A Sensitive LC–MS Bioanalytical Method for Simultaneous Quantification of Structurally Similar Cyclic Tetrapeptides at the Picogram Level

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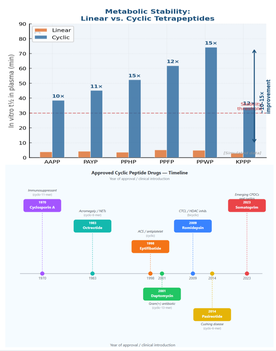
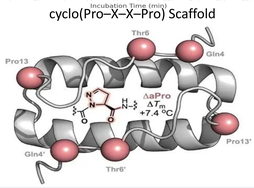
ABSTRACT

Cyclic tetrapeptides represent the smallest cyclic peptide framework that preserves sequence-dependent physicochemical behavior while benefiting from conformational rigidity imparted by cyclization. This structural constraint reduces flexibility, enhances chemical and metabolic stability, and alters charge distribution, making these compounds valuable both as model systems for structure–property investigations and as emerging therapeutic scaffolds. However, their high structural similarity and ultra-low abundance in biological matrices pose significant challenges for chromatographic resolution and sensitive quantification. To address this, an optimized LC–MS workflow was developed enabling picogram-level detection of 40 cyclic tetrapeptides across diverse biological matrices. Fine control of mobile-phase acidity proved critical for chromatographic discrimination and stable ionization, particularly for peptides differing by a single residue or positional isomerism. Basic-residue-containing peptides exhibited predictable charge-state behavior, enabling consistent Q1-based quantification without fragmentation-dependent sensitivity. Compared to conventional MS/MS approaches, this strategy provided substantial signal gains while maintaining selectivity through high-resolution chromatographic separation and retention-time alignment. The project is aimed to quantify designed cyclic peptides from rat plasma.





CHALLENGES & SOLUTIONS

⚠ **C1 — Ultra-Low Concentration (~0.1 pg/µL):** In vivo levels approach the ESI sensitivity floor; conventional flow insufficient.

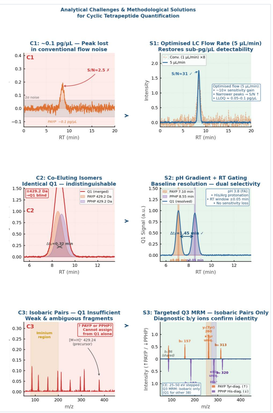
✓ **S1 — Nanoflow LC (1000 nL/min):** 50–100× ESI efficiency gain; narrows peak width; achieves LLOQ ~0.05–0.1 pg/µL.

⚠ **C2 — Similar Structure & Retention Time:** Positional isomers co-elute (Δt$_R$ <0.3 min) under generic RP gradients; impossible to resolve by mass alone.

✓ **S2 — pH Gradient + Q1 RT Gating:** Optimized FA pH modulates His/Lys/Arg protonation; strict ±0.05 min RT windows provide dual orthogonal selectivity without MS/MS sensitivity penalty.

⚠ **C3 — Isobaric MS Interference (e.g. PAYP / PPHP, 429.24 Da):** Q1 cannot distinguish isobaric pairs when chromatographic co-elution occurs; misassignment risk.

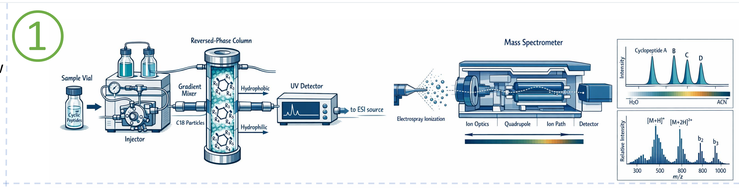
✓ **S3 — Targeted Q3 MRM for Isobaric Pairs Only:** Sequence-specific b/y ions (stepped CE 25–50 eV) unambiguously confirm identity; Q1 retained for all other 38 peptides.



BIOANALYTICAL METHOD DEVELOPMENT FOR SIMULTANEOUS QUANTIFICATION OF 40 CYCLIC TETRAPEPTIDES

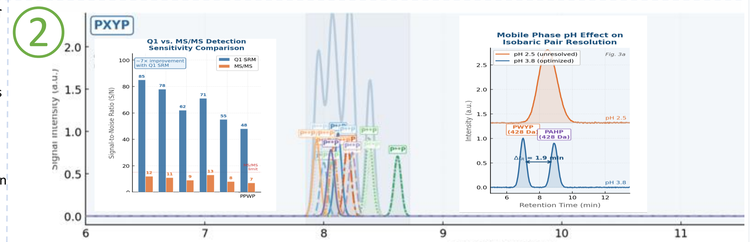
Mass Spectrometric Detection Strategy
All analyses were performed on a SCIEX 6500+ triple quadrupole mass spectrometer coupled to a nanoflow UHPLC system.

Flow rate is 5ul/min to increase the sensitivity



Chromatographic Separation of 40 Structurally Similar Cyclic Tetrapeptides

- Q1 full-scan quantification was therefore adopted as the primary acquisition mode, with targeted Q3 MRM reserved as a confirmatory channel for isobaric and co-eluting peptide pairs.
- The majority of the 40 cyclic tetrapeptides exhibited near-identical chromatographic retention times. Particular attention was directed to pairs sharing the same nominal Q1 m/z (e.g., PAHP and PLHP, [M+H]⁺ = 429.2 Da) where mass-based discrimination alone was insufficient.

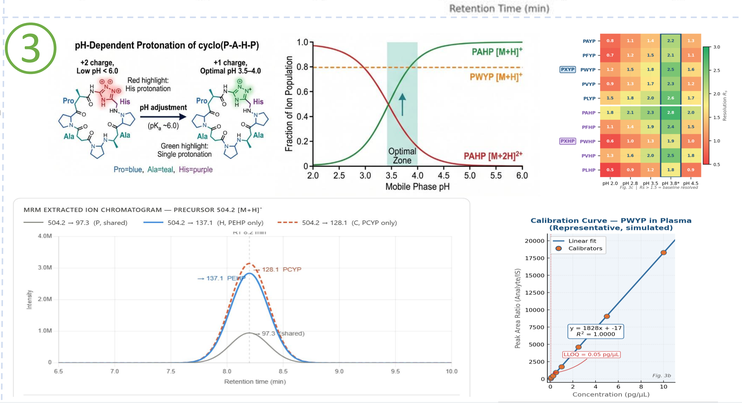


Mobile Phase pH Optimisation: Charge-State Control and Ion-Suppression Mitigation

- Modifying mobile-phase pH enhanced chromatographic resolution; however, pH also governs the equilibrium between [M+H]⁺ and [M+2H]²⁺ charge states for His/Arg-containing peptides, as well as the degree of ion-suppression from residual salt adducts.
- The final gradient conditions represent a carefully balanced optimum among three competing parameters — chromatographic resolution, charge-state distribution, and matrix-derived ion suppression.

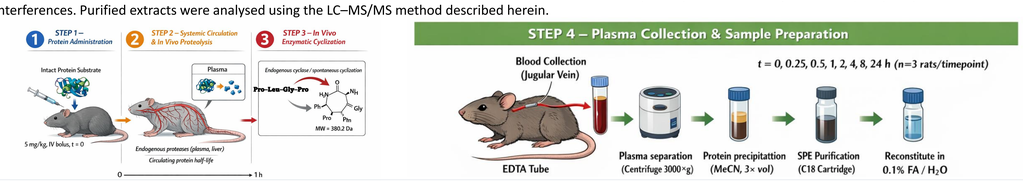
MS/MS Structural Confirmation and Quantitative Calibration

- isobaric species requires sequence-level identity confirmation via diagnostic b- and y-type product ions. Independent eight-point calibration curves (1/x² weighting) were constructed for each of the 40 analytes using stable-isotope-labelled internal standards.



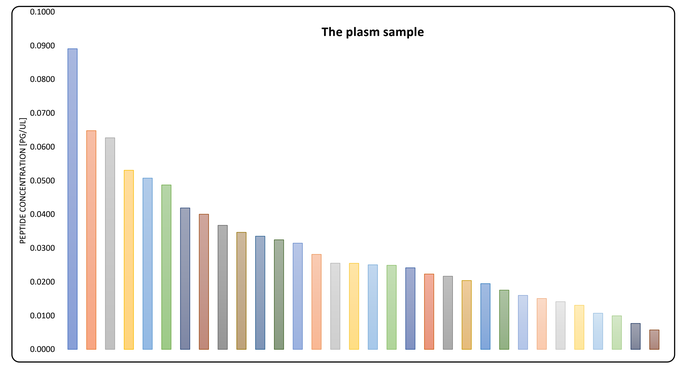
SAMPLE PREPARATION AND PEPTIDE EXTRACTION FROM MOUSE PLASMA

- The parent protein mixture was administered to rats via subcutaneous injection. Following systemic absorption, endogenous proteases enzymatically cleaved the protein substrate to generate the panel of cyclic tetrapeptide analytes in vivo.
- Serial blood samples were collected via cardiac puncture at defined post-injection time points; plasma was isolated by centrifugation. The plasma matrix contains target cyclic tetrapeptides at low concentrations against a complex background of endogenous proteins, phospholipids, and co-eluting small molecules.
- Plasma samples were processed through a validated multi-step extraction protocol (protein precipitation followed by solid-phase extraction) to remove phospholipids and reduce matrix interferences. Purified extracts were analysed using the LC–MS/MS method described herein.



IN VIVO PHARMACOKINETICS OF CYCLIC TETRAPEPTIDES

- Quantitative analysis of rat plasma samples yielded measurable signal for approximately 36 of the 40-target cyclic tetrapeptides.
- Ranked abundance profiles across the peptide panel provide direct insight into the proteolytic digestion pathways of the parent protein substrate and identify candidate cyclic peptide biomarkers reflective of in vivo enzymatic activity.



CONCLUSIONS

- **pH-Controlled Selectivity:** Fine control of mobile-phase pH (formic acid modifier) was essential for achieving simultaneous baseline chromatographic resolution of positional isomers and near-isobaric species, while stabilising analyte ionisation across the full 40-member panel.
- **Q1-Based Quantification:** Q1 full-scan quantification consistently outperformed conventional MS/MS-based MRM in signal intensity for cyclic tetrapeptides, without compromising analytical selectivity when combined with pH-optimised chromatographic resolution and strict retention-time acceptance windows (±0.05 min).
- **Picogram Sensitivity:** Picogram-per-microlitre sensitivity was achieved across all 40 cyclic tetrapeptide analytes
- **Multi-Matrix Robustness:** Consistent method performance was demonstrated across five biologically distinct matrices (rat plasma, liver homogenate, kidney homogenate, brain homogenate, and urine), with matrix-induced ion suppression maintained below the FDA-recommended 15% threshold for Q1-mode acquisition in all tested tissues.
- **Biotransformation Profiling:** Application of the validated method to rat plasma following subcutaneous protein administration enabled quantification of approximately 36 cyclic tetrapeptides in vivo, with ranked abundance profiles providing mechanistic insight into the proteolytic cleavage preferences of the parent substrate and identifying peptide candidates as potential pharmacodynamic biomarkers.
- **Broadly Applicable Framework:** The pH-tuned Q1/Q3 hybrid acquisition strategy described herein is broadly applicable to other structurally constrained peptide scaffolds and low-abundance cyclic peptide therapeutic candidates, establishing a transferable bioanalytical framework for preclinical DMPK and PK/PD support in drug discovery.

Cyclic tetrapeptides represent the smallest cyclic peptide framework that preserves sequence-dependent physicochemical and analytical behavior while benefiting from conformational rigidity. This structural constraint reduces flexibility, enhances chemical and metabolic stability, and alters charge distribution, making these compounds valuable models for investigating structure–property relationships. Beyond their utility as analytical probes, cyclic tetrapeptides are increasingly explored as therapeutic scaffolds, where subtle sequence variations can lead to pronounced functional differences. Their high structural similarity and ultra-low abundance in biological matrices pose significant challenges for chromatographic resolution and reproducible quantification. We developed a sensitive, pH-optimized LC–MS framework enabling picogram-level quantification of a panel of 40 structurally related cyclic tetrapeptides across diverse biological matrices.

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