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Fast, general-purpose metabolome analysis by mixed-mode liquid chromatography–mass spectrometry

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Comprehensive metabolomics requires robust and efficient analytical techniques capable of addressing the chemical diversity, complexity, and high sample throughput demands characteristic of large-scale studies. We introduce a rapid, mixed-mode liquid chromatography method that uniquely integrates anion exchange and hydrophobic interactions within a single stationary phase. Employing an optimized ternary gradient, our method achieves comprehensive separation of diverse metabolite classes over a wide range of polarities within only 4 minutes per run. The performance was tested with standards for ca. 1000 metabolites. For two-thirds of 94 isomeric sets, we could achieve a separation of 2 or more seconds, which is sufficient for correct identification. We demonstrate robustness over 500 consecutive injections of bacterial extracts and with the analysis of complex matrices like plasma, cecum extracts, and urine. Throughout, retention time drifts were <1 s. Our mixed-mode LC-MS approach offers a routine throughput of 360 samples per day per instrument and is ideally suited for studies that require rapid and comprehensive metabolic profiling.

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Introduction

The chemical analysis of biological or environmental samples presents significant challenges. Such samples are particularly complex due to their immense diversity: they can contain thousands of structurally distinct molecules, with concentrations varying by orders of magnitude and chemical properties differing greatly because of natural structural heterogeneity.^{1,2} The intrinsic diversity of these samples calls for approaches that integrate versatility, dynamic range, and a refined ability to discern subtle structural differences that influence bioactivity. Mass spectrometry (MS) is the preferred technique for analyzing complex samples.³ It has become the predominant technique because it is well-suited for detecting molecules of virtually any size, class, or polarity. In addition to its flexibility, MS stands out for excellent sensitivity, speed, and the ability to resolve fine compositional or structural variants by high-resolution detectors and tandem mass spectrometry, respectively.⁴

In standard practice, MS detectors are preceded by a separation technique that is essential for enhancing overall analytical performance. This can occur through various means, such

as separating isomers that remain indistinguishable in the MS, removing salt or other interfering agents, reducing interferences in the ionization process needed to obtain gaseous molecular ions before MS analysis, or timing the occurrence of analytes to facilitate the acquisition of tandem MS data for numerous analytes. The challenge with separation lies in its reliance on the differential physicochemical properties of the analytes of interest. In contrast to MS, however, the versatility is narrower, as each separation method tends to depend on specific properties such as charge, hydrophobicity, size, and others.

In the realm of liquid chromatography–MS, the hyphenated technique most commonly used in metabolomics, the field has long been divided between the use of either reversed-phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC). RPLC relies on hydrophobic interactions with the stationary phase, consisting of C18 or C8 alkyl chains and additional functional groups that enhance stability and selectivity.⁵ The elution is induced by reducing the aqueous content of the mobile phase in favor of organic solvents that disrupt the interaction. As the latter is also beneficial for the ionization process at the interface with the MS, RPLC-MS is a perfect match. RPLC is robust, reproducible, and ideally suited for separating non-polar compounds like bile acids, aromatic metabolites, fatty acids, and all lipids. RPLC fails, however, to retain polar compounds, including key primary metabolites such as amino and organic acids or

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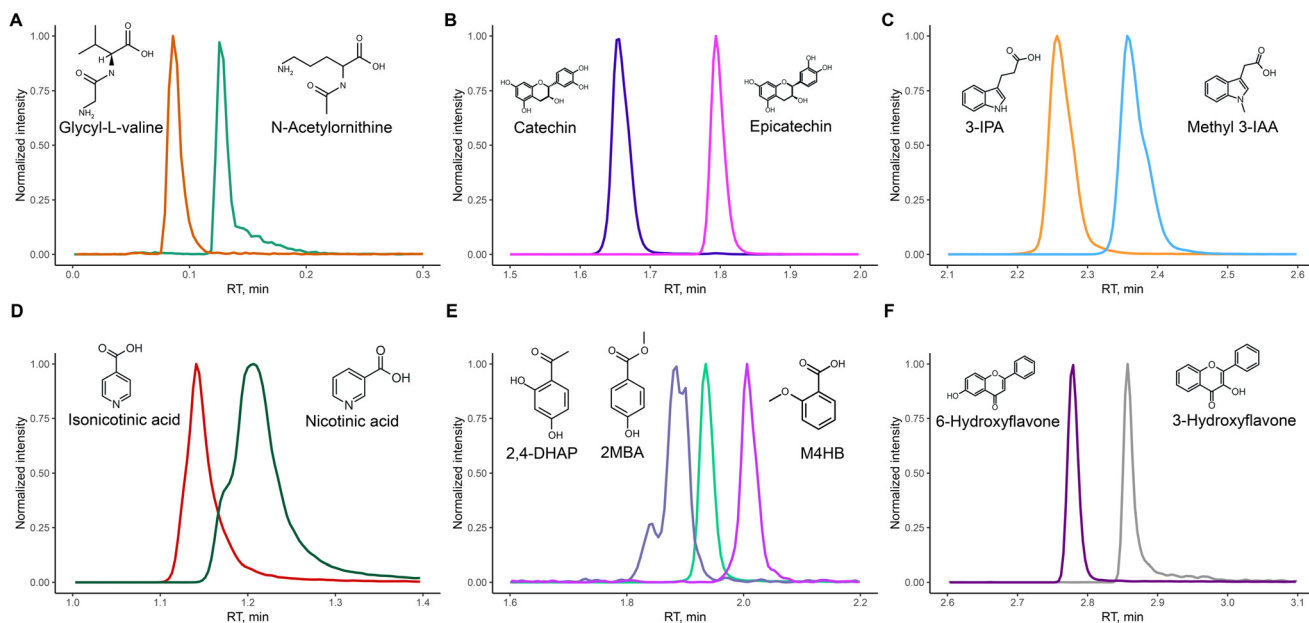


Fig. 2 Separation of 6 sets of isomeric molecules present in the testing library. All data represented is the extracted ion chromatogram acquired in negative mode, with normalized intensity.

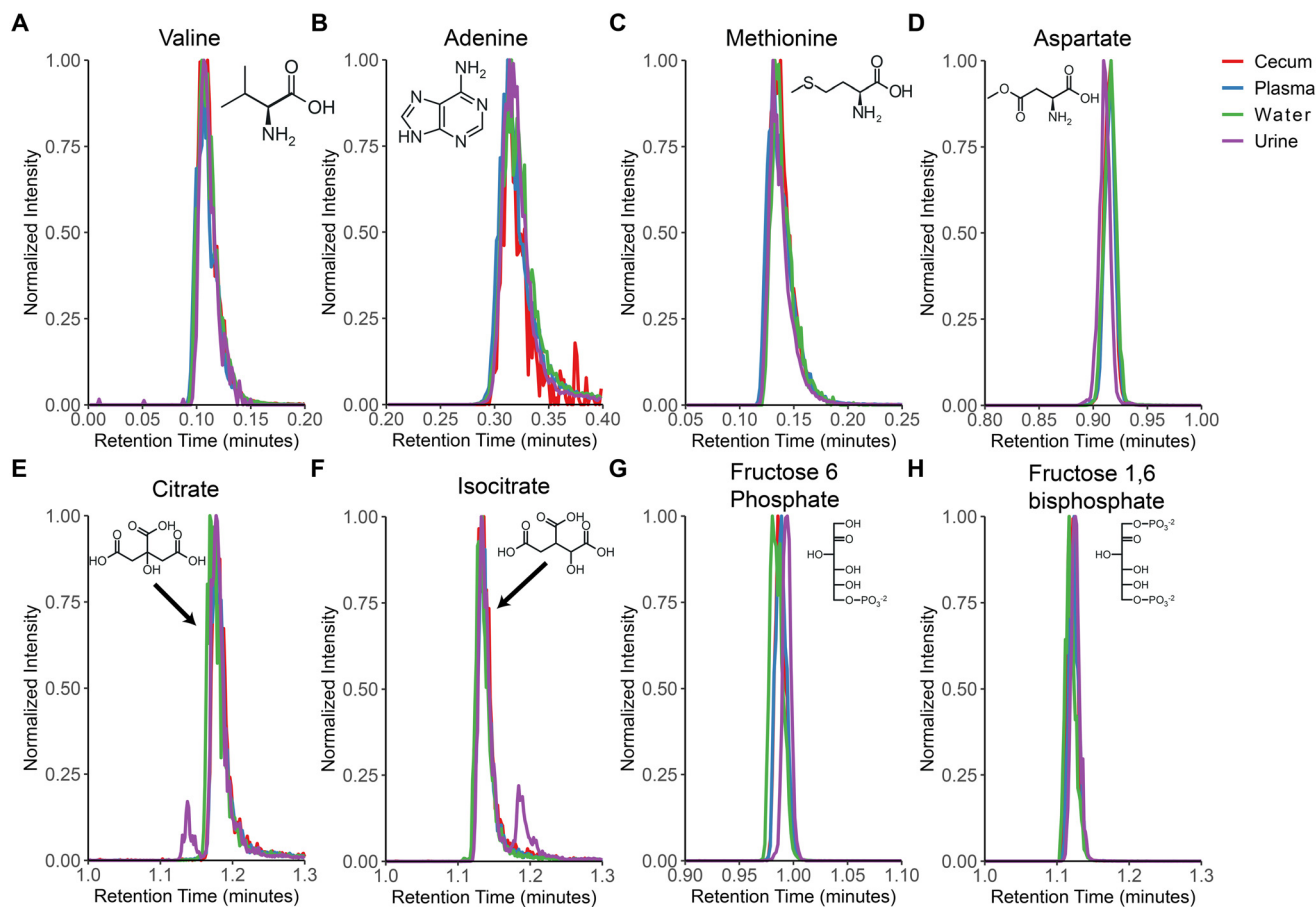


Fig. 3 Extracted ion chromatograms (EICs) in four different matrices.



Chromatographic stability

To test the method's stability in routine analysis, we repeatedly analysed a bacterial extract of *Escherichia coli* for 500 consecutive injections on the same column. The total running time was 36 hours. The back pressure profile was constant throughout the sequence, correctly relaxing to the initial pressure during the final equilibration (Fig. S1A). The initial pressure increases by ~3% between the first and the 500th injection. Excellent chromatographic stability was also confirmed by the total ion chromatogram profiles (Fig. S1B). The only observable drift is in the intensity of the hydrophobic compounds that elute during the wash and equilibration. At the level of single compounds, both retention time and peak shape were stable throughout the test (Fig. S1C–F). For a set of ~60 metabolites that we could detect in *E. coli* extracts, the average retention time drift between the first and the last injection was less than 0.5 s on average (Table S3). The average shift in retention time between different columns or different batches of solvents was less than 0.5 s. No particular issues were observed during the past 18 months, during which the method was routinely employed in our lab. The coefficient of variation of the integrated area was 5–8% for the compounds eluting in the first 6 seconds, and <5% for the rest, which is well suited for untargeted metabolomics.

Robustness to matrix

Finally, we tested the robustness of the method against complex matrices. We extracted plasma, cecum, and urine samples using our routine procedure and added pure standards of compounds with diverse chemical properties and, consequently, varying elution times. The spiked samples were analysed with our mixed-mode method to evaluate the robustness of chromatography. Across all tested compounds, the shifts in retention were less than 1 second (Fig. 3). In the case of citrate (Fig. 3E) and isocitrate (Fig. 3F), we observed the isobaric peaks caused by the endogenous isocitrate and citrate, respectively. The peak shape was similar between water and matrix samples. Peak tailing remained comparably low, even for the phosphorylated compounds that are prone to binding cations. This robustness against salt is primarily due to adding medronic acid to solvent A.

Conclusion

We introduced a rapid and versatile mixed-mode chromatography method tailored for comprehensive metabolomic analysis. Our development builds on recent work by others that clarified why mixed reversed-phase/weak-anion-exchange stationary phases such as Atlantis BEH C18 AX provide excellent selectivity for anions, compatibility with 100% aqueous starts, a wide usable pH window (~2–10), strong batch-to-batch reproducibility, and a certain level of tunability between the two modes.¹⁶ The versatility of the stationary phase was previously exploited for the analysis of diverse compound classes, such as fatty acids,¹⁸ amino acids,¹⁹ or NAD metabolites,²⁰

upon optimization of binary gradients for each class. For multi-class analyses as requested in untargeted metabolomics, binary gradients fail to exploit multimodality.⁵ As the two retention mechanisms are not mutually exclusive, we overlapped the two modalities in a ternary gradient, which resulted in robust and efficient separation across classes within a notably brief 4-minute analysis cycle. This method significantly increases sample throughput, enabling the processing of up to 360 injections daily. Although it is possible to analyze a sample in both polarities, we recommend avoiding polarity-switching because of the spray instability that affects quantification precision; thus, two separate injections would be required.

Compared to RPLC, our method improves the separation of polar to moderately non-polar compounds while maintaining robustness. Compared to HILIC and binary gradients using mixed-mode stationary phases, the presented ternary gradient provides increased speed, robustness, and coverage across diverse classes. It is possible to further enhance the separation of hydrophobic lipids in the second half of the method by reducing the steepness of the gradient through slowing the solvent gradient in that phase. However, this would reduce throughput. Since hydrophilic and hydrophobic compounds are typically fractionated during sample preparation, we believe there is little practical advantage in a slower method attempting to cover all polarities.

Although short, the ternary mixed-mode chromatography method allows sufficient time to intercalate MS² spectra and support metabolite identification. The observed chromatographic peak width (FWHM) ranges between 2 and 4 s. Assuming that seven points are necessary for quantification, the minimum MS cycle time is about 300 ms. This is sufficient to include 3–10 data-dependent MS² scans of 5–20 ms in each cycle, that is ~10–30 MS² scans per second. Over the course of a single injection, it's possible to acquire MS² data for >1000 independent precursors and obtain deep fragmentation.

One aspect that requires further experiments is the sensitivity to matrix effects, which are common in fast LC-MS analyses of complex samples. While we tested the reproducibility of the method across repeated measurements and matrices, we did not evaluate the method's sensitivity to variations in sample composition. Since the extent of coelution is inversely related to the length of the gradient, it is plausible to suggest that fast gradients may be more susceptible to matrix effects. On a positive note, the reversed-phase mode ensures that lipophilic analytes, which are a major source of ion suppression, elute later and do not interfere with the ionisation of polar compounds. These, however, are purely theoretical considerations that should be carefully verified for the specific types of samples.

Our mixed-mode chromatography requires a ternary gradient, and the simplest solution is to use a quaternary pump. Most quaternary pumps initially mix solvents under low pressure before using a single pump head to increase flow through the column. Unlike systems with multiple pump heads that accurately adjust the flow for each channel before



mixing, such as many binary pumps for HILIC and RPLC, quaternary pumps have two main drawbacks. First, their mixing is less accurate due to timed channel switching. Second, they exhibit an increased dead volume between the mixer and the column. However, in our setup, employing a turbulent flow of 1.2 mL min^{-1} alleviates both issues, enabling excellent reproducibility with a cost-effective quaternary pump.

Conflicts of interest

There are no conflicts to declare.

Data availability

All compound data is described in the supplementary tables. Extraction of retention time was confirmed manually for all compounds. Chromatographic data can be provided upon request.

Supplementary information (SI): supplementary figure, which shows the chromatographic stability for TIC, pressure, and three metabolites. It also contains three supplementary tables outlining the metabolites tested to validate the method, the retention time stability results, and the isomer analysis. See DOI: <https://doi.org/10.1039/d5an00641d>.

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References

- 1 E. M. Harrieder, F. Kretschmer, S. Böcker and M. Witting, Current State-of-the-Art of Separation Methods Used in LC-MS Based Metabolomics and Lipidomics, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2022, **1188**, 123069, DOI: [10.1016/j.jchromb.2021.123069](https://doi.org/10.1016/j.jchromb.2021.123069).
- 2 Y.-F. Xu, W. Lu and J. D. Rabinowitz, Avoiding Misannotation of In-Source Fragmentation Products as Cellular Metabolites in Liquid Chromatography–Mass Spectrometry-Based Metabolomics, *Anal. Chem.*, 2015, **87**(4), 2273–2281, DOI: [10.1021/ac504118y](https://doi.org/10.1021/ac504118y).
- 3 The Human Serum Metabolome (HUSERMET) Consortium, W. B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-McIntyre, N. Anderson, M. Brown, J. D. Knowles, A. Halsall, J. N. Haselden, A. W. Nicholls, I. D. Wilson, D. B. Kell and R. Goodacre, Procedures for Large-Scale Metabolic Profiling of Serum and Plasma Using Gas Chromatography and Liquid Chromatography Coupled to Mass Spectrometry, *Nat. Protoc.*, 2011, **6**(7), 1060–1083, DOI: [10.1038/nprot.2011.335](https://doi.org/10.1038/nprot.2011.335).
- 4 T. Cajka and O. Fiehn, Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics, *Anal. Chem.*, 2016, **88**(1), 524–545, DOI: [10.1021/acs.analchem.5b04491](https://doi.org/10.1021/acs.analchem.5b04491).
- 5 S. Girel, D. Guilleme, S. Fekete, S. Rudaz and V. González-Ruiz, Investigation of Several Chromatographic Approaches for Untargeted Profiling of Central Carbon Metabolism, *J. Chromatogr., A*, 2023, **1697**, 463994, DOI: [10.1016/j.chroma.2023.463994](https://doi.org/10.1016/j.chroma.2023.463994).
- 6 P. Jandera, Stationary and Mobile Phases in Hydrophilic Interaction Chromatography: A Review, *Anal. Chim. Acta*, 2011, **692**(1–2), 1–25, DOI: [10.1016/j.aca.2011.02.047](https://doi.org/10.1016/j.aca.2011.02.047).
- 7 W. Lu, M. F. Clasquin, E. Melamud, D. Amador-Noguez, A. A. Caudy and J. D. Rabinowitz, Metabolomic Analysis via Reversed-Phase Ion-Pairing Liquid Chromatography Coupled to a Stand Alone Orbitrap Mass Spectrometer, *Anal. Chem.*, 2010, **82**(8), 3212–3221, DOI: [10.1021/ac902837x](https://doi.org/10.1021/ac902837x).
- 8 R. Zhang, D. G. Watson, L. Wang, G. D. Westrop, G. H. Coombs and T. Zhang, Evaluation of Mobile Phase Characteristics on Three Zwitterionic Columns in Hydrophilic Interaction Liquid Chromatography Mode for Liquid Chromatography-High Resolution Mass Spectrometry Based Untargeted Metabolite Profiling of Leishmania Parasites, *J. Chromatogr., A*, 2014, **1362**, 168–179, DOI: [10.1016/j.chroma.2014.08.039](https://doi.org/10.1016/j.chroma.2014.08.039).
- 9 K. Klavins, H. Drexler, S. Hann and G. Koellensperger, Quantitative Metabolite Profiling Utilizing Parallel Column Analysis for Simultaneous Reversed-Phase and Hydrophilic Interaction Liquid Chromatography Separations Combined with Tandem Mass Spectrometry, *Anal. Chem.*, 2014, **86**(9), 4145–4150, DOI: [10.1021/ac5003454](https://doi.org/10.1021/ac5003454).
- 10 S. Wernisch and S. Pennathur, Evaluation of Coverage, Retention Patterns, and Selectivity of Seven Liquid Chromatographic Methods for Metabolomics, *Anal. Bioanal. Chem.*, 2016, **408**(22), 6079–6091, DOI: [10.1007/s00216-016-9716-4](https://doi.org/10.1007/s00216-016-9716-4).
- 11 B. Van De Velde, D. Guilleme and I. Kohler, Supercritical Fluid Chromatography – Mass Spectrometry in Metabolomics: Past, Present, and Future Perspectives, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2020, **1161**, 122444, DOI: [10.1016/j.jchromb.2020.122444](https://doi.org/10.1016/j.jchromb.2020.122444).
- 12 G. L. Losacco, O. Ismail, J. Pezzatti, V. González-Ruiz, J. Boccard, S. Rudaz, J.-L. Veuthey and D. Guilleme, Applicability of Supercritical Fluid Chromatography–Mass Spectrometry to Metabolomics. II–Assessment of a Comprehensive Library of Metabolites and Evaluation of Biological Matrices, *J. Chromatogr., A*, 2020, **1620**, 461021, DOI: [10.1016/j.chroma.2020.461021](https://doi.org/10.1016/j.chroma.2020.461021).
- 13 M. Xu, J. Legradi and P. Leonards, Cross Platform Solutions to Improve the Zebrafish Polar Metabolome Coverage Using LC-QTOF MS: Optimization of Separation Mechanisms, Solvent Additives, and Resuspension Solvents, *Talanta*, 2021, **234**, 122688, DOI: [10.1016/j.talanta.2021.122688](https://doi.org/10.1016/j.talanta.2021.122688).
- 14 M. Lämmerhofer, M. Richter, J. Wu, R. Nogueira, W. Bicker and W. Lindner, Mixed-mode Ion-exchangers and Their



- Comparative Chromatographic Characterization in Reversed-phase and Hydrophilic Interaction Chromatography Elution Modes, *J. Sep. Sci.*, 2008, **31**(14), 2572–2588, DOI: [10.1002/jssc.200800178](https://doi.org/10.1002/jssc.200800178).
- 15 A. A. Ammann and M. J.-F. Suter, Multimode Gradient High Performance Liquid Chromatography Mass Spectrometry Method Applicable to Metabolomics and Environmental Monitoring, *J. Chromatogr., A*, 2016, **1456**, 145–151, DOI: [10.1016/j.chroma.2016.06.001](https://doi.org/10.1016/j.chroma.2016.06.001).
 - 16 T. H. Walter, B. A. Alden, J. A. Field, N. L. Lawrence, D. L. Osterman, A. V. Patel and M. A. DeLoffi, Characterization of a Highly Stable Mixed-mode Reversed-phase/Weak Anion-exchange Stationary Phase Based on Hybrid Organic/Inorganic Particles, *J. Sep. Sci.*, 2021, **44**(5), 1005–1014, DOI: [10.1002/jssc.202001136](https://doi.org/10.1002/jssc.202001136).
 - 17 L. K. Pino, B. C. Searle, J. G. Bollinger, B. Nunn, B. MacLean and M. J. MacCoss, The Skyline Ecosystem: Informatics for Quantitative Mass Spectrometry Proteomics, *Mass Spectrom. Rev.*, 2020, **39**(3), 229–244, DOI: [10.1002/mas.21540](https://doi.org/10.1002/mas.21540).
 - 18 M. Mattarozzi, N. Riboni, M. Maffini, S. Scarpella, F. Bianchi and M. Careri, Reversed-Phase and Weak Anion-Exchange Mixed-Mode Stationary Phase for Fast Separation of Medium-, Long- and Very Long Chain Free Fatty Acids by Ultra-High-Performance Liquid Chromatography-High Resolution Mass Spectrometry, *J. Chromatogr. A*, 2021, **1648**, 462209, DOI: [10.1016/j.chroma.2021.462209](https://doi.org/10.1016/j.chroma.2021.462209).
 - 19 T. Kipura, M. Hotze, A. Hofer, A.-S. Egger, L. E. Timpen, C. A. Opitz, P. A. Townsend, L. A. Gethings, K. Thedieck and M. Kwiatkowski, Automated Liquid Handling Extraction and Rapid Quantification of Underivatized Amino Acids and Tryptophan Metabolites from Human Serum and Plasma Using Dual-Column U(H)PLC-MRM-MS and Its Application to Prostate Cancer Study, *Metabolites*, 2024, **14**(7), 370, DOI: [10.3390/metabo14070370](https://doi.org/10.3390/metabo14070370).
 - 20 O. Nacham, J. W. Brown, M. M. Maneshi, V. Kurschner, M. Sheehan, R. Sadowski, C. Ling, N. Talaty, R. Johnson and A. M. Swensen, A Mixed-Mode LC-MS-Based Method for Comprehensive Analysis of NAD and Related Metabolites from Biological Sample Matrices, *Sci. Rep.*, 2025, **15**, 14187, DOI: [10.1038/s41598-025-97834-2](https://doi.org/10.1038/s41598-025-97834-2).

