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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.

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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sugars. This led to the development of several methods tailored to the analysis of polar compounds, which include a variety of stationary phases for HILIC,[6] as well as ion pairing[7] and ion exchange chromatography,[8] all of which necessitate rather lengthy separation and equilibration periods and are accompanied by specific drawbacks.

To achieve broad coverage of the metabolome, the established practice is to run parallel or sequential RPLC and HILIC methods,[9,10] with the rising demand for instrumentation and measurement time required for large-scale studies. This demand for rapid yet general-purpose methods in metabolomics has sparked a renaissance in supercritical-fluid chromatography (SFC),[11] which demonstrated the possibility to separate several compound classes within a 10-minute method, with some limitations with nucleotides.[12] SFC uses carbon dioxide as a solvent and can be adapted to several stationary phases, making it a highly versatile technique. However, it also requires dedicated instrumentation for compound separation, leading to lower overall laboratory utilization.

Chromatographic columns with stationary phases that have more than one mode of separation have been described and used previously.[13–15] However, in most cases, the separation is not optimized, as typically only one mode of separation is employed. The use of a quaternary chromatographic pump enables full utilization of this technology by employing different modes of separation within the same chromatography run.

We present a straightforward system for a fast yet comprehensive metabolome analysis. Our strategy employs a stationary phase that combines different modalities, namely anion exchange and hydrophobic interactions. The benefits of the hybrid stationary phase have been characterized in isocratic[16] conditions and binary gradients.[5] However, these configurations do not fully utilise its multimodal retention mechanism. Instead, we optimised a ternary gradient that allows for simultaneous but controlled elution of both retention mechanisms, thereby improving sample throughput. We demonstrate robust separation of a wide range of metabolite classes in just 4 minutes, enabling a throughput of 360 samples per day per instrument. We investigated the suitability of the method to characterize a broad range of species and its robustness over time and in complex matrices.

Materials and methods

Chemicals

All chemicals were LC-MS grade and purchased from Merck, except for medronic acid, which was purchased as a 100 : 1 stock solution from Agilent (Part. no. 5191-3940). For optimization and testing of the method, we used the Mass Spectrometry Metabolite Library of 600 standards supplied by IROA (Merck, MSMLS-1EA) and the Human Endogenous Metabolite Compound Library of 1000 standards (Selleck Chemicals, Art. No. L4500). Standards from the IROA library dissolved according to the manufacturer's guidelines with water (for plates 1–5) and methanol (plates 6–7). The Selleck library was diluted 1 : 10 with ethanol to a concentration of 100 μM. Compounds were mixed in batches of 10, and the compound mixtures were further diluted with 90 μL of water for LC-MS analysis.

LC-MS

We used an Agilent Infinity 1290 II LC stack including a quaternary pump (G4204A), autosampler (G1767B), and a column oven (G7116B), connected by stainless steel capillaries with 0.12 and 0.17 mm inner diameter. The column was an Atlantis BEH C18 AX 1.7 μm, 2.1 × 30 mm, equilibrated at 40 °C. The solvents were (a) 5 μM medronic acid in water; (b) 0.1% v/v formic acid in methanol; and (c) 5 mM ammonium formate and 0.1% v/v formic acid in water. The flow rate was constant at 1.2 mL min^{-1}. The gradient is described in Table 1.

The mass spectrometer was a SCIEX 7600 ZenoTOF System, operated with SciexOS version 3.0.3. The following settings were used for electrospray ionization: curtain gas 45, CAD gas 7, ion source gas 1 70 psi, and gas 2 70 psi, the source temperature 700 °C, declustering potential 80 V, spray voltage −3500 V in negative mode and 5500 V in positive mode. The MS was operated in full scan mode, acquiring data from 50 to 1750 Th with an accumulation time of 75 ms (~13 Hz) and a collision energy of 10, which is the default to ensure the transmission of ions to the detector.

Sample extraction

We grew *Escherichia coli* in shake flasks to an OD600 of 0.5. 1 mL aliquots were extracted with an ice-cold mixture of acetonitrile : methanol : water (40 : 40 : 20). The mixture was vortexed for 15 seconds and left at −20 °C for at least 2 hours. Then, extracts were centrifuged for 1 min at 15 000 rpm. We collected the resulting supernatant and dried it in a vacuum concentrator (Christ AVC 2–33 CO plus) equipped with a freeze dryer (Christ Alpha 2–4 LSCbasic). The dried pellets were reconstituted in 100 μL of water. For robustness tests in different matrices, we extracted serum, urine and cecum. Serum and urine extraction were precipitated with a four-time ice-cold extraction solvent of methanol : acetonitrile : water (40 : 40 : 20). After two hours at −20 °C, the samples were centrifuged at 15 000 rpm, and the supernatant was collected and dried as above. The pellet was resuspended in 100 μL of water.

Table 1 Chromatographic gradient

Time/min	A/%	B/%	C/%
0	100	0	0
0.4	100	0	0
0.8	40	0	60
1.5	5	40	55
2.05	5	40	55
2.5	0	100	0
2.9	0	100	0
3	100	0	0
4	100	0	0

For the cecum samples, cecum content was extracted with hot water (80 °C) for 3 minutes with shaking. The samples were centrifuged at 15 000 rpm, and the supernatant was collected and diluted before mass spectrometric measurement. The final pellet was resuspended with 200 μL of water.

Data analysis

Raw MS data were converted to centroided *.mzML with Proteowizard and quantified using Skyline.[17]

Results and discussion

Coverage

The mixed-mode liquid chromatography method was designed to separate a wide range of biologically relevant compounds, cover all polarities, and enable both positive and negative ionization. Optimization and testing were based on *ca.* 1000 pure standards comprising metabolites across chemical classes (Table S1) and led to a ternary LC gradient that can be subdivided into four phases (Fig. 1A). The first 0.4 minutes (in blue) are run with an isocratic phase with pure water. The mobile phase also contains medronic acid to scavenge cations and reduce the impact of residual salts on the initial phases of separation that rely on hydrophilic interactions. The injection void elutes after *ca.* 5 seconds and includes neutral sugars, alanine, glycine, and polyamines. Several attempts were made to increase the retention of these compounds: we tested the use of precolumn or a pH gradient with a fourth solvent channel, all of which negatively affected robustness and column stability. Since most of these coeluting compounds can be resolved by accurate mass, except for sugars, we decided to adhere to the proposed ternary method because it

provided the best reproducibility. During the isocratic phase, we observe the elution of non-acidic compounds that are weakly retained on the lipophilic column. These include nucleobases and aromatic amines as exemplified by adenine, inosine, guanosine and uridine (Fig. 1B).

The isocratic phase is followed by a ramp in formate, which leads to the release of the acidic metabolites bound to the cationic groups on the column. The ramp ends at 0.8 minutes when the third phase with methanol starts and proceeds for 1.2 minutes. During this period, we observe the sequential release of organic acids, followed by phosphorylated compounds, bile acids, polyphenols, indoles, and then non-polar compounds. At this stage, analytes are bound to the column through hydrophobic interactions. Following this is the fourth and final stage, involving a ramp to 100% methanol, which is equivalent to RPLC and induces the elution of any lipids present in the samples, including glycerophospholipids and triglycerides. The total cycle time, including equilibration, is 4 minutes. The method can be performed on both polarities without the need to adjust chromatographic conditions. In negative ionization mode, it is common to detect medronic acid multimers at the beginning of the run, but we have not observed any adverse effects on the measurement.
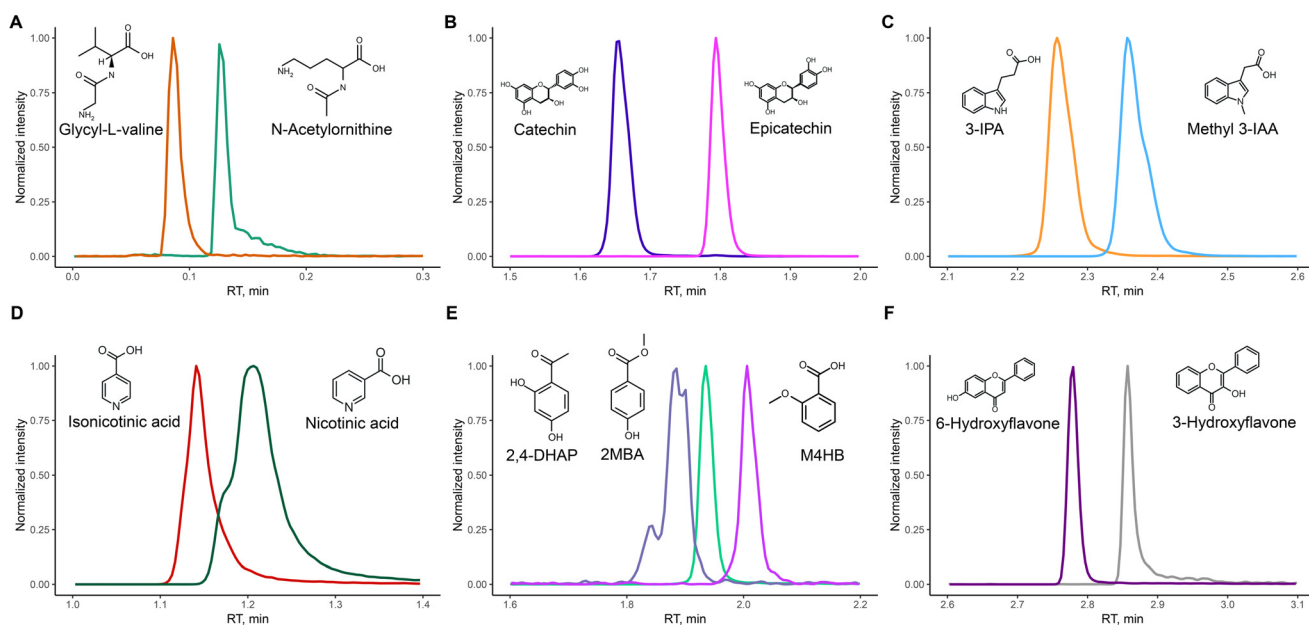
Separation of isomers

An important function of the chromatographic step is the temporal separation of isomers that are difficult to distinguish by mass spectrometry. To assess the suitability of mixed-mode chromatography, we overlaid the extracted chromatograms for six sets of isomers from various compound classes (Fig. 2). In these examples, we observed distinctly recognizable differences in retention times for all isomers and, in four cases, baseline separation of the chromatograms, which would allow for the quantification of individual isomers regardless of their relative abundance. Overall, our development dataset contained a total of 94 isomeric sets (Table S2). Of these 94 sets, our mixed-mode method failed to separate 31 combinations, with 17 being sugars and compounds that eluted within the first 10 seconds. The remaining 63 combinations of isomers exhibited at least 2 seconds of separation between them, which we deem to be sufficient for correct identification.

A few examples are of particular interest. The first is the clear separation of small metabolites, such as *N*-acetylornithine and glycyl-L-valine (Fig. 2A), which is surprising because it occurs in the initial 15 seconds of the method during the isocratic phase. The second example is the sharp separation of the diastereomers (+)-catechin and (−)-epicatechin (Fig. 2B). They differ in the chirality of carbon 3, which determines whether the attached hydroxyl group and the catechol-like ring bound to carbon 2 are in the *cis* or *trans* configuration. As illustrated in this example, the mixed-mode method can separate isomers with different orientations of individual polar groups, especially hydroxylic and carboxylic groups, even in larger molecules (Fig. 2C–F). These examples illustrate the added value that the anion exchange brings about, even in the case of hydrophobic compounds.
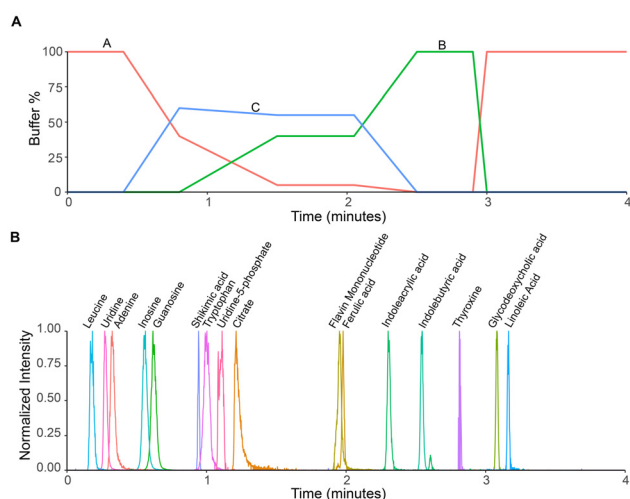

Fig. 1 Representation of mixed-mode chromatographic gradient. (A) Gradient profile with the relative concentrations of solvent A (red): 5 μM medronic acid in water, solvent B (green): 0.1% v/v formic acid in methanol; and solvent C (blue): 5 mM ammonium formate and 0.1% v/v formic acid in water. (B) Examples of chromatographic peak shapes.

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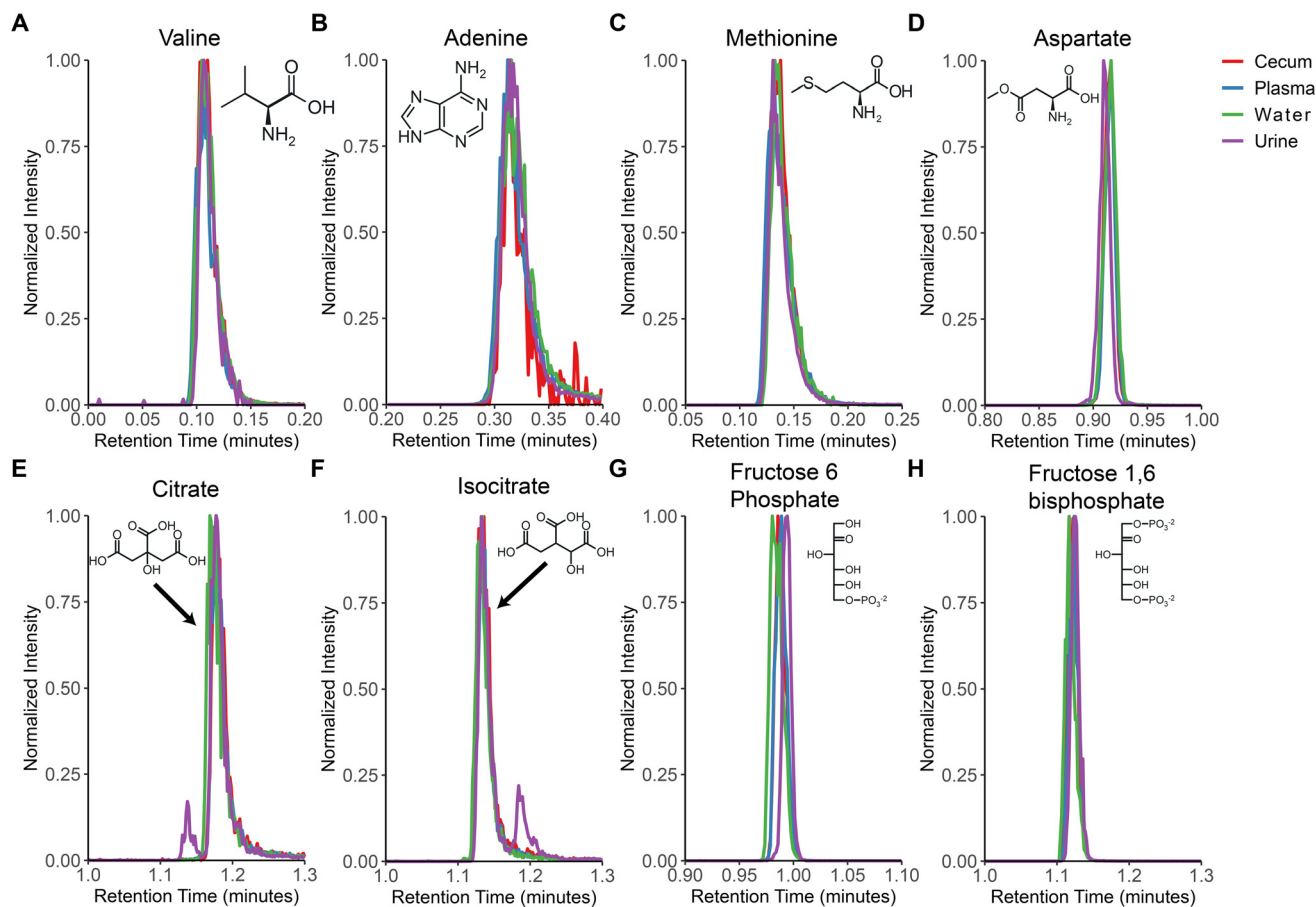
Fig. 3 Extracted ion chromatograms (EICs) in four different matrices.

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 MS^2 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 MS^2 scans of 5–20 ms in each cycle, that is ~10–30 MS^2 scans per second. Over the course of a single injection, it's possible to acquire MS^2 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.

Acknowledgements

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.

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.

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.

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.

5 S. Girel, D. Guillarme, 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.

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.

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.

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.

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.

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.

11 B. Van De Velde, D. Guillarme 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.

12 G. L. Losacco, O. Ismail, J. Pezzatti, V. González-Ruiz, J. Boccard, S. Rudaz, J.-L. Veuthey and D. Guillarme, 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.

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.

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.

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.

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.

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.

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.

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.

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.