

Novel approach for differentiating mRNA fingerprints with two-dimensional liquid chromatography using a YMC Accura Bio Pro IEX QF column

The rapid expansion of mRNA-based therapeutics has created a growing need for fast and reliable quality control methods to confirm mRNA sequences. Currently, available approaches rely on labour-intensive sequencing workflows or extensive analysis of RNase digests using

liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). As a result, simple and robust analytical methods for comprehensive mRNA characterisation remain limited, highlighting the need for novel strategies.



Ion-pair reversed-phase (IP-RP) chromatography has emerged as a powerful tool for nucleic acid analysis. Ion-pairing agents such as alkylamines interact with the negatively charged phosphate backbone of mRNA, enabling retention of these highly hydrophilic analytes on reversed-phase columns. In addition to IP-RP, anion-exchange chromatography (AEX) is a valuable alternative for nucleic acid separation. However, large nucleic acids such as mRNA require high salt concentrations for elution due to their strong overall charge, which can pose practical limitations. To address these challenges, a novel separation mode combining key aspects of IP-RP and AEX has

been developed. This ion-pair mediated anion-exchange (IPAX) approach introduces weak ion-pairing cations that associate with the negatively charged nucleic acid. By partially neutralising the analyte's charge, IPAX enhances both retention and resolution, offering a promising and versatile new avenue for efficient mRNA analysis.

This Technical Note, based on the publication by Carstensen et al., presents a two-dimensional analytical approach combining IPAX and IP-RP chromatography with UV detection [1]. The method enables comprehensive characterisation of mRNA digests through detailed fingerprinting.

First-dimension method development using a YMC Accura BioPro IEX QF column

To evaluate whether IPAX offers advantages for two-dimensional mRNA characterisation, optimised IPAX and AEX methods are developed and compared. Therefore, a systematic screening is performed using temperatures of 20, 40, and 60 °C and flow rates of 0.05 and 0.10 mL/min in both AEX and IPAX separation modes. In salt-mediated

AEX, sodium chloride serves as the eluting salt, whereas tetramethylammonium chloride (TMAC) is used in IPAX mode. A bioinert-coated YMC Accura BioPro IEX QF column is used to prevent undesired interactions between the negatively charged mRNA and metal ions typically found in standard column hardware.

Table 1: Chromatographic conditions [1].

| | |
|--------------|--|
| Column: | YMC Accura BioPro IEX QF (3 µm) 50×2.1 mm ID |
| Part No.: | QF00S03-05Q1PTC |
| Eluent: | A) 25 mM TRIS-HCl (pH 7) B) 25 mM TRIS-HCl + 2 M TMAC (pH 7) or 2 M NaCl (pH 7) |
| Gradient: | 0% B (0-5 min), 5-70% B (5-125 min) Equilibration: 15 min at 0.2 ml/min |
| Flow rate: | 0.05 mL/min or 0.1 mL/min |
| Temperature: | 20 °C, 40 °C or 60 °C |
| Injection: | 5 µl |
| Detection: | UV at 260 nm, 40 Hz |
| Sample: | CleanCap® eGFP mRNA digested with RNase T1 |

Temperature has a strong influence on resolving power for both eluents, with 40 °C emerging as the optimal compromise between peak capacity and analysis time (Figure 1). While the retention of mRNA fragments increases at higher temperatures with NaCl as eluent, TMAC shows a

slight decrease in retention time at elevated temperatures indicating much greater retention-time stability across changing temperatures. For both eluents, a flow rate of 0.10 mL/min improves separation without excessively prolonging the run.

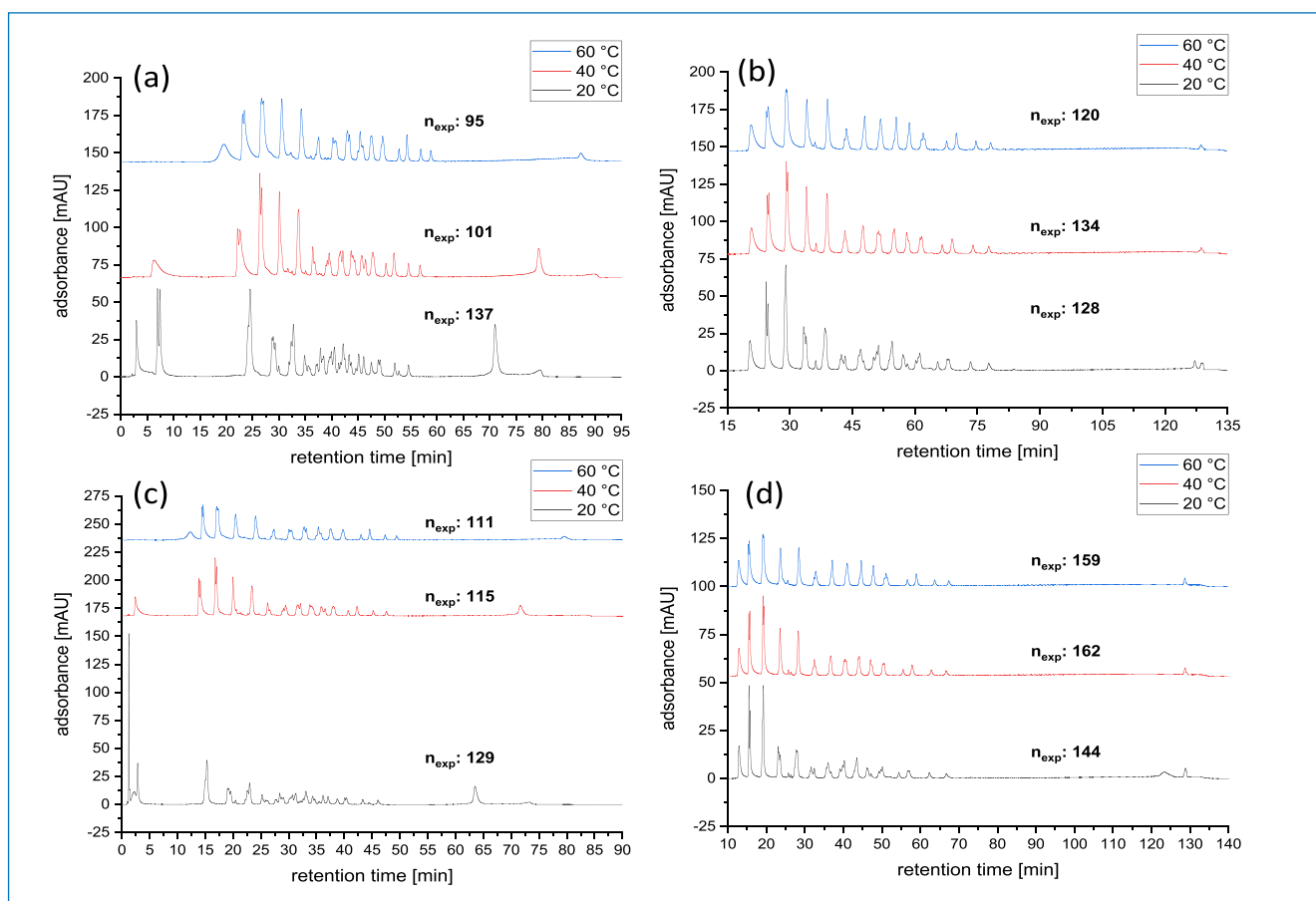


Figure 1: Optimisation of the first-dimension separation of an eGFP digest using the YMC Accura BioPro IEX QF column with NaCl (a, c) and TMAC (b, d) as eluents at flow rates of 0.05 mL/min (a, b) and 0.10 mL/min (c, d) [1].

The TMAC-based IPAX method consistently results in higher peak capacities compared to the AEX approach due to its ability to separate the mRNA fragments more effectively according to size. Under TMAC conditions, larger fragments exhibit stronger retention, widening the separation window and spreading fragment groups more effectively across

the gradient (Figure 2). This behaviour might be linked to disruption of secondary structures by TMAC, allowing more anionic charge sites on the RNA backbone to interact with the stationary phase surface of the YMC Accura BioPro IEX QF column.

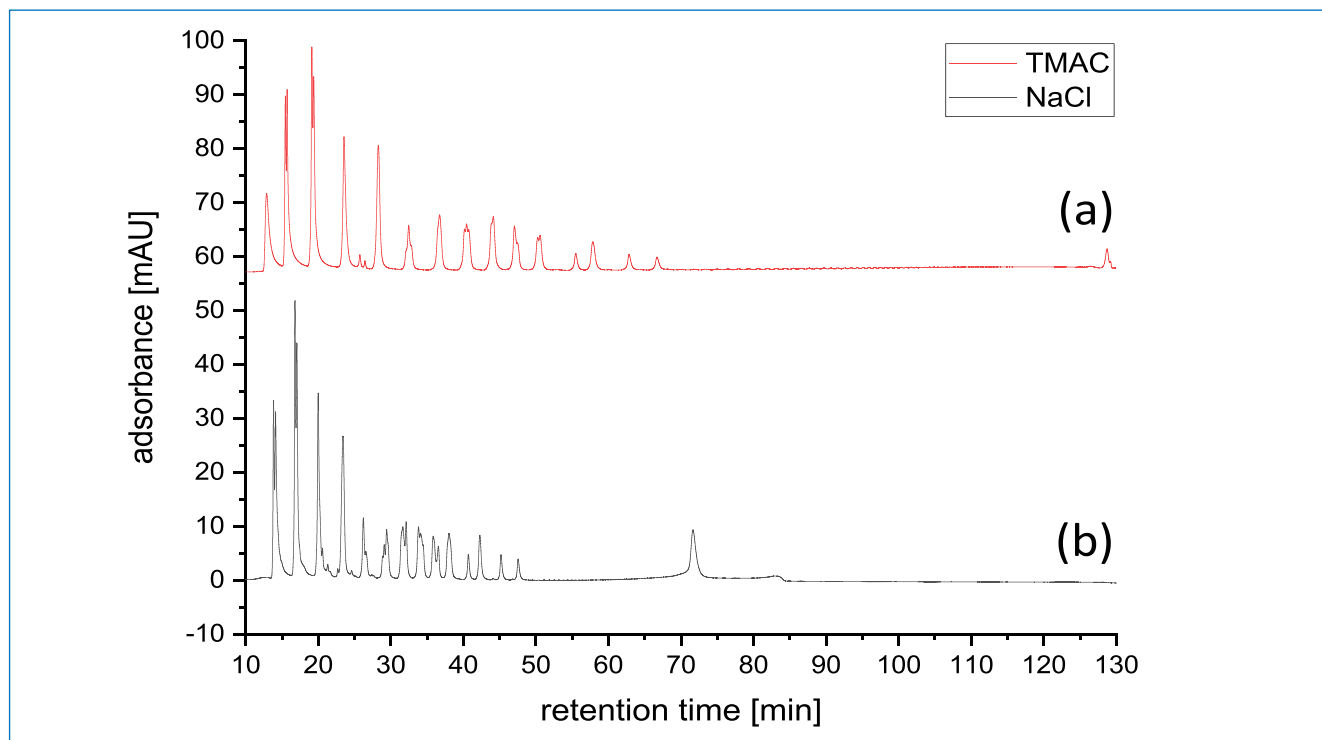


Figure 2: Direct comparison of an eGFP digest using the YMC Accura BioPro IEX QF column with 2 M TMAC (a) and 2 M NaCl (b) under optimised conditions [1].

NaCl results in a narrower elution range with less pronounced grouping of fragment sizes, limiting the overall resolving power. Although adequate for traditional AEX applications, the NaCl elution mode provides less differentiation between closely sized fragments and therefore offers lower orthogonality when combined with reversed-phase separation in the second dimension. Using

TMAC as ion pairing agent, the digested mRNA fragments elute in large peak groups, which seems to be advantageous for two-dimensional separation. Combined with its superior compatibility with IP-RP, IPAX is therefore a more suitable choice for first-dimension separation in two-dimensional mRNA fingerprinting than AEX.

The optimised method parameter of 40 °C and 0.10 mL/min flow rate are selected for mRNA fingerprinting.

Two-dimensional separation of two digested mRNAs

In the second dimension, an optimised IP-RP method uses 100 mM tripropylammonium acetate (TPAA) as ion pairing agent. Combined with IPAX, the method is subsequently applied to the LC×LC fingerprinting. Two representative mRNA samples: eGFP mRNA and Raxtozinameran (the active ingredient in the Comirnaty® XBB 1.5 vaccine) are used. Figure 3 displays the resulting contour plots generated using TMAC-based IPAX in the first dimension and IP-RP applying TPAA in the second dimension.

The fingerprints show clear differences between the two mRNA drug substances. The eGFP mRNA exhibits a single, long polyA-tail of approximately 100–120 nucleotides, which appears as a distinct, shooting-star-shaped feature in the upper retention region of the contour plot, with its intensity maximum near 108 minutes. Raxtozinameran, by contrast, displays a split polyA-tail composed of a 30-nucleotide fragment and a larger 70-nucleotide fragment appearing at around 95 minutes.

Table 2: Chromatographic conditions [1].

| | ¹ D | ² D |
|--------------|--|---|
| Column: | YMC Accura BioPro IEX QF (3 μm) 50×2.1 mm ID | C18 column |
| Part No.: | QF00S03-05Q1PTC | |
| Eluent: | A) 25 mM TRIS-HCl (pH 7) B) 25 mM TRIS-HCl + 2 M TMAC (pH 7) | A) 100% water + 100 mM tripropylammonium acetate (pH 5.2) B) 75% ACN + 100 mM tripropylammonium acetate (pH 5.2) |
| Gradient: | 0% B (0-5 min), 5-80% B (5-125 min) Equilibration: 15 min at 0.2 ml/min | 5%B (0-0.05 min), 5-95%B (0.05-0.4 min), 5%B (0.4-0.45 min) |
| Flow rate: | 0.1 mL/min | 2.5 mL/min |
| Temperature: | 40 °C | 60 °C |
| Injection: | 5 μl | Loop filling: 75% |
| Detection: | UV at 260 nm, 40 Hz | UV at 260 nm, 160 Hz |
| Sample: | CleanCap® eGFP mRNA and Vaccine (Raxtorinameran) mRNA digested with RNase T1 | CleanCap® eGFP mRNA and Vaccine (Raxtorinameran) mRNA digested with RNase T1 |

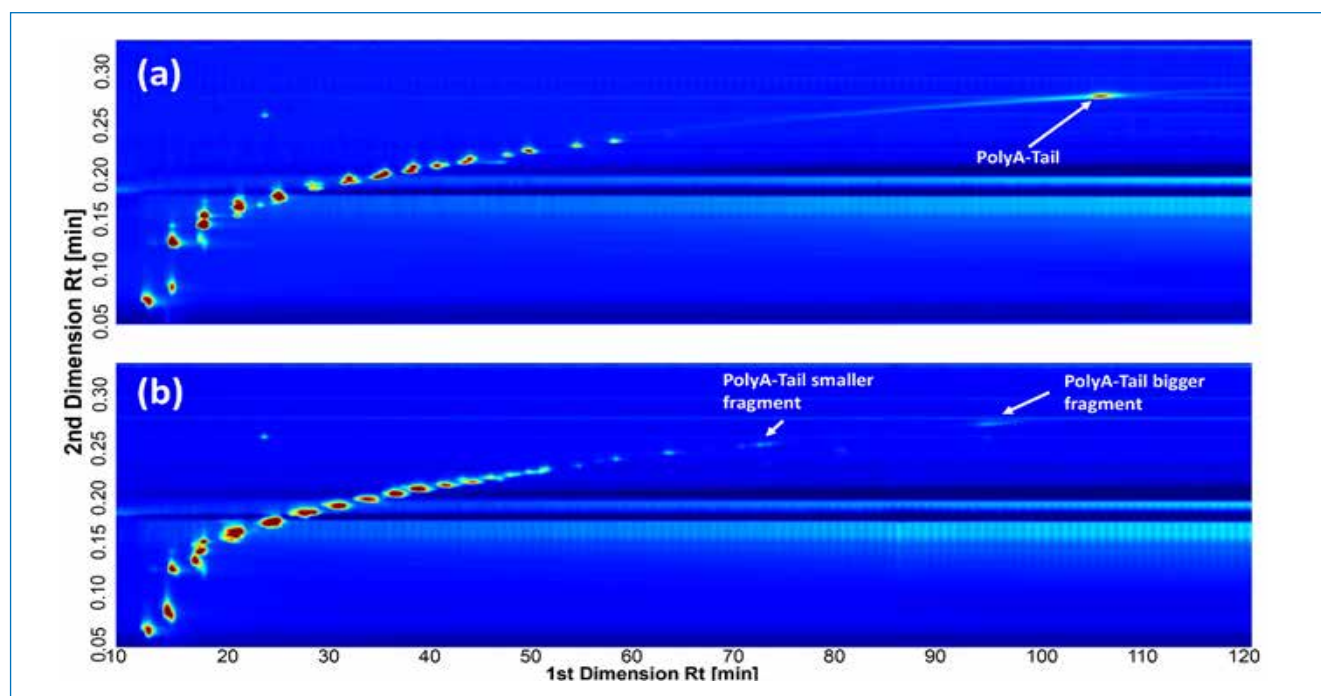


Figure 3: Direct comparison of mRNA fingerprints from digested eGFP (a) and Raxtozinameran (b) obtained using the IPAX × IP-RP two-dimensional separation method [1].

Beyond these large structural differences, the two mRNA species also display distinct patterns in the smaller digest fragments. Their distribution in both dimensions varies in terms of retention time, resolution, and signal intensity,

reflecting the unique sequences of each mRNA. This demonstrates the effective separation of smaller mRNA fragments as well as larger characteristic structures such as the polyA-tail by the developed IPAX × IP-RP method.

Conclusions

The IPAX method using the YMC Accura BioPro IEX QF column provides more effective separation of mRNA digests than conventional AEX, when applied to two-dimensional separation. Overall, the results demonstrate the successful fingerprinting of two complex mRNA samples using the developed IPAX × IP-RP method. Thereby, the mRNAs could be distinguished based on a single RNase digestion. This makes the method a powerful option for mRNA quality control, offering even greater fragment resolution when integrated with mass spectrometry-based sequencing.

References

[1] N. Carstensen, R. Karongo, A. Mengel, M. Lämmerhofer, Full comprehensive ion-pair-mediated anion exchange chromatography × ion-pair reversed-phase liquid chromatography fingerprinting of digested messenger ribonucleic acid drug substances, *J. Chromatogr. Open*, 2025, 8, 100271