



OPEN Optimizing nanoLC-TIMS-TOF and lipidomics sample preparation for low volume of plasma

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Lipidomics provides critical insights into disease mechanisms, biomarker discovery, and precision medicine, but conventional LC-MS workflows often require sample volumes that limit their application in minimally invasive studies. Here, we evaluated a nanoflow liquid chromatography workflow coupled with trapped ion mobility spectrometry and time-of-flight mass spectrometry (nanoLC-TIMS-TOF) for positive ion lipid profiling from as little as 1 μ L of plasma. Two commonly used single-phase extraction protocols, MeOH: MTBE and IPA: H₂O, were scaled to microsample volumes using NIST[®] SRM[®] 1950 plasma. The effects of plasma input volume and mobile-phase ammonium formate concentration (2, 5, and 10 mM) on feature detection and chromatographic performance were evaluated. Descriptive comparisons revealed extraction-, volume-, and buffer-associated differences in the detected feature profiles, while putative lipid annotations were evaluated using MS/MS and ion-mobility information, where available. The optimized workflow enabled broad positive ion lipid class profiling and detection of low intensity features from only 1 μ L of starting material. The 1 μ L extracts retained the dominant annotated lipid classes observed at higher input volumes, although profiling depth and feature distributions remained dependent on plasma input volume. Among the tested mobile phase conditions, 5 mM ammonium formate produced the highest detected feature count while limiting pressure drift during extended acquisition. These results support the feasibility and practical scalability of positive ion nanoLC-TIMS-MS lipid profiling for sample-limited applications under the evaluated conditions.

Keywords NanoLC, Lipidomics, timsTOF, Ion mobility, Ultra-low volume sampling

Lipidomics allows a comprehensive analysis of lipids within a biological system. It provides the necessary tools to understand lipid metabolism, discover biomarkers, and identify underlying mechanisms and perturbations in the lipid metabolic pathway during disease onset and progression^{1,2}. Most lipidomics pipelines that involve biological matrices often detect and identify lipid species using high-resolution mass spectrometry (HRMS) coupled to liquid chromatography (LC)¹. Direct infusion or shotgun lipidomics is an alternative approach; however, it can be limited by factors such as ion suppression, which consequently obscures signals from low ionizing and low abundance molecules³, and the inability to distinguish between isobaric and isomeric lipid species³. The latter constraint is also seen in hyphenated workflows. Still, these molecules can be resolved at the MS/MS level by incorporating alternative fragmentation techniques such as electron-activated dissociation (EAD)⁴, or the integration of ion mobility spectrometry (IMS) into LC-MS instruments⁵.

To enable higher throughput, most established lipidomics workflows utilize chromatographic columns that operate in the micro- to milliliter flow rates. However, high flow rates make these regimes incompatible with sample-restricted analyses such as tissue microbiopsies, small cell subpopulations, or even single cells due to dilution effects⁶. In recent years, nanoflow liquid chromatography (nanoLC) coupled with HRMS has emerged as a more sensitive and efficient approach, particularly in proteomics.

Operating at nL/min flow rates, nanoLC offers improved sensitivity and peak resolution⁶, making it attractive for lipidomics applications where sample availability is limited^{7,8}. When combined with HRMS and IMS, nanoLC-IMS-MS enables deeper profiling of low abundance lipids and enhanced structural elucidation through orthogonal separation⁹.

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A crucial component in any LC-MS-based lipidomics is the type and concentration of buffers and additives used in the chromatography eluents. Analytical flow methods commonly use ≥ 10 mM buffers to stabilize peak shapes and enhance signals from low-abundance lipids. However, these high buffer concentrations can be problematic in micro- and nanoflow setups¹⁰, as they can lead to signal suppression, high column back pressure, and clogging of columns and capillaries.

To validate lipidomics protocols, reference materials such as the Standard Reference Material[®] (SRM) 1950 human plasma from the National Institute of Standards and Technology (NIST) are frequently used. SRM[®] 1950 is a well-characterized benchmark for inter-lab comparison, method development, and quality control in lipidomics^{11,12}. Numerous protocols have been evaluated for lipid recovery, including traditional biphasic systems such as chloroform/methanol/water (Folch¹³, Bligh and Dyer¹⁴) and methyl tert-butyl ether (MTBE)/methanol/water (Matyash)¹⁵. While effective for class-specific coverage¹⁶, these biphasic systems can limit LC-MS compatibility, reduce throughput, and are difficult to scale for low-volume samples. In addition, chloroform-based methods may cause unwanted adduction, especially in negative ion mode which can complicate data analysis and isomer/isobar separation. In contrast, MTBE-based extractions are generally more LC-MS friendly but can produce an unstable interphase, complicating lipid layer separation¹⁸. Monophasic, chloroform-free protocols such as MTBE/methanol¹⁹ and 2-propanol/water²⁰, have practical advantages: they are easier to scale down for low-volume extractions and obtained extracts can be injected directly.

This study focuses on positive ion nanoLC-trapped ion mobility spectrometry (TIMS)-MS/MS profiling of low-volume plasma extracts under defined extraction and buffer conditions. The workflow was designed to evaluate practical feasibility, lipid-class coverage, chromatographic stability, and putative lipid annotation confidence, rather than to provide full quantitative recovery, formal sensitivity validation, or complete lipidome coverage across both ionization modes. Negative ionization mode was not evaluated; therefore, the effects of plasma input volume, extraction protocol, and buffer concentration observed in positive ion mode cannot be assumed to extend to negative ion lipid profiling. Direct comparison with previously reported analytical flow or microflow datasets was not attempted because the studies differed in acquisition and instrument conditions and data processing workflows.

Using NIST[®] SRM[®] 1950, we evaluated positive ion feature detection and lipid-class representation achievable using these miniaturized methods. Our results support the feasibility and practical scalability of a nanoLC-TIMS-MS workflow for profiling lipids from as little as 1.0 μ L of plasma, enabling lipidomics from minimally invasive sampling techniques under the tested conditions.

Results

Reference protocol selection and modifications to the methods

The lipid extraction protocols employed in the methodology were modified to accommodate three primary considerations: the first was scalability in sample volumes. To enable compatibility with limited sample volumes, protocols were required to maintain low procedural complexity and eliminate steps that could compromise yield at small scales. Consequently, methods involving filtration, open-column chromatography, or solid-phase extraction were excluded. Additionally, at reduced volumes, biphasic solvent systems present challenges in achieving effective phase separation, as the separation might not be visible with low volumes, further necessitating simplified approaches.

Second was the suitability for high(er)-throughput extractions. Given the study's objective to evaluate the feasibility of low-volume sampling for clinical implementation, extraction methods were prioritized based on their compatibility to high(er)-throughput workflows. Protocols employing minimal solvent systems and requiring limited reagent handling were favored to facilitate automation and reproducibility.

Lastly, potentially necessary drying steps should be flexibly and seamlessly integrated into the extraction methods. To preserve analyte integrity, especially during downstream reconstitution and prolonged storage, protocols were evaluated for their flexibility in incorporating solvent evaporation steps.

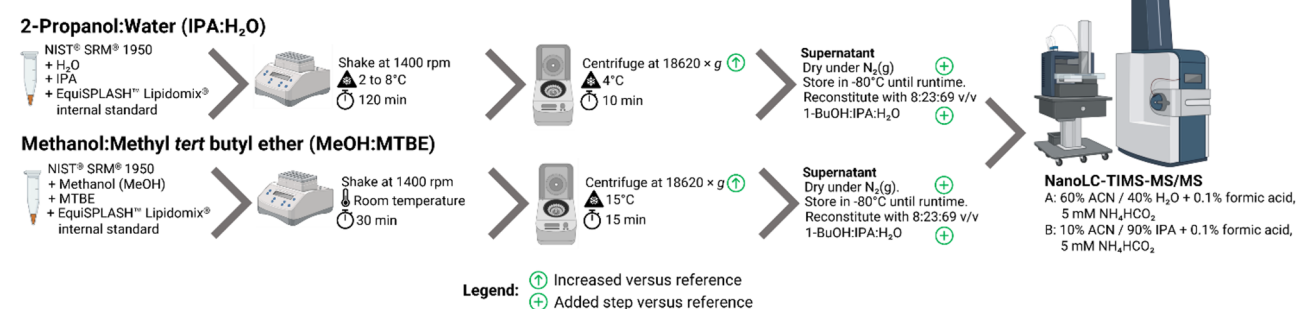
Based on these prerequisites, two single-phase extraction protocols (MeOH: MTBE and IPA: H₂O) were selected for investigation and further optimization. The general schematics of each are shown in Fig. 1a. Both protocols were modified to use higher *g*-force for centrifugation and to include a drying step with nitrogen gas (Fig. 1a). Centrifugation parameters were increased from the references to promote faster sedimentation and more efficient separation of precipitates from the supernatant²¹ at low volumes. High-speed centrifugation also serves as a practical function by removing larger particles that could otherwise obstruct the nanocolumn and the nanoLC. Each condition included three extraction replicates and three technical injection replicates. These replicates were used to qualitatively assess within-run consistency in feature detection and chromatographic behavior, particularly at lower plasma input volumes.

Comparison of single-phase lipid extraction protocols and scaling

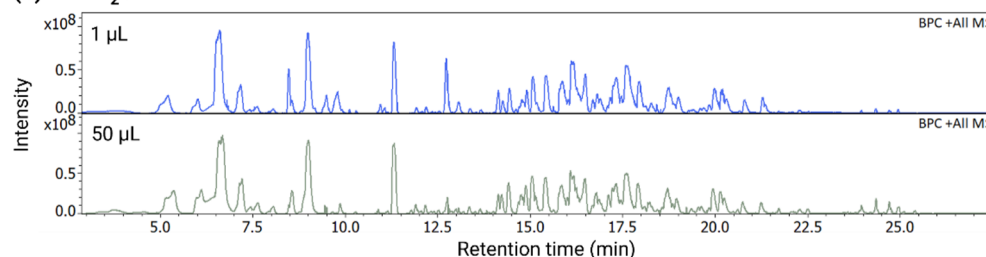
Two widely used lipidomics extraction protocols were compared (Fig. 1a). Multivariate analysis (MVA) of 36 data files, including blanks, revealed a clear separation of blanks from plasma extracts. Base peak chromatograms from the 1 μ L and 50 μ L plasma extraction volumes (Fig. 1b, c), along with intermediate volumes (25, 10, and 5 μ L; SI Figures S1, S2) show qualitatively similar signal and chromatographic profiles across the replicate measurements.

MVA was used as an exploratory quality control assessment after blank subtraction and feature filtering. Because nanoLC can enhance both analyte and background signals, blanks were retained in these models to visualize background separation and identify potential background-related contributions. However, because the sample-versus-blank contrast may substantially influence the variance, loading, and discriminatory structures, the blank-containing models were not treated as inferential evidence for differences among the experimental plasma groups.

(a) Selected extraction protocols



(b) IPA:H₂O



(c) MeOH:MTBE

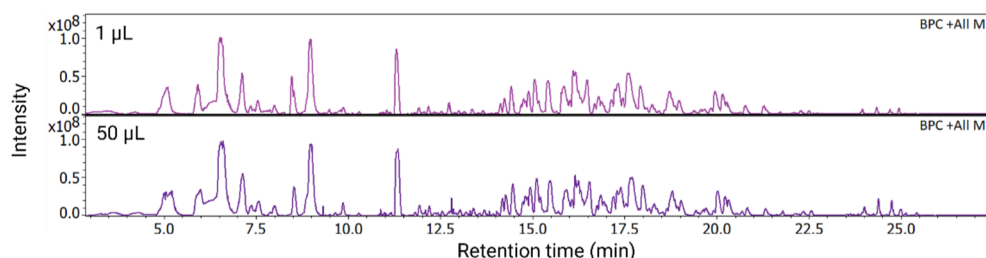


Fig. 1. Miniaturized lipidomics workflow based on NIST® SRM® 1950. **(a)** Sample preparation and analytical pipeline for the two protocols selected, with upward arrows (↑) indicating parameters increased relative to the original method and plus signs (+) indicating steps added. **(b,c)** Normalized representative base peak chromatograms (BPC) for 1 µL and 50 µL starting plasma volumes for the IPA: H₂O and MeOH: MTBE setups, respectively.

The exploratory principal component analysis (PCA) containing both plasma extracts and blanks showed clear separation of the blank measurements from the plasma samples (Fig. 2a). The positions of the extraction and input-volume groups within this model are shown descriptively and were not used to establish statistical differences or equivalence among the plasma conditions.

The corresponding loadings, partial least squares-discriminant analysis (PLS-DA), and variable importance plots (VIP) are retained as exploratory visualizations of features contributing to the blank-containing models (Fig. 2b–d and SI Figure S3a). The labelled phosphocholines (PC), lysophosphocholines (LPC), and sphingomyelin (SM) features are presented as descriptive contributors to these models rather than statistically established markers of differences among the plasma groups or evidence of lipid-class enrichment. In the class-count summaries, LPC, PC, and SM were the most frequently represented putative annotation classes across both extraction protocols and the evaluated input volumes (Fig. 2e). The relative intensity summary of the shared putatively annotated features likewise showed that these classes remained the largest contributors across the low-volume conditions (Fig. 2g). These findings are descriptive and do not establish statistical equivalence between the extraction protocols or the input-volume conditions.

Across the low-volume plasma inputs, LPC, PC, and SM remained the most frequently represented classes among the putatively annotated features. Both extraction workflows were dominated by LPC, PC, and SM annotations across 1, 5, 10, and 25 µL (Fig. 2e), indicating that reduced starting volume did not cause a complete deviation of class-level lipid representation. Because annotation counts do not capture changes in relative feature intensity, these results should not be interpreted as evidence that abundance profiles were unchanged or equivalent across conditions. The MVA further indicated volume- and extraction-dependent differences in the detected lipid feature profiles, with 50 µL samples separating most strongly from the lower-volume conditions. The putative annotations were cross-referenced against the compiled NIST® SRM® 1950 lipid list after nomenclature harmonization (Fig. 2f). The number and proportion of matching annotations varied with plasma input volume. A substantial fraction of the annotations did not match an entry in the compiled reference list, and

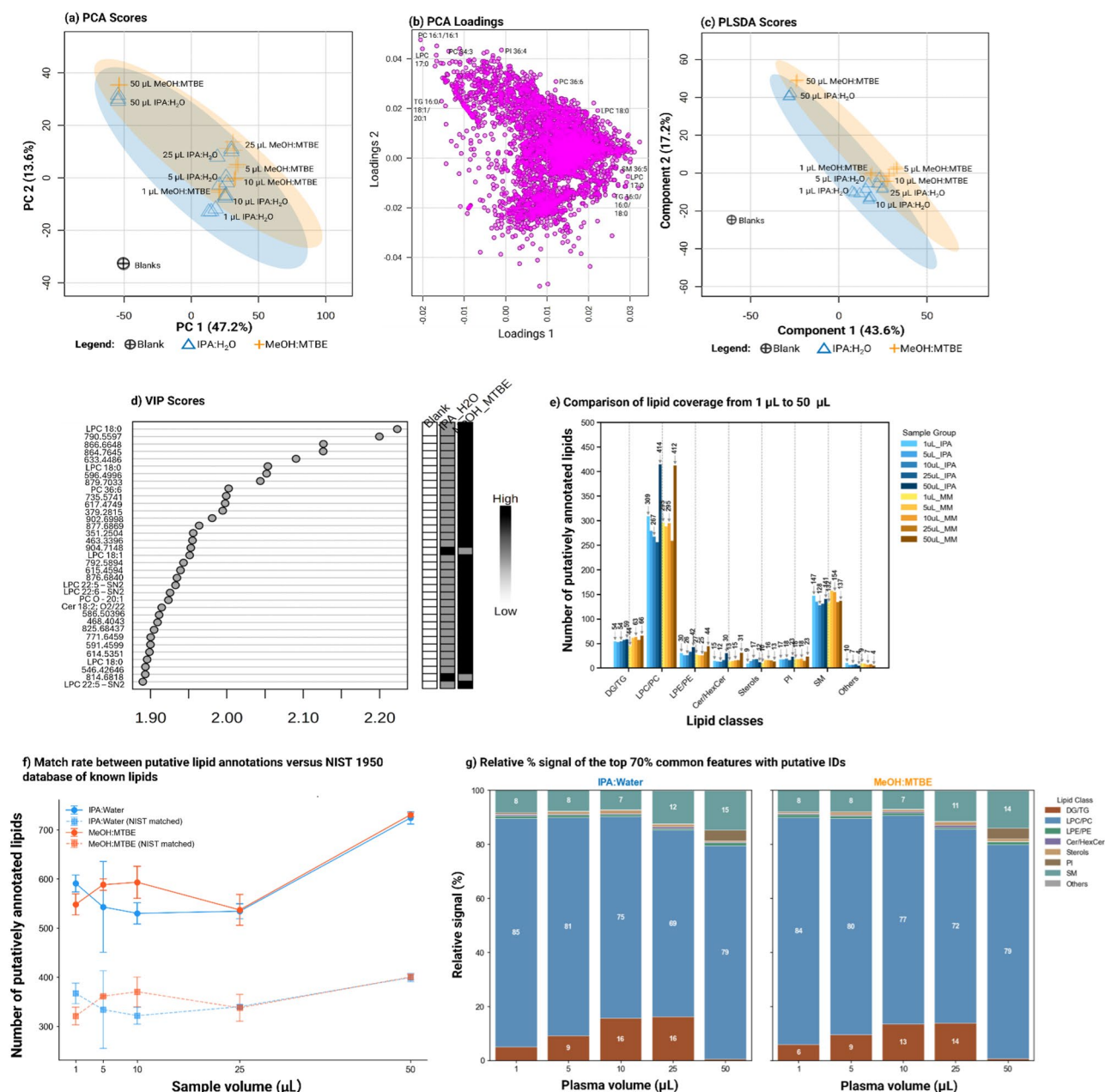


Fig. 2. Effect of plasma input volume and extraction protocol on low-input nanoLC-TIMS-TOF lipidomics performance. **(a)** PCA scores plot of plasma extracts and blanks prepared using IPA: H₂O and MeOH: MTBE across the tested input volumes. **(b)** PCA loadings structure showing putatively annotated lipid features contributing to group separation. Labels indicate selected high-contributing features. **(c)** PLS-DA scores plot showing separation of extraction protocols and plasma input volumes. **(d)** Variable importance in projection (VIP) scores for selected features contributing to the model, with adjacent heatmap indicating relative feature signal across extraction and volume conditions. **(e)** Lipid class annotation counts across extraction protocols and plasma input volumes. **(f)** Total putatively annotated lipid features and NIST SRM 1950 database-matched annotations detected across input volumes for each extraction protocol. **(g)** Relative class-level signal contribution calculated from normalized intensities of the common putatively annotated lipid features across the volume series. Lipid class summaries are interpreted as class-level trends in putative lipid-feature recovery and signal contribution, rather than as confirmed lipid-identification counts. **(e,g)** provide descriptive comparisons of lipid-class annotation counts and relative class-level signal contributions, respectively. These summaries were not subjected to formal equivalence testing.

this unmatched fraction showed greater variation at lower input volumes. Because the unmatched annotations were not further characterized according to abundance, annotation confidence, or detection consistency, the reason for this pattern cannot be determined from the present comparison. Unmatched status was not interpreted as evidence of either a novel lipid or a false-positive annotation.

To complement the annotation-count summaries, the relative class-level signal contributions of the shared putatively annotated features were examined across the evaluated sample volumes (Fig. 2g). LPC, PC, and SM accounted for substantial proportions of the shared-feature signal across the conditions, but the relative distributions were not uniform. The 50 μ L condition showed a higher relative contribution from phosphoinositol (PI)-associated features and a lower contribution from tri- and diacylglyceride (TG/DG)-associated features than several lower-volume conditions. These observations indicate that plasma input volume influenced the relative class-level signal distribution, even when the analysis was restricted to features shared across conditions.

Effects of mobile phase buffer concentration on feature coverage and nanoLC performance

In addition to sample preparation, consistent and stable measurement conditions are important for extended nanoLC-MS acquisition. A common challenge in nanoLC-MS workflows is the rapid increase in system back pressure during extended operation. Unlike conventional analytical flow LC, nanoLC systems employ columns with very narrow inner diameters (typically 75 to 150 μ m)^{22–24}, rendering them more sensitive to blockages and fouling. Consequently, small changes in column permeability translate into large pressure shifts.

NanoLC performance differed among the tested NH_4HCO_2 concentrations (SI Figure S4). Representative chromatograms for each condition are shown in Figure S5. Compared with the 2 and 5 mM conditions, the 10 mM condition showed a higher baseline, greater back pressure, and broader peak profiles for some earlier eluting signals. These chromatographic differences were evaluated qualitatively and were not independently quantified as ion suppression.

Pressure changes were monitored over 12 h of continuous acquisition (SI Figure S4). Between the first and sixteenth injections, the 10 mM condition showed increases of 56 bar in pump A and 52 bar in pump B. In comparison, the pressure drift observed with the 2 and 5 mM conditions was no greater than 13 bar. Under the tested conditions, the lower buffer concentrations were therefore associated with reduced pressure drift and more stable baselines relative to 10 mM, without an apparent loss of peak resolution.

Similar to the previous section, the PCA and PLS-DA models containing plasma extracts and blanks are presented as exploratory quality control visualizations (Fig. 3a and SI Figure S6). Buffer-associated observations were instead interpreted descriptively using pressure behavior, detected feature counts, annotation-count summaries, and relative class-level signal contributions.

The class-count summary showed comparatively small differences in the numbers of cholesteryl esters (CE), ceramides (Cer), PI, and TG/DG annotations across the tested buffer conditions, whereas larger descriptive differences were observed for PC/LPC and SM annotations (Fig. 3c). These values represent counts of putatively annotated features assigned to each class and do not indicate class abundance or relative signal intensity. Because no class-level hypothesis testing was performed, it cannot be determined whether these differences represent systematic buffer-associated effects or variation among the evaluated measurements.

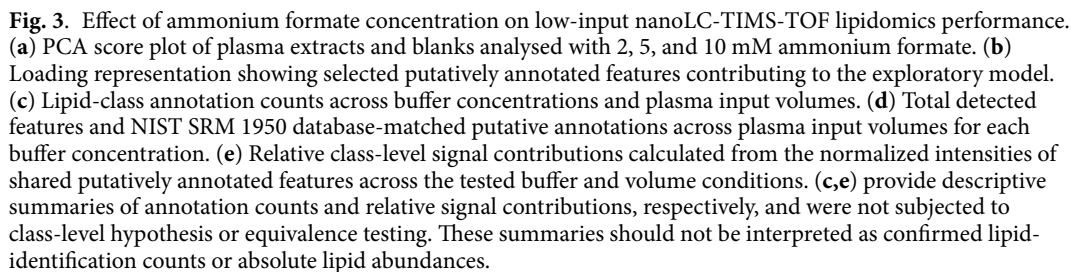
Across the 1, 5, and 10 μ L plasma inputs, LPC, PC, and SM were the most frequently represented classes among the putatively annotated features (Fig. 3c). The 10 mM condition yielded fewer total detected features and fewer NIST[®] SRM[®] 1950 database-matched putative annotations than the 2 and 5 mM conditions (Fig. 3d). The proportions of database-matched features showed less variation than the corresponding absolute counts.

The relative class-level signal contributions of the shared putatively annotated features were evaluated separately from the annotation counts (Fig. 3e). Although the number of TG/DG annotations showed comparatively small differences in Fig. 3c, their relative signal contribution varied among the buffer conditions in Fig. 3e. These findings are not necessarily contradictory because a similar number of annotated features can produce different aggregate signal contributions when their measured intensities vary. Because Fig. 3e presents relative contributions from shared features, the observed TG/DG pattern does not demonstrate the appearance of additional TG/DG species. It also cannot distinguish an increase in TG/DG-associated signal from a proportional reduction in signals assigned to other classes.

Figure S5 and Fig. 3 provide different representations of the data. Figure S5 shows representative overall chromatographic signal patterns, whereas Fig. 3c and e summarize processed annotation counts and relative signal contributions after class aggregation. Consequently, differences among earlier eluting signals visible in the representative chromatograms may not produce corresponding changes in class level annotation counts or relative contributions. The apparent reduction of some earlier-eluting signals was not quantified independently and is therefore interpreted only as a qualitative chromatographic observation.

The 5 mM condition produced the highest detected feature count, with 3,606 features, compared with 2,909 at 10 mM. Further reduction of the ammonium formate concentration to 2 mM did not improve feature detection under the tested conditions. Together with the lower pressure drift observed at 5 mM relative to 10 mM, these supported the selection of 5 mM ammonium formate as a practical operating condition for the present nanoLC-TIMS-MS setup. Because the class level comparisons were descriptive and were not subjected to extensive validation and recovery studies, the results do not establish 5 mM as a generally optimal buffer concentration for lipid coverage.

Mobile phase salt concentration can influence chromatographic behavior, electrospray ionization, adduct formation, and other system-level processes. However, the present study was not designed to distinguish between these mechanisms. The observed differences in pressure stability, baseline behavior, detected feature counts, and class-level signal contributions are therefore described as buffer-associated trends under the tested conditions rather than evidence of a specific mechanism.



Stability at low volumes

To evaluate the stability of low-volume lipid extracts over time in autosampler storage conditions, BPCs were compared for plasma samples extracted with MeOH: MTBE (left, Figure S7) and IPA: H₂O (right, Figure S7) at three time points ($t = 0$ h, 14 h, and 30 h).

At $t = 0$ h, both extraction methods yielded chromatographic profiles containing numerous lipid features across the retention time (RT) window (Figure S7). Differences in extract stability emerged at later time points. After 14 h, approximating an overnight run, MeOH: MTBE extracts exhibited a marked reduction in signal intensity, with only a subset of peaks remaining, indicating substantial degradation or loss of lipid species. In contrast, IPA: H₂O extracts retained greater chromatographic complexity, with only moderate intensity loss relative to the initial profile. After 30 h, roughly corresponding to a weekend run, MeOH: MTBE extracts were nearly depleted of detectable lipid features, whereas IPA: H₂O extracts continued to display multiple well-defined peaks, albeit at reduced abundance.

The rapid loss of lipid signals in MeOH: MTBE extracts may reflect the susceptibility of specific lipid classes to oxidative or hydrolytic degradation in organic-rich environments, whereas the aqueous component of IPA: H₂O may confer greater stability. An alternative explanation involves enzymatic activity, as IPA: H₂O extractions required prolonged incubation to ensure complete precipitation of proteins and even nucleic acids. The superior stability of IPA: H₂O extracts suggests that this method is preferable for studies involving extended storage or delayed analysis.

Ion mobility and compound annotation

The nanoLC-TIMS-TOF workflow enabled broad positive mode lipid profiling from only 1 μ L of plasma, demonstrating effective low-input performance under the tested conditions. This performance arose from the complementary contributions of both nanoLC and TIMS-TOF. Previous studies have described sensitivity-related advantages of low-flow electrospray, including the formation of smaller droplets and more efficient ion transfer^{25,26}. These properties may be particularly relevant to low-abundance features and neutral lipid classes such as CEs and TGs, which can be challenging to detect using electrospray ionization^{27–29}. Parallel accumulation-serial enhanced fragmentation (PASEF) has also been reported to increase precursor utilization by sampling multiple mobility-separated precursors within each duty cycle^{30,31}. These established principles provide analytical context for the present workflow; however, ionization efficiency, ion-transmission efficiency, and signal-to-noise ratios were not independently quantified in this study.

The orthogonal separation provided by TIMS reduced spectral congestion and enhanced annotation confidence. As illustrated in Fig. 4, plotting collisional cross section (CCS) versus m/z yields well-defined, class-specific trendlines for major lipid categories, such as phospholipids, sphingolipids, and neutral lipids. This separation minimizes spectral overlap that typically complicates MS-based lipidomics, particularly for isobaric and near-isobaric species. PC and SM annotations occupied partly distinguishable regions, although substantial overlap remained. Because positivemode PC and SM annotations can be affected by shared phosphocholine fragments, adduct-related mass relationships, isotope overlap, and incomplete feature deconvolution, acronym, IM differences were treated as supporting descriptors rather than definitive evidence of distinct molecular identities.

The CCS- m/z distribution provided an additional means of examining class-level organization among the putatively annotated features. Several neutral lipid annotations, including TGs and CEs, followed recognizable class-associated trends, while deviations from these trends highlighted features requiring cautious interpretation. Such deviations may reflect lower-confidence assignments, unresolved isomers, adduct-related features, alternative gas-phase conformations, or incomplete feature deconvolution. Accordingly, CCS- m/z relationships were used to support feature-level interpretation and identify potential annotation outliers, rather than to establish unique molecular identities or confirm novel lipid species.

Discussion

The choice of sample preparation and extraction protocols is a critical determinant in ultra-low-volume analysis. Given the restricted availability of sample material, uncomplicated procedures with minimal and simplified clean-up steps are required to maximize extraction efficiency and analytical yield. In the single-phase lipidomics protocols evaluated here, high-speed centrifugation represents the only step that may additionally function as a clean-up procedure. However, higher g -force could cause the precipitation of some lipid species^{32,33}. This can be circumvented by keeping the polarity of extraction solvents low enough to ensure that the lipids remain soluble and by using shorter centrifugation times.

While both methods referenced in this study were also designed for immediate LC-MS analysis, the inclusion of a drying step is also important for both chemical and practical reasons. Drying extracts with a nitrogen stream offers the advantages of easier and safer storage, reduced risk of contamination and oxidation^{34,35}, and minimization of chemical and enzymatic activity³⁵. Prior to analysis, these samples were then reconstituted with 8:29:63 v/v 1-butanol:2-propanol:water solution, as this combination yielded better and sharper peak shapes in nanoflow LC²⁵. Changes in each protocol were done on the same steps to control extraction variables that could affect downstream analyses. Because this study was designed as a qualitative workflow assessment, replicate injections were used to evaluate consistency of low-input feature detection and class-level trends rather than to establish formal quantitative reproducibility. Future targeted or semi-quantitative validation studies would be required to define recovery, linearity, limits of detection, limits of quantification, and quantitative precision for specific lipid species.

As shown in the results, PCs, LPCs, and SMs are the largest sources of variance driving the separation in Fig. 2. These species, being among the most abundant plasma lipids^{36,37}, scale disproportionately with increased plasma extraction volume, thereby exerting greater influence on the principal components.³⁸ This could be due

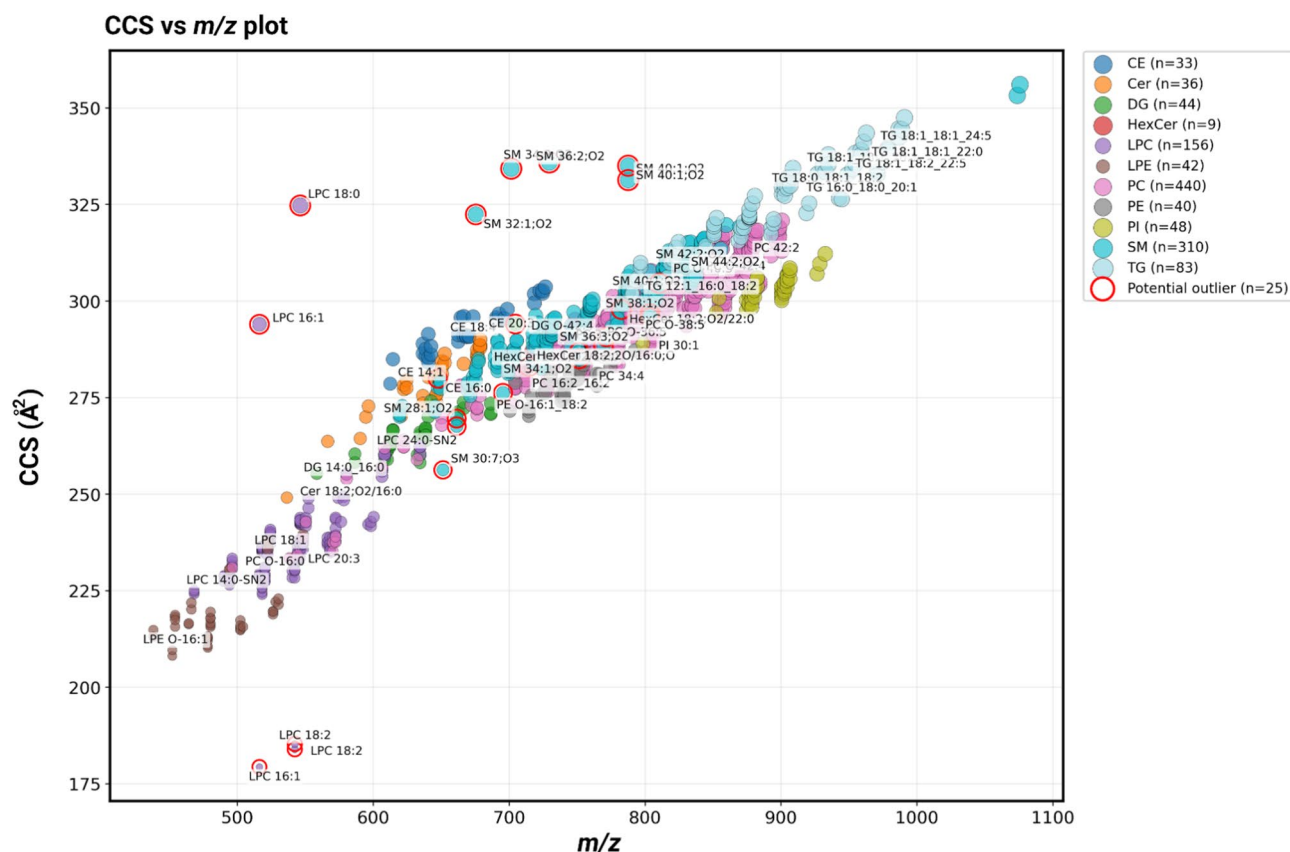


Fig. 4. CCS- m/z distribution of putatively annotated lipid features measured using TIMS. The scatter plot shows collision cross section (CCS, $\text{\AA}^2$) versus m/z , with points coloured by putative lipid class. Red open circles and selected labels indicate illustrative features with atypical positions in CCS- m/z space. Because of the high feature density and resulting marker overlap, the plot is intended as a qualitative overview of class-level organization rather than a systematic assessment of within-class trends or individual outliers. CCS values are presented as complementary descriptors and not as definitive validation of the automated lipid assignments. Labels reproduce the automated candidate annotations generated during data processing; displayed acyl-chain or sn -positional detail was not considered structurally validated or used in the interpretation.

to better extraction efficiency and recovery at higher volumes, which could be biased by lipid class chemistry^{39,40}. Polar and amphiphilic lipids were also noted to stick to untreated surfaces, such as plastic and glass⁴¹. Moreover, as shown by single-cell experiments, adsorption effects or surface losses^{40,42}, including pipetting error increase in every step^{43,44} with the scaled down samples.

PC, LPC, and SM annotations accounted for the largest class-level contributions in the 1 μL extracts from both protocols. Descriptive differences were observed across protocols and input volumes, including a lower SM annotation count in the 1 μL MeOH: MTBE extracts. No formal equivalence testing was performed. Despite the reduced profiling depth, the 1 μL extracts retained the dominant annotated lipid classes at higher input volumes. This similarity was limited to class-level representation and should not be interpreted as equivalence of the complete feature or abundance profiles. By contrast, larger input volumes may accentuate matrix effects and disproportionately weight abundant lipid species, complicating quantitative interpretation and cross-study comparability. In addition to annotation-count comparisons, intensity-based evaluation of the shared putatively identified lipid features showed that the relative class-level signal contribution was broadly maintained across the lower sample-volume conditions. This strengthens the conclusion that low-input nanoLC-TIMS-TOF preserves the major features of the class-level lipid profile. Nevertheless, because the analysis was restricted to commonly detected putative features and summarized at the class level, it should not be interpreted as evidence that the complete lipid feature profile is unchanged.

Meanwhile, the absence of a match to the compiled NIST[®] SRM[®] 1950 reference list does not by itself indicate an incorrect annotation. Unmatched entries may arise from differences in dataset composition, annotation resolution, nomenclature, available adduct or CCS information, or failure to satisfy the matching criteria applied here. The aggregate relationships between total annotations and reference list matches across plasma input volumes and buffer conditions are shown in Figs. 2f and 3d, respectively. Although the total annotation count decreased more strongly than the number of referencelist matches at lower plasma input volumes, the identities and class distributions of the annotations lost with decreasing input volume were not separately evaluated. Similarly, the matched and unmatched annotations were not examined individually across buffer

conditions. It therefore remains unclear whether the observed changes preferentially affected the unmatched fraction. Abundance, detection robustness, and annotation confidence were not systematically compared between the matched and unmatched annotations. Consequently, the analytical basis of these differences cannot be determined from the present analysis.

Some putative lipid annotations, particularly LPC and lysophosphoethanolamine (LPE) assignments, exhibited RT behavior inconsistent with the expected elution order in reversed phase chromatography. RT and CCS provide complementary but non-interchangeable information; therefore, agreement with a predicted CCS value alone is insufficient to resolve an implausible chromatographic assignment or establish a distinct molecular identity. Similarly, multiple detected features assigned the same lipid sum composition should not be interpreted as definitive evidence of chromatographically or IM-resolved isomers. Such multiplicity may arise from adducts, isotopes, in-source fragments, alternative candidate annotations, partially resolved chromatographic signals, or multiple gas-phase conformations of the same analyte. These assignments should be regarded as lower-confidence putative annotations pending confirmation with authentic standards or additional diagnostic structural evidence.

The automated annotation workflow was not designed to provide a fully RT-curated lipid-identification library. In reverse phase lipidomics, retention behavior is expected to follow class-associated trends related to headgroup polarity, acyl-carbon number, and degree of unsaturation, and assignments that deviate from these trends require cautious interpretation. This limitation is particularly relevant to positive-ion MS/MS data, which may support lipid-class or sum-composition assignments without providing sufficient diagnostic fragments to confirm exact fatty-acyl composition or individual isomeric structures. Negative-ionization performance may differ because ion formation, adduct behavior, matrix effects, extraction selectivity, and the influence of mobile-phase composition can be ionization-mode dependent. Accordingly, the observed effects of plasma input volume, extraction protocol, and ammonium formate concentration should not be extrapolated to negative-ion lipid profiling without direct experimental evaluation.

Because the two extraction protocols produced broadly similar class-level feature summaries under the tested conditions, practical considerations such as total experiment time and ease of execution were also considered. In this respect, MeOH: MTBE offers a throughput advantage, as its shorter workflow enables more rapid sample processing compared with IPA: H₂O. The latter requires a two hour incubation step to ensure complete extraction and protein precipitation, but this extended protocol inevitably reduces throughput. These differences, however, were not reflected in the nanoLC-MS results. Thus, depending on the specific application and cohort or batch size, one extraction protocol may provide greater utility than the other. For this study, successive analyses were done using the MeOH: MTBE method.

Meanwhile, pressure management remains a major bottleneck in the adaptation of nanoLC for lipidomics. Nanoflow analyses inherently operate at ultrahigh pressures due to the use of columns with extremely narrow internal diameters. When combined with the high-viscosity solvents and salt-containing mobile phases commonly employed in lipidomics, this results in accelerated back-pressure buildup. Several factors contribute to this: first is the use of high buffer concentrations (e.g., ≥ 10 mM ammonium formate or acetate) that can promote salt precipitation⁴⁵ in capillaries or within emitter regions during gradient elution, and particularly under nanoliter flowrate conditions. Second, plasma- and tissue-derived extracts often contain residual matrix components (e.g., proteins, salts) that cannot be removed completely during sample cleanup, gradually accumulating on the stationary phase and restricting flow. Third and last, carryover of particulates from sample vials and autosampler injectors, especially with small injection volumes (< 2 μ L), can further accelerate or exacerbate clogging.

Rapid increases in back pressure have practical consequences for both chromatographic reproducibility and instrument uptime. Sudden or abrupt pressure changes increase mechanical stress on pump seals and valves, and may shorten column lifetime⁴⁶. More importantly, the associated deterioration in RT stability and peak shape directly compromises accuracy in lipidomics studies. In severe cases, abrupt pressure spikes can lead to system shutdowns, interrupting long batch sequences.

Some mitigation strategies include lowering buffer concentrations (e.g., 2 to 5 mM ammonium salts) to minimize precipitation, improving sample cleanup or filtration where applicable, and performing column maintenance such as backflushing or guard column replacement. However, such maintenance is generally impossible for nanoLC columns, making buffer composition and sample preparation hygiene the most effective and accessible means of preventing blockages.

Another limitation of nanoLC analysis is the extended chromatographic and acquisition times often required. In lipidomics, high chromatographic resolution is essential for improving the separation of isomeric species; however, prolonged run times may compromise the stability of ultra-low-volume samples. During stability assessment of the 1 μ L plasma extracts, solvent-dependent effects were pronounced. MeOH: MTBE extracts showed a rapid loss of detectable features, whereas IPA: H₂O extracts maintained signal complexity over time. Differences between the MeOH: MTBE and IPA: H₂O extracts were visually apparent in the representative BPCs. These chromatograms provide a qualitative comparison of signal complexity over the evaluated period; formal repeatability or statistical assessment was not performed. Under the evaluated conditions, the IPA: H₂O extracts showed greater qualitative retention of detectable signal complexity over time than the MeOH: MTBE extracts. This also raises an important consideration for quality control, as reduced sample volumes may limit the feasibility of reinjecting controls in long analytical sequences, potentially compromising the robustness of data validation. For high-throughput applications, splitting extracts and aliquoting pooled QCs can mitigate limitations associated with the stability issues. Additionally, utilizing the built-in re-dissolving function in some LC models provides an automated way to maintain sample integrity during extended batch runs.

Chromatographic separation of isomeric species is not always guaranteed, particularly in untargeted lipidomics workflows.

Structural elucidation in this workflow is limited by the acquisition and fragmentation strategy and is independent of plasma input volume, extraction protocol, or buffer concentration. PASEF was used as a DDA-based MS/MS acquisition strategy, but it does not provide the electron-based fragmentation required for confident double-bond localization. Therefore, lipid annotations, particularly for classes dominated by headgroup fragments in positive mode, should be interpreted at the class, sum-composition, or putative molecular-species level according to the available spectral evidence. acronym IM-derived CCS values provide an additional descriptor for visualizing lipid-feature organization in *CCS-m/z* space, but they were not used as comprehensive validation criteria for all automated assignments.

Although reference CCS values from authentic standards remain limited, the observed *CCS-m/z* distributions provided supporting information about class-level feature organization. Predicted CCS agreement and class-associated mobility trends were not treated as definitive validation of individual automated assignments. The annotations should therefore be regarded as putative unless supported by authentic standards or additional diagnostic structural evidence.

Conclusions

Both single-phase extraction protocols enabled detection of putatively annotated features from the dominant positive ion lipid classes under the tested conditions. Notably, IPA: H₂O extracts demonstrated stable signal complexity over time, an essential advantage for large-scale studies. Both extraction strategies are scalable with respect to input volume, though higher centrifugal forces are particularly critical at low volumes to prevent clogging of nanoLC columns.

Among the conditions tested, 50 μ L of plasma extraction volumes yielded the broadest feature coverage, underscoring input volume as a stronger determinant of profiling depth than solvent polarity. Notably, 1 μ L extraction volumes still recovered over 80% of the lipid features detected at high volumes (50 μ L), achieving this with 98% less sample material. This feature-level overlap did not indicate equivalence of class-level signal distributions or complete lipid profiles across input volumes. These findings support the feasibility of positive ion lipid profiling from microliter-scale plasma samples using the evaluated nanoLC-TIMS-MS workflow.

Buffer concentration was associated with differences in nanoLC pressure stability and detected feature counts under the evaluated conditions. The 5 mM ammonium formate condition produced the highest detected feature count and lower pressure drift than the 10 mM condition and was therefore selected as a practical operating condition for this setup. Class-level annotation counts and relative signal contributions varied descriptively among the buffer conditions, but no class-level hypothesis testing was performed. These findings should therefore not be interpreted as establishing a generally optimal buffer concentration for lipid coverage.

Overall, these results establish practical guidelines for balancing extraction efficiency, input volume, and buffer composition in nanoLC-MS lipidomics. The demonstrated compatibility of 1 μ L plasma extractions with high-sensitivity platforms such as nanoLC-TIMS-MS/MS opens opportunities for minimally invasive sampling strategies. Applications may include pediatric studies, longitudinal monitoring, and microsampling workflows where sample volumes are inherently limited. Continued optimization of extraction efficiency, nanoLC stability, and data processing strategies will further expand the scope of lipidomics in precision medicine and translational research.

Methods

Reagents and chemicals

LC-MS grade (LiChrosolv[®]) water, 2-propanol (IPA), methanol (MeOH), acetonitrile, HPLC-grade methyl *tert*-butyl ether (MTBE), 1-butanol, and ammonium formate solution (LiChropur[™] 10 M in H₂O) were purchased from Supelco[®] (Merck KGaA, Darmstadt, Germany). LC-MS grade formic acid (Optima[™]) was supplied by Fisher Scientific GmbH (Schwerte, Germany). EquiSPLASH[™] LIPIDOMIX[®] deuterated lipid internal standard was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA).

Human plasma samples

Method development and optimization were conducted using Standard Reference Material (SRM[®]) 1950 developed by the National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA).

2-Propanol/water method

This method was adapted and modified from the protocol for lipid analysis in human plasma and serum²⁰ developed by the National Phenome Center at Imperial College London, London, United Kingdom. Volume scaling of plasma material, including the extraction solvents is detailed in Table S1.

NIST[®] SRM[®] 1950 samples were thawed on ice for 30.0 min and shaken for 1.0 min with a vortex shaker. Samples were aliquoted to 1.5 mL clear microcentrifuge tubes according to the volumes specified in Table S1. LC-MS grade H₂O was added to each aliquot according to Table S1. Using a shaker (Eppendorf ThermoMixer[®] Compact, Eppendorf, Hamburg, Germany), samples were mixed at 1400 rpm for 5.0 min at 8 °C and centrifuged for 10.0 min at 4.0 °C and 18,620 $\times$ g.

After centrifugation, the corresponding volumes for 2-propanol and 1:50 dilution of internal standard/methanol were added to each aliquot (see Table S1). Samples are once again shaken at 1400 rpm for 120 min at 8 °C. This was followed by centrifugation for 15.0 min at 4.0 °C and 18,620 $\times$ g. Specific volumes of supernatant (see Table S1) were collected and transferred to 1.5 mL microcentrifuge tubes. Extracts were dried under nitrogen gas and stored in -80.0 °C until use.

Methanol/MTBE method

This method was adapted from Villaseñor et al., 2014¹⁹. Volume scaling of plasma including the extraction solvents is detailed in Table S2. Like the first method, plasma or whole blood samples were thawed on dry ice for 30.0 min. Samples were then vortexed for 2.0 min. Aliquots of the samples were prepared in 1.5 mL microcentrifuge tubes according to Table S2. Cold (around -20 °C) methanol was added, followed by an equivalent amount of room temperature MTBE. Samples were then spiked with the deuterated internal standard mix according to Table S2. Samples were shaken for 30.0 min at room temperature at 1400 rpm. This was followed by centrifugation for 15.0 min at 15.0 °C and 18,620 × *g*. Specific volumes of supernatant were collected (see Table S2) and transferred to microcentrifuge tubes. Extracts were dried under nitrogen gas and stored in -80.0 °C until use.

NanoLC-MS/MS analysis

Dried SRM[®] 1950 extracts were reconstituted with the appropriate volume (see Tables S1 and S2) of 8:23:69% v/v 1-butanol:2-propanol: H₂O solvent mixture³⁰ and transferred to 1.0 mL total recovery LC-MS vials (Waters[™] Corporation, Milford, Massachusetts, USA). Samples were then analyzed in the positive ion mode using a Bruker nanoElute 2 coupled to Bruker timsTOF HT with a CaptiveSpray ion source (Bruker Daltonics, Bremen, Germany). For chromatographic separation, 1.0 µL of the extract was injected into a generation 3 Aurora Ultimate[™] C18 (25 cm × 75 µm ID, 1.7 µm) UHPLC column (IonOpticks, Melbourne, Victoria, Australia). The autosampler temperature was set to 7.0 °C.

Separation was performed using a linear multistep gradient with two eluents: eluent A (40:60 v/v water: acetonitrile) and eluent B (90:10 v/v 2-propanol: acetonitrile). Both eluents were supplemented with 0.1% formic acid and 5.0 mM ammonium formate (NH₄HCO₂). The column temperature was maintained at 60.0 °C throughout the run. The gradient began with 10% B at a flow rate of 0.35 µL/min, held for 2.0 min. From 2.0 to 7.0 min, B was increased to 40%, then ramped to 60% from 7.0 to 12.0 min. It was further increased to 70% over the next 5.0 min. At 17.0 min, B reached 70%, and then gradually increased to 99% over 10.0 min. This composition was held isocratically for 5.0 min before returning to 10% B for re-equilibration. The nanoLC gradient and MS acquisition window were both 30.0 min. The full method duration was 45.71 min, with the additional time allocated to sample loading, column washing, and re-equilibration before the next injection.

To study the effects of buffer concentration, both A and B solvents were prepared with 2.0 mM and 10.0 mM NH₄HCO₂, while maintaining 0.1% formic acid. In all setups, the gradient, TIMS-MS, and MS/MS parameters were kept the same. All samples were injected in triplicate.

MS analysis was conducted with the following instrument settings: 1.5 kV capillary voltage, 3.0 L/min desolvation gas flowrate, and 180 °C source temperature. Ion mobility measurements were performed through a TIMS device, with 1/*K*₀ range of 0.5 to 1.8 Vs/cm², 100 ms TIMS ramp and accumulation time at 100% duty cycle, and ramp rate of 9.43 Hz. Ion Charge Control (ICC) was set to a target value of 7.5 M. Sodium formate (1.0 mM) and ESI Tune Mix (Agilent Technologies, Inc. California, USA) were infused for *m/z* (high precision calibration mode) and mobility (linear mode) calibration, respectively.

TIMS-MS/MS was scanned using data dependent acquisition-parallel accumulation serial fragmentation (DDA-PASEF) mode with one MS and two PASEF ramps, targeting the *m/z* range from 100 to 1500. The maximum charge state was set to +1. Precursors must meet a target intensity of 4000 and active exclusion was enabled. Fragmentation was done with 30.0 eV collision energy.

Data analysis

Raw data files were processed using Bruker Compass DataAnalysis 6.1 and MetaboScape[®] (version 2024b). MetaboScape[®] enabled processing of native (unconverted) IM-MS data for batch analyses, while Compass DataAnalysis was used to manually recalibrate each file before import into MetaboScape[®]. For the extraction protocol comparison, 36 files were acquired. An additional 36 files were generated in a separate experiment to evaluate the effects of mobile phase buffer concentration. Each dataset set included triplicate sample injections along with corresponding blanks. A single feature table was set up by grouping blanks, IPA: H₂O, and MeOH: MTBE samples, separately.

The parameters used for MetaboScape[®] T-Rex 4D positive mode analysis were as follows: four minimum number of features for extraction, four minimum number of features for results, 300 *m/z* minimum, 1500 *m/z* maximum, 1800.0 s maximum RT, 6.0s minimum RT, 7500 seed intensity threshold, 250 minimum cluster size for targeted extraction, 0.05 Å² mobility delta, 0.015s RT delta, with enabled recursive feature extraction. Primary ion was set to [M + H]⁺, with [M + H - H₂O]⁺ as common ion, and [M + Na]⁺ and [M + NH₄]⁺ as seed ions. Under expert configuration, the previous *m/z* and mobility calibrations conducted via Compass DataAnalysis were applied. This step ensures that MetaboScape does not overwrite the user-defined calibration, as the dedicated calibration segment was removed from the runtime to improve reproducibility.

All annotation methods (rule-based lipid species, spectral library, and Smart Formula) were conducted using MetaboScape[®]. A custom target list annotation was applied for the annotation and detection of the EquiSPLASH[™] LIPIDOMIX[™] internal standards. Blank signals were subtracted from the extracted features before normalization according to the internal standards detected. Following automated annotation, features without MS/MS and assignments with a deviation greater than 3.0% from the rule-based predicted CCS value were excluded. The CCS threshold was applied as an exclusionary quality control filter to remove assignments showing substantial disagreement with the predicted value. Agreement within this tolerance was treated as supporting evidence rather than confirmation of molecular identity, because prediction performance may vary among lipid subclasses according to the composition and representativeness of the underlying training data. Systematic evaluation of CCS behavior across homologous lipid series was not performed.

RT plausibility was assessed qualitatively using the available internal-standard elution windows and expected reverse phase elution behavior. Particular attention was given to LPC and LPE annotations eluting within the

RT region of DGs and to glycosphingolipid annotations showing an elution order inconsistent with their degree of glycosylation. Systematic homologous-series RT mapping was not performed. RT information was therefore interpreted as qualitative supporting evidence rather than definitive confirmation of individual assignments. Features showing implausible RT behavior were regarded as lower-confidence putative annotations. RT interpretation also considered nanoLC performance because of pressure instability, partial flow restriction, changes in emitter or column performance, and gradient fluctuations can affect chromatographic behavior.

MS/MS, RT behavior, and predicted CCS matching were treated as complementary but non-interchangeable sources of annotation evidence. In positive ion mode, MS/MS spectra may support lipid-class or sum-composition assignments through headgroup- and adduct-related fragments without providing sufficient diagnostic evidence to confirm exact fatty-acyl composition. Neither the presence of MS/MS nor agreement within the predicted CCS tolerance was considered sufficient by itself to establish a unique molecular identity or override implausible RT behavior. The resulting assignments were therefore retained as putative annotations, and confirmation of exact molecular identities would require authentic standards or additional diagnostic structural evidence.

The final feature table was exported as a .csv file and re-formatted for statistical analysis and visualization via MetaboAnalyst 6.0⁴⁷. All plots were log-transformed and Pareto-scaled within MetaboAnalyst. Blanks were included in the MVA to evaluate background contribution, which is particularly critical in nanoLC-MS due to the high sensitivity of the system and increased susceptibility to carryover, column bleed, and solvent-derived contaminants that can contribute significantly to detected signals. When blanks were included in the PCA or PLS-DA visualizations, the resulting models were interpreted as exploratory QC analyses rather than definitive models of sample-group discrimination. To avoid blank-driven interpretation of the lipid profiles, discriminatory features were restricted to sample groups, and blank-associated features were not used to support extraction-, volume-, or buffer-dependent conclusions.

Ion mobility plots (CCS versus m/z) were generated with Python 3.11. CCS values were used to support visualization of lipid-feature distributions in the CCS- m/z space and to provide additional annotation context. No post hoc CCS-trend-based rejection was applied across all lipid subclasses or sum-composition series.

Matching to known NIST® SRM® 1950 lipids

For comparison with previously reported NIST® SRM® 1950 lipids, a reference list was manually compiled from the Mandal et al.⁴⁸ and the NIDDK/NIST Human plasma sample project⁴⁹. The nanoLC-TIMS-TOF annotations and the reference list were normalized to a shared LIPID MAPS nomenclature using LipidLynxX⁵⁰. Nomenclature normalization was performed solely to enable comparison at a common reporting level. Duplicated records were not assumed to represent the same molecular entity solely because they shared the same normalized name, sum composition, RT, or CCS. This is to avoid removing unresolved structural isomers, including double-bond positional isomers that may share these properties. Entries were merged only when the available evidence supported with high confidence that they represented the same detected feature.

Matching was performed using a Python-based parsing script. Harmonized lipid names and precursor m/z agreement within the tolerance of $\Delta m/z < 0.005$ were applied as the initial matching gate. Adduct information was considered in both datasets. Where CCS values were available for both entries, agreement within a relative deviation of less than 3.0% was required as corroborating evidence. Entries satisfying the applicable criteria were assigned to a single pass category, no tiered annotation-confidence was applied.

MS/MS spectra was not compared during the cross-dataset matching step because the NIST® SRM® 1950 reference list does not provide corresponding spectra. MS/MS evidence had instead been evaluated upstream during the annotation of the nanoLC-TIMS-TOF data in MetaboScape® 2024b. RT was likewise not used as a cross-dataset matching criterion because corresponding RT information was unavailable for the reference list. The matching procedure therefore only assessed concordance between the harmonized annotations and did not constitute independent spectral validation, confirmation of molecular identity, or resolution of structural isomers.

Data availability

All data tables are available from Zenodo ([https://zenodo.org/records/18342204] (https://zenodo.org/records/18342204)). Raw data files are available from MaSSIVE (accession: MSV000100612).

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Author contributions

Conceptualization – MW, AB; Original draft writing – KR; Experimental Design – KR, MW, LA, AB; Data curation and investigation – KR; Technical support – LA, AB; Writing (review and editing) – KR, LA, AB, MW; Supervision – AB, MW; Funding and resources – MW All authors read, reviewed, and approved the manuscript.

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Declarations

Competing interests

Authors LA and AB are employed by Bruker Corporation or one of its subsidiaries (“Bruker”). Bruker produces and sells the instruments and software used in this study. All other authors declare no competing financial interests.

Ethics approval

The used reference plasma NIST 1950 is a commercially available reference standard commonly used in metabolomics and lipidomics. It can be obtained from different vendors such as NIST directly or Merck/Sigma-Aldrich. The use of this plasma does not require ethical approval.

Additional information

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