Developing and Evaluating Electrospray Ionization Mass Spectrometry Methods for the Discovery and Quantitative Analysis of Cyclic Peptide

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Highlights

- Developed a sensitive LC-MS/MS method for detecting Microcystin and its variants in environmental samples.
- Evaluate a method for sequential injection volume effects on quantitative accuracy in LC-MS analysis.
- Analyzed retention times and fragmentation pattern of microcystin variants using HR-MS.
- Developed a method to evaluate ionization efficiency and method reproducibility using Bacitracin.

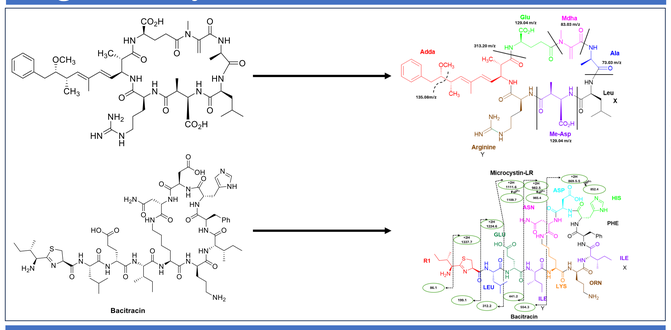
Introduction

- Cyclic peptides are bioactive molecules and can act as therapeutic agents or environmental toxins.
- Structural diversity among cyclic peptide creates challenges for quantitative LC-MS analysis.
- Microcystin and its variants are toxic cyclic peptides produced by cyanobacteria in freshwater environment.
- Over 250 microcystin variants exist, most without synthetical standards.

Materials and Method

- Standards: Leucine Enkephaline, Microcystin LR, Microcystin YR, Bacitracin.
- Instruments: Vanquish Core: UHPLC + Orbitrap Exploris 120.
- Ionization Mode: ESI
- LC-MS: Full scan & MS2; C18 column with Acetonitrile and water spiked with formic acid gradient.
- QC analysis: Included blanks, duplicates, and matrix spikes.

Targeted Analyte Structure



Dual Injection Flow Chart

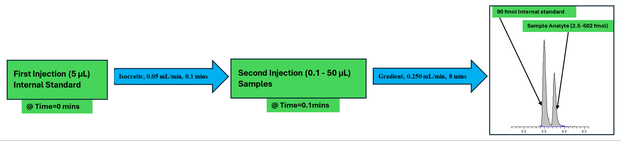


Figure 1: Schematic representation of the dual injection method used for LC-MS quantification of microcystins with Leucine Enkephalin as the internal standard. The first injection (5 µL) delivers the internal standard at time zero, followed by an isocratic flow (0.05 mL/min for 0.1 min). The second injection (0.1–50 µL) introduces the analyte (Microcystin) at 0.1 minutes and gradient elution is then applied at 0.250 mL/min for 8 minutes.

High-Quality Quantification and Isotopic Confirmation of MC-LR by LC-HRMS

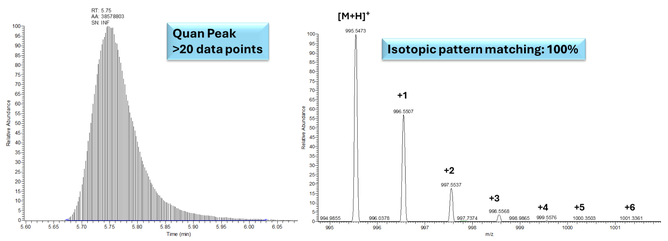


Figure 2: Extracted ion chromatogram showing a sharp quantitation peak of Microcystin-LR with over 20 data points and a retention time of 5.75 minutes, supporting reliable quantification. The observed isotopic pattern matches the theoretical distribution with 100% accuracy, confirming the molecular identity of Microcystin-LR.

EIC for the Internal Standard and Microcystin at low level ppb

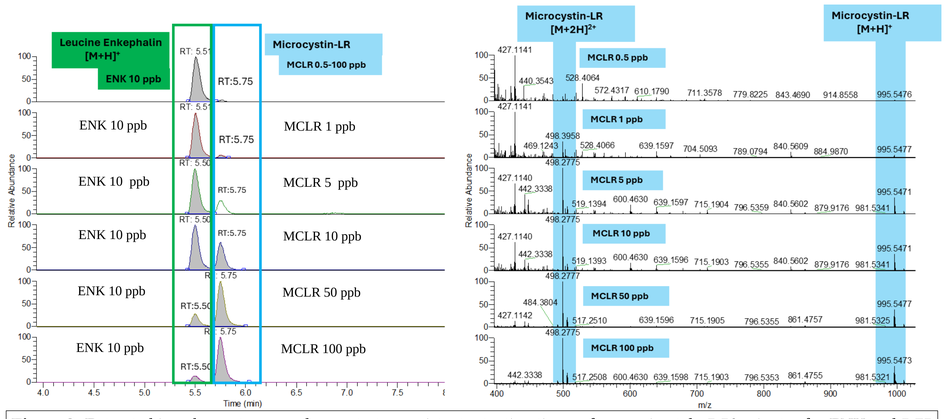


Figure 3: Extracted ion chromatograms demonstrate consistent retention times of approximately 5.50 minutes for ENK and 5.75 minutes for MCLR, confirming stable chromatographic performance and resolution across the calibration range. The clear separation and consistent elution profile support reliable peak area ratio calculations for quantitative analysis

Comparison of LOQ for Microystcin-LR with and without Co-eluting

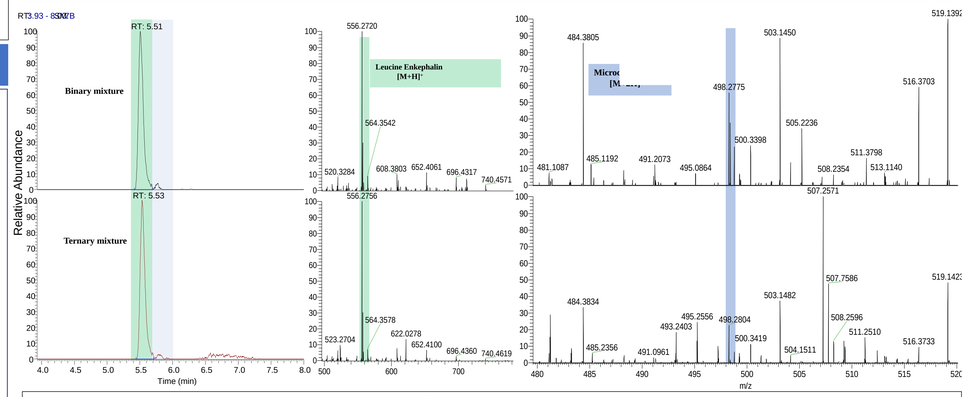
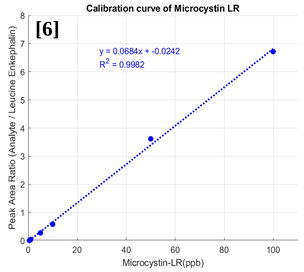


Figure 4: LC-HRMS analysis comparing the limit of detection of Microcystin-LR (MCLR) in two experimental conditions: MCLR and Leucine Enkephalin (ENK, m/z 556.27) in a binary mixture. The extracted ion chromatograms and mass spectra confirm clear detection of MCLR (m/z 498.27 and 995.55) with a retention time of 5.75 min. Ternary mixture containing MCLR, ENK, and Microcystin-YR. The presence of MCYR tests for potential ion suppression or chromatographic interference. The signal for MCLR and ENK remains stable, indicating it is less sensitive to co-eluting matrix effects

Normal Injection Method Calibration Curve of Microcystin-LR



Dual Injection Method For low concentration range

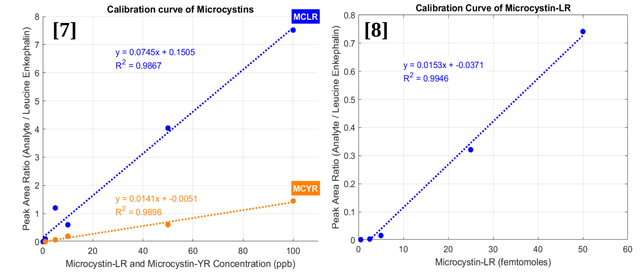


Figure 6: Calibration curve of Microcystin-LR using Leucine Enkephalin as the internal standard. Peak area ratios (MC-LR/ENK) were plotted against MC-LR concentrations ranging from 0.1 to 100 ppb. **Figure 7**: Calibration curves for Microcystin-LR and Microcystin-YR in the presence of Leucine Enkephalin as internal standard. Both analytes were quantified in a co-mixed solution using peak area ratio (analyte/ENK) over a concentration range of 0.1–100 ppb.

Figure 8: Calibration curve of Microcystin-LR using the dual injection method at low femtomole concentrations.

Limit of Detection and Quantification in all Methods

Microcystin Variants	LOD (ppb)	LOQ (ppb)
MC-LR	0.05	0.1
MC-YR	0.1	1.0

Identify Cyclic Peptide Homologs: Bacitracin Case

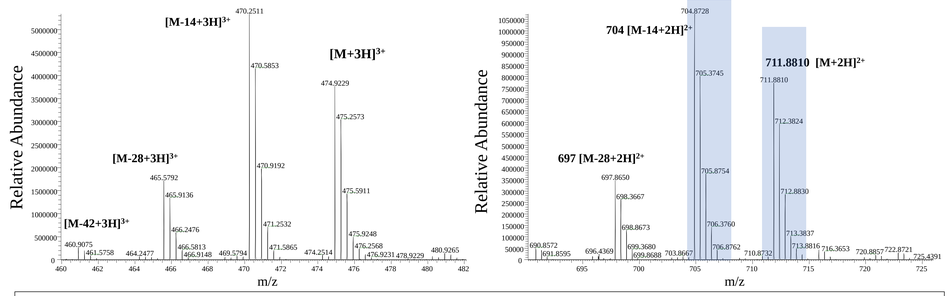


Figure 9: Bacitracin standard (1000 ppb) analyzed in positive ion mode. The right panel covers the mid-mass region (690–725 m/z), with major ions observed at m/z 704.8732, 711.8810, and 712.3824. The presence of multiple analogs across both regions reflects the inherent heterogeneity of the Bacitracin sample.
The observed exact mass difference **14.0154**, matched that of the CH_2 (**14.0157**). In comparison, the exact mass of N is 14.0031. This sample likely had **demethylation** during its production. This type of post-translational modification (PTM) could alter the peptide's properties and function.

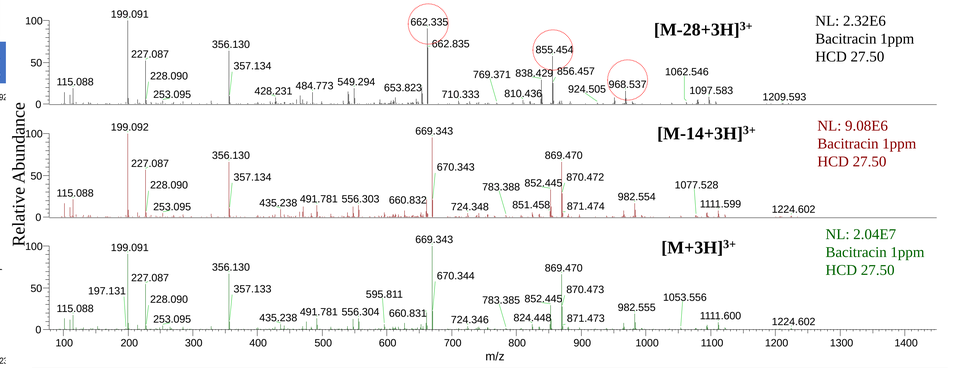


Figure 10. The two impurity molecules (-14 and -28) were identified to be structural homolog of Bacitracin by their highly similar MS/MS spectra.

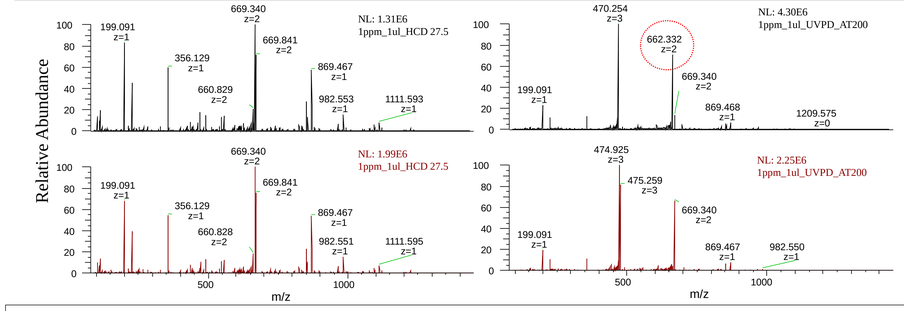


Figure 11: UVPD unique *MS/MS spectra.* May suggest the structure of the -14Da analog.

Conclusions

- An efficient, 8 minutes LC-HRMS method was developed and applied for the analysis of microcystin.
- An efficient, and fast LC-HRMS method was developed with UVPD and HCD with application for the analysis of bacitracin standard to clearly identify different isoforms.
- All target ions (MS1) and confirmation ions (MS2) met mass accuracy requirements within ±5 ppm, ensuring high-confidence identification.
- Retention time reproducibility across injections was within ±0.1 minutes
- LOQ: MCLR-0.1 ppb, MCYR-1 ppb; LOD: MCLR-0.05 ppb, MCYR-0.1 ppb
- Dual-injection strategy with internal standard separation by flow profile
- The sensitivities of different microcystin variants vary depending on the injection mode and the composition of the solution mixture.

Acknowledgements

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References

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