plasma - UV



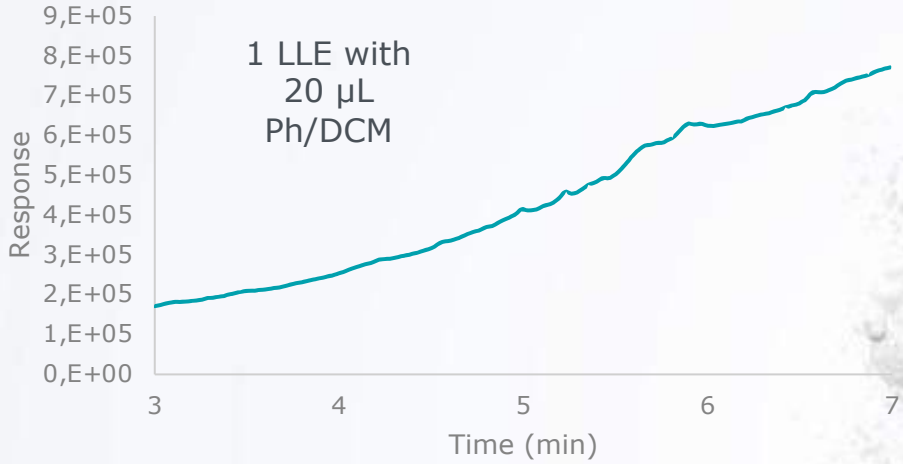
Protein precipitation with ACN - UV

Reversed phase gradient
0.1% FA in water and
ACN on a Waters
BioResolve column

test plasma sample - UV



test plasma sample - UV



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HOW TO SOLVE PEAK SHAPE PROBLEMS

Accept columns going bad over 240-300 samples, changing precolumns regularly

- 1 column
- 4 precolumns
- Total 1600 EUR

Easy, straight forward and robust

Set up a clean-up method after LLE

- C18 SPE plates, if it sticks on c18 columns it is assumed to stick on C18 SPE material

- 4 96-well SPE plates
- Total 1700 EUR

- More time required for sample preparation
- More dilute samples
- More steps in the sample preparation
- More solvents and waste

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CONCLUSIONS

- Succesfull method development for oligonucleotides
- Division of MS signal over adducts and charge states reduced
- An effective extraction method for plasma samples was set up
- Pre-validation passed the acceptance criteria

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AKNOWLEDGEMENTS

- I would like to thank Hrisula Teodorakidu for her hard work in this project as a master thesis.

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THANK YOU!

QUESTIONS?

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