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Disentangling method-induced differences in metabolomics: single- *versus* dual-phase extraction

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Efficient metabolite extraction is a key determinant of metabolome coverage and data quality in metabolomics. In this study, we systematically compare two widely used metabolite extraction strategies in untargeted metabolomics, including methanol-based single- and methyl tertiary-butyl ether (MTBE) based dual-phase solvent systems, and evaluate their performance across common urine and plasma, in liquid chromatography-mass spectrometry (LC-MS)-based metabolomics. Through a rational experimental design, we disentangled the multifactorial differences between single- and dual-phase extractions into three analytically distinct components: pipetting, partitioning, and matrix effects. We further employ a comprehensive panel of isotopically labelled internal metabolite standards derived from ^{13}C-labeled yeast extract to quantitatively characterize extraction-induced metabolic changes. Our results indicate that pipetting-induced variability is minimal, as polar standards exhibit fold changes close to unity between single- and dual-phase extraction protocols. In contrast, partitioning effects are strongly governed by solvent composition and phase volume ratios. For example, in plasma dual-phase extraction, where aqueous and organic phases are approximately equal, partitioning losses are limited. However, in urine dual-phase extraction, a higher organic-to-aqueous volume ratio promotes substantial redistribution of nonpolar metabolites into the organic phase. In addition, matrix effects further contribute to selective differences between the two methods, with ion suppression observed for subsets of compounds within specific retention time regions. Ultimately, the observed net response of each metabolite reflects the combined influence of partitioning behavior and matrix effects. Collectively, these findings highlight the complex, multifactorial nature of extraction-driven differences in LC-MS-based metabolomics. The framework established here can be readily extended to future method development, enabling systematic and mechanistic evaluation of method-induced metabolic changes.

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Introduction

Metabolomics is a cornerstone of omics technology, aiming to comprehensively characterize small molecule metabolites within biological systems.[1,2] Owing to their extensive chemical diversity and frequently low abundances, the success of untargeted metabolomics critically depends on robust and reproducible sample preparation.[3–5] Among these steps, metabolome extraction is particularly pivotal, as it dictates which metabolites are recovered, their relative abundances, and the overall analytical reproducibility.[6] Following extraction, liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) has become the most widely adopted platform for metabolite and lipid detection, quantification, and identification.[7,8] Nevertheless, the depth, coverage, and quantitative reliability of metabolomics data are ultimately governed by how effectively metabolites are transferred from complex biological matrices into the analytical system, making the strategy of metabolome extraction a central performance determinant of metabolomics.

Among extraction methods, single- and dual-phase protocols represent two divergent but widely used approaches. Single-phase extraction employs a single organic solvent, most commonly methanol or acetonitrile, to induce protein precipitation. Following centrifugation, the soluble metabolite fraction is collected by transferring the clarified supernatant to a new vial.[9–11] Its advantages include procedural simplicity, speed, and compatibility with high-throughput workflows. However, single-phase extraction primarily recovers polar metabolites and yields poor extraction efficiency for nonpolar lipids. Consequently, when comprehensive coverage of both

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polar metabolites and nonpolar lipids is required, an additional aliquot of the same biological sample must be processed separately for lipid extraction and downstream LC-MS analysis.[12–14] This practice not only doubles sample-preparation effort but also requires twice the amount of biological material. In situations where sample volume is limited, such as archived biofluids from biobanks or samples obtained from costly experimental models (*e.g.*, gene-knockout mice), single extraction becomes impractical.

Conversely, parallel or simultaneous metabolomics and lipidomics is an integrated analytical strategy in which polar metabolites and nonpolar lipids are extracted, measured, and analyzed from the same biological sample.[15–20] In this combined workflow, solvent mixtures are used to partition the sample into an aqueous phase enriched in polar metabolites and an organic phase enriched in lipids. In this manuscript, we refer to this strategy as dual-phase extraction, to distinguish it from the conventional single-phase extraction approach. Such one-sample preparation is highly promising for real-world applications, as it reduces biological sample consumption and has been demonstrated across a wide range of metabolomics studies in biological systems.[21–25]

Despite its conceptual advantages of high throughput, dual-phase extraction may hypothetically yield lower concentrations of target analytes compared with single-phase extraction. This reduction may arise from multiple factors, including greater loss of the metabolite layer during careful pipetting, required to avoid disturbing the interphase between the aqueous and organic layers, as well as partial partitioning of analytes between the two phases. Beyond these valid concerns, systematic studies aimed at understanding the differences between single- and dual-phase extraction strategies remain limited. Existing work in this area has largely focused on specific classes of compounds and has not extended to comprehensive, omics-level analyses.[26,27]

One difficulty of such comparison study arises because differences in metabolite abundance can originate from several distinct sources: unavoidable technical losses during pipetting, chemical partitioning of metabolites according to their polarity, and ion suppression due to coeluting matrix components. Without explicitly disentangling these influences, true method performance cannot be accurately assessed. Accordingly, this study employs a comprehensive set of isotopically labeled chemical standards derived from ^{13}C-cultured yeast cells and focuses on three mechanistically distinct factors hypothesized to drive differences between single- and dual-phase extractions: (1) pipetting effect, (2) partitioning effect, and (3) matrix effect. Together, these factors govern the effective concentration of analytes entering the mass spectrometer and ultimately determine the observed signal intensity. By systematically isolating and quantifying each contribution, this work establishes a framework for objectively evaluating extraction protocols, thereby guiding researchers in balancing methodological simplicity with analytical robustness according to their specific experimental objectives.

Experimental

Internal standards preparation

Internal Standards (IS) and Long-Term Reference Standards (LTRS) were obtained from the IROA TruQuant IQQ Workflow Kit (Sigma-Aldrich, St Louis). The IS consist of 95% U–^{13}C-labeled compounds. IS were dissolved in 1200 μL of ACN/H_2O (1 : 1, v/v) and later spiked into individual blanks and biological samples. A list of the IS compounds detected in our experiments is provided in Table S1. The LTRS is a 1 : 1 mixture of paired U–^{13}C-labeled compounds (95% and 5%) and serves as a reference for building compound libraries used to annotate the IS. LTRS were dissolved in 40 μL of ACN/H_2O (1 : 1, v/v) for quality control.

Single- and dual-phase extraction

Urine single-phase extraction was performed by mixing 40 μL of urine with 160 μL of ice-cold methanol (MeOH).[28] Samples were stored at −20 °C for 4 hours to promote protein precipitation, centrifuged at 14 000 rpm for 10 min at 4 °C, and the metabolite-containing supernatant was transferred. The extracts were dried in a CentriVap Concentrator (Labconco, USA) at ambient temperature for 120 min per batch and reconstituted in 40 μL of ACN/H_2O (1 : 1, v/v) for LC-MS analysis. For dual-phase extraction, protein precipitation followed the same steps as single-phase extraction. After precipitation, 450 μL of MTBE was added, and the samples were shaken for 20 min on a Fisher Scientific microplate shaker.[29] Phase separation was induced by adding 85 μL of water, followed by centrifugation at 14 000 rpm for 10 min at 4 °C. The metabolite-containing layer was carefully collected, dried, and reconstituted in 40 μL of ACN/H_2O (1 : 1, v/v) for LC-MS analysis.

Plasma single-phase extraction was performed by mixing 40 μL of plasma with 320 μL of ice-cold methanol, and stored at −20 °C for 4 hours.[30] Samples were then centrifuged at 14 000 rpm for 10 min at 4 °C, and the metabolite-containing supernatant was transferred. For dual-phase extraction, protein precipitation followed the same steps as single-phase extraction. After precipitation, 187 μL of MTBE was added, and the samples were shaken for 20 min.[31] Phase separation was achieved by adding 133 μL of water, followed by centrifugation at 14 000 rpm for 10 min at 4 °C. The metabolite-containing layer was collected, dried, and reconstituted in 40 μL of ACN/H_2O (1 : 1, v/v).

To assess pipetting and partitioning effects, urine and plasma were replaced with an equivalent volume of IS solution. In more details, the IS mixture was dried down, re-solvated in water, and processed following the same experimental procedure as the urine and plasma samples. To assess matrix effect, IS mixture was spiked into the dried extract of biological samples. Lastly, to assess the combined influence of all three effects, 40 μL of IS mixture was dried down and then added to an equivalent volume of urine or plasma, and processed following the protocols. For each experiment, five experimental replicates were performed to assess reproducibility.

LC-MS analysis

LC-MS analysis was conducted using a Bruker Impact II ultra-high resolution Qq-time-of-flight (UHR-QqTOF) mass spectrometer (Bruker Daltonics Billerica, MA) coupled with a Thermo Vanquish Horizon UHPLC system (Thermo Scientific, Waltham, MA, USA). Hydrophilic interaction liquid chromatography (HILIC) separation was achieved using Atlantis Premier z-HILIC column (150 mm × 2.1 mm, 2.5 μm, 95 Å) in both electrospray ionization (ESI) positive and negative modes. In ESI positive mode, mobile phase A consisted of 95/5 H_2O/ACN containing 10 mM NH_4Ac, adjusted to pH 4.8 with acetic acid. In ESI negative mode, mobile phase A consisted of 95/5 H_2O/ACN containing 10 mM NH_4AC, adjusted to pH 9.8 with ammonium hydroxide. For both modes, 95/5 ACN/H_2O was used as mobile phase B. The gradient started at 95% B, decreased to 50% B at 18 min, rapidly to 5% B at 18.1 min, held at 5% B until 20 min, then returned to 95% B at 21 min and maintained for column re-equilibration. The flow rate was 0.30 mL min^{-1}. A 1 μL injection volume was used for both modes and sample types. Samples were run in data-dependent acquisition (DDA) mode.

Reverse-phase liquid chromatography (RPLC) separation was achieved using a Waters Acquity UPLC BEH C18 column (100 mm × 1 mm, 1.7 μm) in ESI positive mode. H_2O with 0.1% formic acid was used as mobile phase A, and ACN with 0.1% formic acid was used as mobile phase B. The gradient elution program was as follows: 5% B at 0 min, ramping to 25% B at 8 min, 70% B at 14 min, 95% B at 20 min, held at 95% B until 23 min, and then returned to 5% B at 23.1 min for column re-equilibration. The flow rate was 0.15 mL min^{-1}. A 2 μL injection volume was used for urine samples, and a 1 μL injection volume was applied for plasma samples.

Data processing and compound annotation

Raw LC-MS data files (.d) were first converted to the .abf format using a file-format converter (Reifycs Inc). The converted files were processed in MS-DIAL (version 4.6) for metabolic peak picking and feature alignment, with each experimental condition analyzed separately.[32] The detailed parameter settings are available in Text S1. MS-DIAL outputs were subsequently filtered in Microsoft Excel to remove metabolites eluting beyond the analytical gradient (>23 min). In addition, any metabolite whose maximum sample intensity was less than three times its corresponding method blank intensity was excluded. After data cleaning, features from the single-phase and dual-phase extractions were aligned based on RT and *m*/*z*, using tolerances of 0.1 min and 0.01, respectively. IS compounds were annotated directly from the raw LC-MS data using ClusterFinder, a software tool designed to process isotopically enriched metabolomics data from the IROA Work Kit. The ^{13}C peaks were confirmed based on their characteristic isotopic patterns. The detailed parameter settings are available in Text S2. A custom Python script was then used to integrate MS-DIAL outputs with the ClusterFinder annotations. Full parameter settings for both MS-DIAL and ClusterFinder are provided in SI. Fold change was calculated by dividing single-phase intensities by dual-phase intensities, and statistical significance was assessed using a non-parametric *t*-test implemented in Python using SciPy (version 1.15.2). False discovery rate (FDR) correction of *p*-values was performed using the Benjamini–Hochberg procedure implemented in statsmodels (version 0.14.4). A fold-change threshold of 1.2 and a *p*-value cutoff of 0.05 were used to evaluate whether the intensity differences between extraction methods were significant.

Results and discussion

Metabolic differences between single- and dual-phase extractions

To assess the overall metabolic difference, we first compared the total number of metabolic features detected across extraction methods, biofluids, and chromatographic modes. As presented in Fig. 1, single-phase extraction consistently yielded a higher number of detected metabolites than dual-phase extraction across all biofluids and chromatographic modes. We further evaluated the overlap in detected metabolites in both extraction methods. Across all the analytical modes, most features were shared between the two extractions. However, each method also contributed unique detections.

In more details, in urine analysis, single- and dual-phase extractions showed substantial overlap across all chromatographic modes. In HILIC+, 3371 features were shared (79% overlap), with 481 and 419 unique to single- and dual-phase extractions, respectively. HILIC− showed a similar trend, with 2539 shared features (81% overlap) and 366 and 217 unique features. RP+ exhibited slightly lower overlap (74%), with 1792 shared features and 336 and 287 unique to single- and dual-phase extractions. In plasma analysis, extraction overlaps varied more broadly across chromatographic modes. HILIC+ showed 2457 shared features (70% overlap), with moderate numbers of unique features for each method. HILIC− exhibited the same 70% overlap, with 1696 shared features and comparable unique feature counts. RP+ showed a much lower overlap of 38%, with 1681 shared features and substantially more features unique to single-phase extraction.

Beyond differences in detection coverage, signal intensities also differed markedly between extraction workflows. As shown in the volcano plots (Fig. S1), a large proportion of overlapping features exhibited significantly higher intensities in single-phase extracts across biofluids and chromatographic modes. Overall, across all evaluated biofluids and chromatographic modes, single-phase yielded not only a greater number of unique features but also higher features intensities relative to dual-phase extraction. Importantly, because the two extraction methods showed comparable RSD performance (Fig. S2), these differences cannot be attributed to variability in quantitative reproducibility. Together, these results highlight that single- and dual-phase extractions can lead to markedly different metabolomic profiles. A comprehensive and systema-

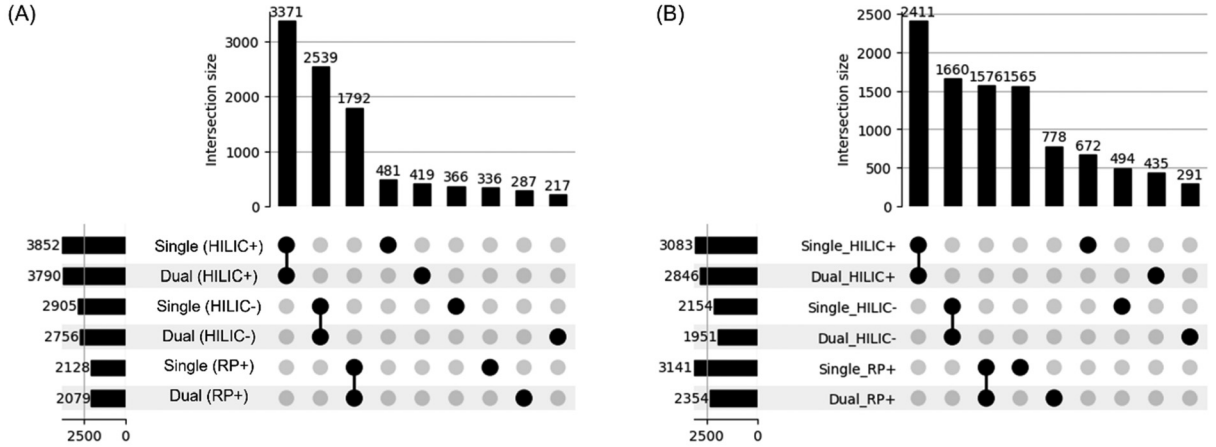

Fig. 1 Number of features detected by each extraction method and their overlap across chromatographic modes. (A) Features detected in urine samples; (B) features detected in plasma samples.

tic investigation is therefore warranted to elucidate the mechanisms underlying these discrepancies.

Rationales on the experimental design

To approach a systematic and mechanistic understanding, we first dissect and recognize the main independent factors that could influence metabolic differences. As presented in Fig. 2, we hypothesize that there are three possible effects that in combination, which could lead to the data discrepancy. Among them, pipetting effect refers to the pipetting volume differences in between single- and dual-phase extractions. In both approaches, a small but unavoidable volume loss occurs because the protein pellet must be avoided when transferring the clarified supernatant to a new vial. In dual-phase extraction, additional volume loss might arise because material near the interphase must be excluded to prevent disruption of the boundary between the metabolite and lipid layers.

Next, partitioning effect refers to the potential distribution of metabolites into the lipid layer during dual-phase extraction, depending on their polarity. Particularly, hydrophobic metabolites are more likely to dissolve in the lipid layer during dual-phase extraction, and thus has decreased concentration in the metabolite layer.[33] This effect represents another mechanism through which metabolite signal intensity can be reduced in dual-phase.

Finally, matrix effect reflects signal suppression caused by coeluting matrix components.[34] The metabolite layer obtained from dual-phase extraction is anticipated to be cleaner than that from single-phase extraction, since certain matrix compounds may also partition into the organic layer depending on their polarity.[35] By separating non-polar compounds into the organic phase, dual-phase extraction reduces the number of interfering species in the aqueous metabolite layer, which in turn minimizes ion suppression.

To investigate these three factors, stable isotopic labelled IS compounds prepared from ^{13}C-labeled yeast extract were spiked at different stages of the metabolomics workflow (Fig. 3). The use of ^{13}C-labeled yeast extract serves two main

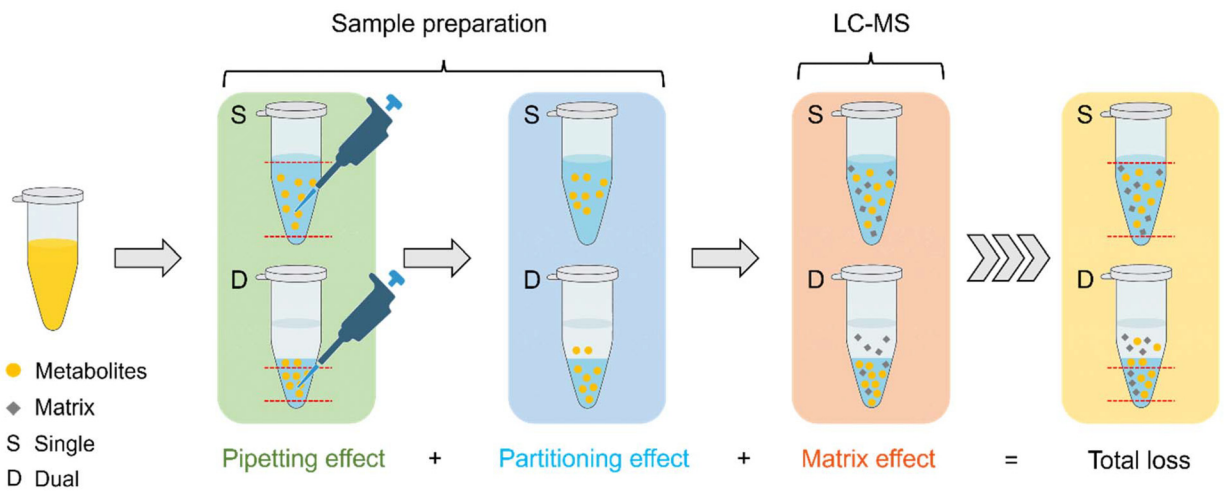

Fig. 2 Visualization of the three effects studied: pipetting, partitioning, and matrix effects.

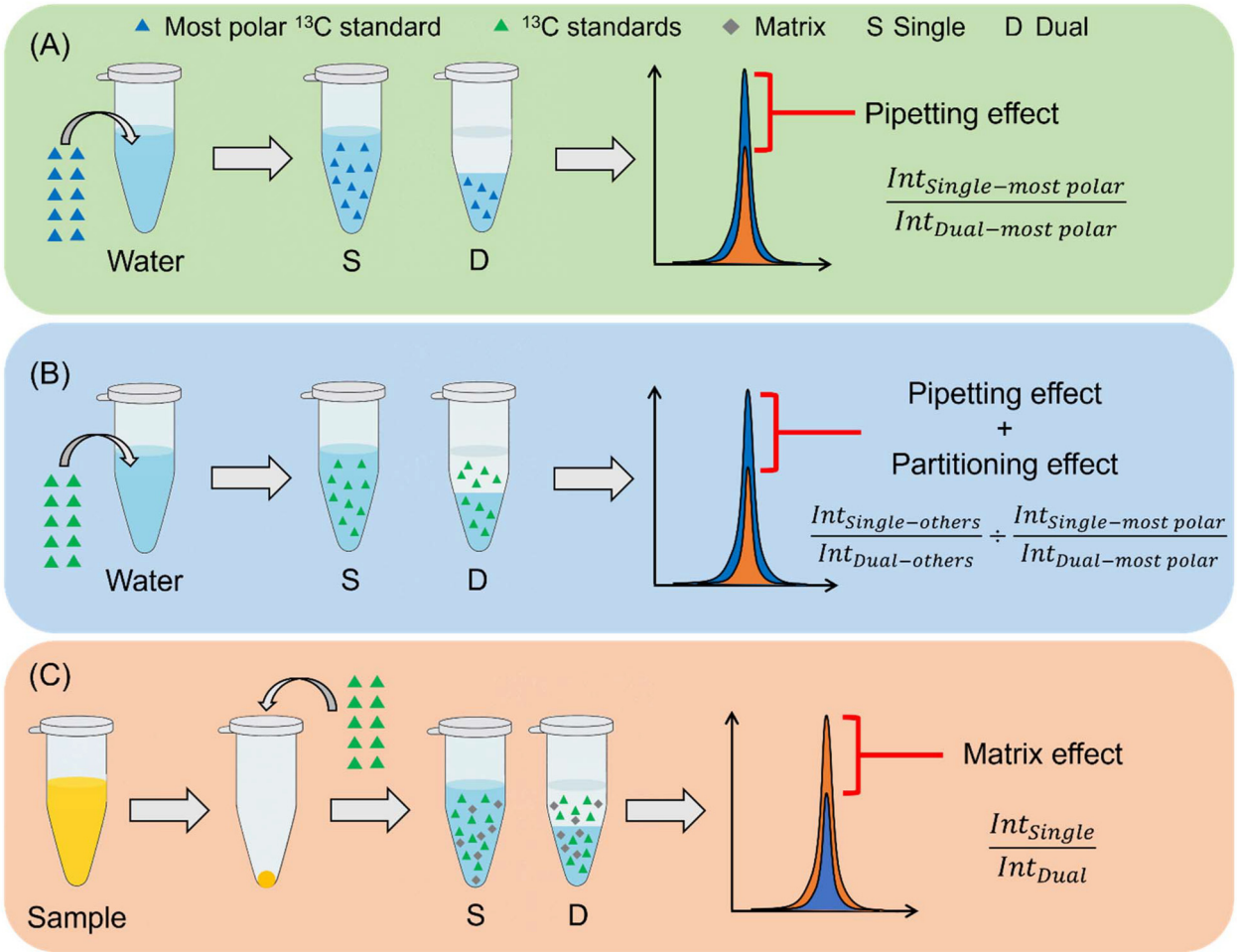

Fig. 3 Schematic representation of the experimental design used to investigate (A) pipetting, (B) partitioning, and (C) matrix effects.

purposes. First, the ^{13}C-labeled metabolites in the yeast extract can function as surrogate internal standards, as they are not naturally present in unlabeled human urine or plasma samples and can therefore be clearly distinguished from endogenous compounds. Second, ^{13}C-labeled yeast extract contains a broad spectrum of endogenous metabolites that are commonly detected in metabolomics analyses. As such, it provides a representative coverage of the metabolome relevant to conventional LC-MS-based metabolomics workflows. However, because the extraction was performed using methanol-based protocols, nonpolar metabolites are not well represented in this study.

To study pipetting and partitioning effects, IS compounds were spiked into water blank and processed using the standard metabolite extraction protocol. LC-MS was used to quantify the intensity fold changes of all IS compounds in between single- and dual extractions. To isolate the contribution of pipetting effect, driven solely by the differences in transferred metabolite layer volume, we examined the fold change of the most polar IS between the single- and dual-phase extractions. This approach relies on two key assumptions. First, we assume that phase partitioning is negligible for highly polar standards, such that their recovery is not substantially affected by distribution between phases. Second, given the broad spectrum of metabolites present in the ^{13}C-labeled yeast extract, we expect to identify suitable highly polar standards that can reliably represent the behavior of polar metabolites in the extraction workflow. After quantifying pipetting effect, we evaluated metabolite-specific partitioning by correcting their observed fold changes accordingly. Finally, to assess matrix effects independently, IS compounds were spiked into the dried sample extracts immediately prior to LC-MS analysis. This approach effectively eliminates contributions from both pipetting and partitioning, allowing matrix-induced ionization effects to be evaluated in isolation.

Pipetting and partitioning effects

In HILIC+ mode, ^{13}C-glutamic acid showed a fold change of 1.0 for both the urine and plasma extractions performed on water blanks. In HILIC− mode, ^{13}C-glycerylphosphorylethanolamine showed fold changes of 1.1 and 1.2 for the urine and plasma protocols, respectively. In RP+ mode, ^{13}C-lysine showed fold changes of 1.0 and 1.2 under the urine and plasma protocols, respectively. Across all protocols, these

standards showed fold-change values that are close to 1, showing that pipetting effect was minimal across both extraction workflows. This confirms that pipetting effect has minimal contribution to the metabolic discrepancy in between single and dual extractions.

After correcting for the pipetting effect, partitioning effect was evaluated (Fig. 4). Compounds were classified as affected by partitioning when their fold change exceeded 1.2 (FDR adjusted p-value < 0.05). The full summary of affected compound counts across all modes, including results obtained using more stringent fold-change thresholds (FC > 1.3 and FC > 1.5), is provided in Table S8. In urine, HILIC+ mode showed that 36% (77/216) of the standards had higher intensities in single-phase extract, whereas less than 1% (1/216) were higher in dual-phase extract using the urine protocol (Table S2). In HILIC− mode, 20% (26/129) of standards were higher in single-phase (Table S3). In RP+ mode, 20% (31/154) of standards showed higher intensities in single-phase extraction (Table S4). The remaining standards showed no significant differences. For plasma, HILIC+ mode resulted in less than 1% of standards (1/252) showing higher intensities in single-phase and 1% (3/252) higher in dual-phase (Table S5). In HILIC− mode, 2% (3/155) were higher in single-phase (Table S6). In RP+ mode, 2% of standards (5/268) were higher in single-phase and 7% (18/268) were higher in dual-phase (Table S7).

Overall, partitioning played a solvent-ratio–dependent role. In plasma, the metabolite and lipid layers in dual-phase extraction were roughly 1 : 1, which limited the redistribution of non-polar metabolites and resulted in relatively minor intensity differences between single- and dual-phase extractions. Furthermore, the dual-phase metabolite layer is more polar than the single-phase extract due to the added water, which could enhance the solubility of certain polar metabolites. This may explain why a subset of standards showed unexpectedly higher intensities in dual-phase, especially in plasma RP+. In contrast, for urine, the dual-phase protocol employed a higher organic (MTBE) volume relative to the aqueous fraction. This increased organic-to-aqueous ratio favoured partitioning of non-polar metabolites into the organic layer or near the interphase, reducing their measured intensities in the aqueous metabolite layer. Consequently, 20–40% of the standards in the urine protocol showed higher responses in single-phase extraction whereas only 2–7% of the standards were higher in the plasma protocol. These results demonstrate that the extent of partitioning is strongly influenced by phase volume ratios.

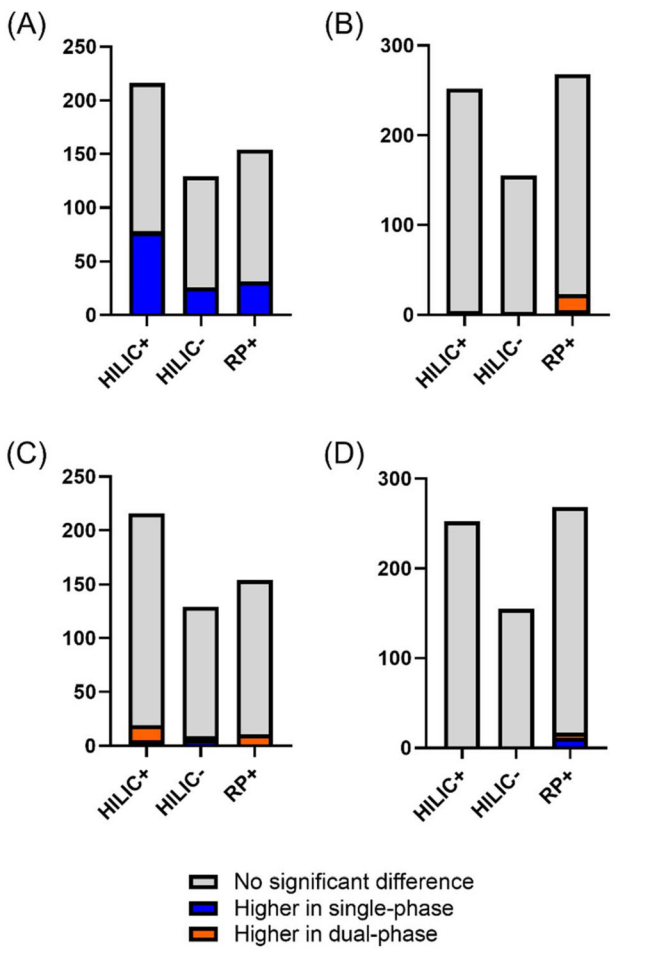

Fig. 4 Partitioning and matrix effects across modes. (A) Partitioning effect from urine protocol. (B) Partitioning effect from plasma protocol. (C) Matrix effect in urine. (D) Matrix effect in plasma.

Matrix effect

Matrix effect was evaluated using the same fold change and statistical criteria (FC > 1.2, FDR adjusted p-value < 0.05). Matrix effect shows certain impact towards the metabolic intensities. In urine, HILIC+ mode showed that 2% of spiked standards (5/216) had higher intensities in the single-phase extract and 6% (14/216) had higher intensities in the dual-phase extract (Table S2). In HILIC− mode, 5% of standards (6/129) exhibited higher signals under single-phase extraction and 2% (3/129) higher under dual-phase extraction (Table S3). In RP+ mode, 1% of standards (1/154) produced higher intensities in the single-phase extract, whereas 8% of standards (10/154) favoured dual-phase extraction (Table S4). For plasma, HILIC+ mode showed fewer than 1% of standards (1/252) with higher intensities in dual-phase extraction (Table S5). In HILIC− mode, 1% (1/155) in dual-phase (Table S6). In RP+ mode, 4% of standards (11/268) yielded higher intensities in single-phase extraction and 2% (6/268) in dual-phase (Table S7). The number of compounds meeting more stringent fold-change criteria (FC > 1.3 and FC > 1.5) is summarized in Table S8.

Overall, matrix effects impacted only a small proportion of spiked standards across all modes and biofluids. In urine, although the affected percentages were low, the few standards that did show differences tended to have slightly higher intensities in the dual-phase extract, especially in positive ESI modes. This is likely because phase partitioning removes some endogenous metabolites into the opposite solvent layer, reducing background and modestly enhancing ionization efficiency. In contrast, in plasma, single-phase extraction pro-

duced a cleaner matrix for more standards than dual-phase in RP+ mode. This is likely due to the aforementioned phase volume distribution, which in our earlier partitioning study limited the redistribution of non-polar standards. Here, the same effect appears to limit the removal of interfering matrix compounds, as the aqueous and organic layers are roughly 1 : 1, restricting transfer of endogenous metabolites away from the aqueous metabolite layer. Furthermore, because most of the standards are relatively polar compounds from yeast extract, they largely remain in the aqueous phase during dual-phase extraction. The addition of water in the dual-phase protocol increases their solubility, and with only minimal partitioning of endogenous metabolites into the organic layer, the aqueous phase becomes relatively enriched in polar compounds compared with a single-phase extract. As a result, these analytes do not benefit from the reduced background that phase partitioning provides, in some cases, may even experience greater matrix interference if additional polar endogenous components are co-enriched in the aqueous layer.

These findings underscore that partitioning and matrix effects influence signal intensities through distinct mechanisms, and their contributions can be decoupled when evaluated using spiked water blanks *versus* spiked samples. Nonetheless, our assessment is constrained by the limited number and chemical diversity of standards, mostly polar metabolites in yeast extract. Future work incorporates a broader panel, especially the nonpolar metabolites, will be essential to generalize these observations. In addition, matrix effects are inherently sample-type dependent. Although our results show the limited contribution of matrix effect, it does not extrapolate directly to other types of biological matrices. Expanding the evaluation to other biological matrices, such as tissues, cultured cells, and fecal samples, would provide a more comprehensive understanding of the performance differences between single-phase and dual-phase extraction strategies.

Representative cases of mixed outcome and future perspectives

To better illustrate the interplay among these factors and their net outcome, we highlight two representative cases. Fig. 5 shows the behaviour of ^{13}C-adenosine in both single- and dual-phase extractions. Overall, ^{13}C-adenosine exhibits substantially higher intensity in the single-phase extraction (Fig. 5A). When we decompose the signal into partitioning and matrix effects, we observe opposing trends: ^{13}C-adenosine shows higher intensity in the partitioning component (Fig. 5B) but lower intensity in the matrix-effect component (Fig. 5C). This pattern suggests that ^{13}C-adenosine undergoes considerable metabolite loss during dual-phase extraction, yet benefits from reduced matrix suppression, which increases its apparent intensity in that workflow. When both factors are considered together, the net outcome remains in favour of the single-phase extraction, which provides higher overall intensity and better sensitivity for ^{13}C-adenosine.

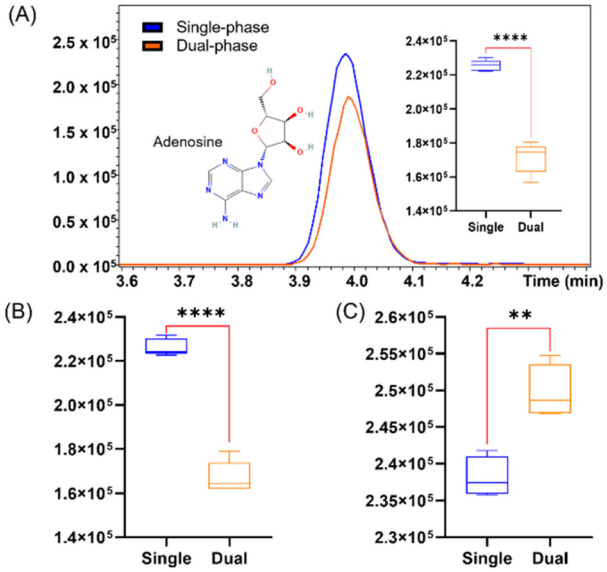

Fig. 5 Representative example illustrating the interplay of partitioning and matrix effects for ^{13}C-adenosine in urine HILIC+ mode. (A) Overall intensity of ^{13}C-adenosine. (B) Contribution of partitioning effect. (C) Contribution of matrix effect.

Another example is from ^{13}C-adenosine monophosphate, which shows overall higher MS signals (Fig. 6A). With the addition of phosphate, adenosine monophosphate is much more hydrophilic, compared to adenosine. As a consequence, the sample loss of partitioning effect during dual extraction is negligible as it is unlikely to partition into the MTBE layer (Fig. 6B). Additionally, with less matrix effect in dual-extrac-

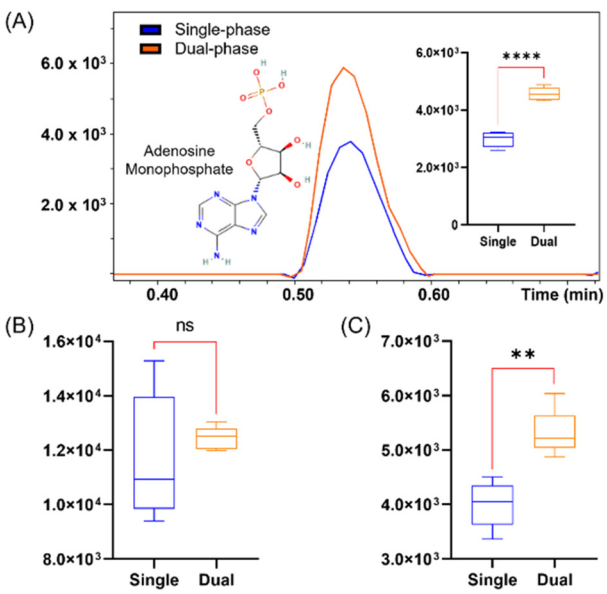

Fig. 6 Representative example illustrating the interplay of partitioning and matrix effects for ^{13}C-adenosine monophosphate in urine RP+ mode. (A) Overall intensity of ^{13}C-adenosine monophosphate. (B) Contribution of partitioning effect. (C) Contribution of matrix effect.

tion, higher MS signal intensity is observed (Fig. 6C). Combing these two factors together, ^{13}C-adenosine monophosphate favours dual extraction.

More broadly, these contrasting behaviours highlight that extraction-induced losses and matrix-driven ionization changes can interact in compound-specific ways, sometimes reinforcing and sometimes offsetting one another. Subtle differences in polarity, charge, and functional groups can shift this balance, meaning that neither single- nor dual-phase extraction is uniformly advantageous across all metabolites. These representative cases underscore the importance of dissecting signal changes into mechanistic components rather than relying on net intensities alone. Looking ahead, expanding the chemical diversity of standards, refining partitioning strategies, and incorporating more systematic evaluations of endogenous background will help establish generalizable principles for choosing or tailoring extraction workflows for different metabolite classes.

Our results demonstrate that the choice between single- and dual-phase extraction has measurable consequences for metabolite coverage, recovery, and susceptibility to matrix effects. From a biological perspective, this means that extraction strategy can influence the metabolite subsets that are preferentially detected and quantified, with downstream impact on pathway interpretation and biomarker discovery. For example, single-phase extraction favoured broader metabolite coverage in both urine and plasma, but certain polar metabolites and standards benefited from dual-phase due to reduced matrix suppression in specific retention windows. Thus, biological conclusions drawn from untargeted datasets may differ depending on the extraction approach, particularly when analytes of interest are concentrated in polarity ranges sensitive to partitioning or matrix effects.

Technically, these findings emphasize the importance of tailoring extraction protocols to study goals. For discovery-driven metabolomics, single-phase protocols provide broad coverage and methodological simplicity, whereas dual-phase protocols may be advantageous when both metabolites and lipids need to be collected from the same sample. Although dual-phase extraction can occasionally reduce localized matrix suppression, our results indicate that overall matrix effects are limited, and both methods produce largely reproducible and interference-free measurements. Furthermore, our systematic use of IS across biofluids and chromatographic modes illustrates a generalizable framework for deconvoluting pipetting effect, partitioning effect, and matrix effect contributions in extraction optimization. This approach can be extended to other sample types and solvent systems, offering a roadmap for method standardization in metabolomics and lipidomics.

However, several limitations should be acknowledged in our experimental design. First, our evaluation focused on urine and plasma matrices, which differ in protein content and polarity but do not capture the full range of biological sample types (*e.g.*, tissues, cells, or fecal extracts). Thus, the generalizability of pipetting effect, partitioning effect, and matrix effect to other biological matrices remains to be validated.

Second, although we employed representative IS spanning different chemical classes, the coverage of standards does not fully reflect the diversity of endogenous metabolites. Some compound classes may therefore be underrepresented in our analysis. Third, we restricted comparisons to methanol-based single-phase extraction and MTBE-based dual-phase extraction. Alternative solvent systems (*e.g.*, acetonitrile, chloroform–methanol mixtures) may alter partitioning dynamics and matrix effects.[36,37] Finally, our analysis was limited to LC-MS detection; the interplay of extraction with other analytical platforms such as nuclear magnetic resonance (NMR) or gas chromatograph-mass spectrometry (GC-MS) was not addressed here.

Conclusion

This study provides a systematic evaluation of single- and dual-phase extraction workflows across common biofluids, such as urine and plasma, and across commonly used chromatographic modes. By decomposing the overall extraction outcome into three independent and measurable factors, pipetting effect, partitioning effect, and matrix effect, and incorporating a broad panel of ^{13}C-labeled IS, we established a rational experimental framework that enables a divide-and-analyze approach to examine each component separately. Our results show that pipetting effect is relatively negligible. In contrast, partitioning effects exert a far broader influence and vary substantially with both metabolite hydrophobicity and the solvent ratios used in single-phase *versus* dual-phase extractions. Matrix effects are generally reduced in the dual-phase workflow comparing to the single-phase workflow. When these factors are combined, the net outcome depends on the balance between partitioning loss and matrix-effect reduction for each metabolite. Taken together, these findings demonstrate that single- and dual-phase extractions do not produce universally equivalent results; their relative performance depends on metabolite polarity, phase-volume ratios, and matrix complexity. While single-phase extraction typically offers broader feature coverage and higher signal intensities, dual-phase extraction can provide reduced matrix interference for specific metabolites and sample types. Understanding the mechanistic origins of these differences enables more informed selection or integration of extraction workflows to maximize metabolomic coverage and data quality. It also facilitates the design of experiments to evaluate the differences between single- and dual-phase extractions in other types of biological samples, such as tissues and cells.

Author contributions

TH: supervision, conceptualization, methodology, formal analysis, writing – review & editing; funding acquisition; EK: meth-

odology, software, validation, formal analysis, data curation, writing – original draft, visualization.

Conflicts of interest

There are no conflicts to declare.

Data availability

Raw data are publicly available on Zenodo (https://zenodo.org/records/18176928).

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6an00025h.

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