Bridging untargeted and targeted 4D-Lipidomics – the dda-PASEF to prm-PASEF workflow

Application of a streamlined, software-enabled workflow translating discovery results to achieve reliable and accurate lipid quantitation.

Abstract

The establishment of robust mass spectrometry (MS)-based lipid quantitation strategies represents a pivotal step towards the integration of lipidomics into routine clinical practice. Accordingly, we created a bridge that allows lipidomics researchers to seamlessly translate discovery into targeted quantitative lipid analysis by combination of Bruker software solutions.

For demonstration, a hydrophilic interaction liquid chromatography (HILIC)-based 4D-Lipidomics™ approach was employed to resolve critical type-II overlaps using high ramp times and achieve trapped ion mobility spectrometry (TIMS)-enhanced quantitation accuracy. Additionally, the discovery-to-targeted quantitation lipidomics pipeline based on parallel accumulation serial fragmentation (PASEF®) in data-dependent acquisition (dda-PASEF®) and parallel reaction monitoring (prm-PASEF®) modes resulted in advanced reliability in (molecular) species quantitation by facilitating lipid MS/MS transition monitoring, which shows potential for clinical lipidomics.

Keywords:
4D-Lipidomics, prm-PASEF®, streamlined workflow, lipid quantitation, HILIC-TIMS-MS/MS

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Introduction

Lipids exhibit numerous diverse functions within the cell, from membrane stabilization to cell signaling, with perturbations causing tremendous effects [1]. Since its introduction, mass spectrometry (MS)-based lipidomics has gained in popularity owing to improvements in measurement performance, data processing, and workflow standardization. MS-based lipidomics is consequently emerging in clinical applications [2,3], where the establishment of individual lipid biomarkers represents a cutting-edge area of research [4].

The accurate quantitation of lipids in complex biological samples is a cornerstone of clinical applications of lipidomics and can be achieved with the use of isotopically-labelled internal standards (IS) as lipid specific calibrators. However, this approach is challenging because of both the vast number of distinct lipid species (Figure 1) and the limited availability of commercial standards. To overcome these challenges, hydrophilic interaction liquid chromatography (HILIC) is a popular solution. In HILIC, lipids elute based on their class-dependent polarity, enhancing selectivity and reducing matrix effects, while preserving class-specific co-ionization and enabling the use of a single internal standard (IS) per lipid class [5]. Still, HILIC is less able to resolve isobaric interferences than reversed-phase chromatography (RPC) which can result in overestimated lipid concentrations. In particular, the type-II overlap caused by the natural isotopic distribution from lipid species with an additional double bond must be considered (Figure 2) [6].

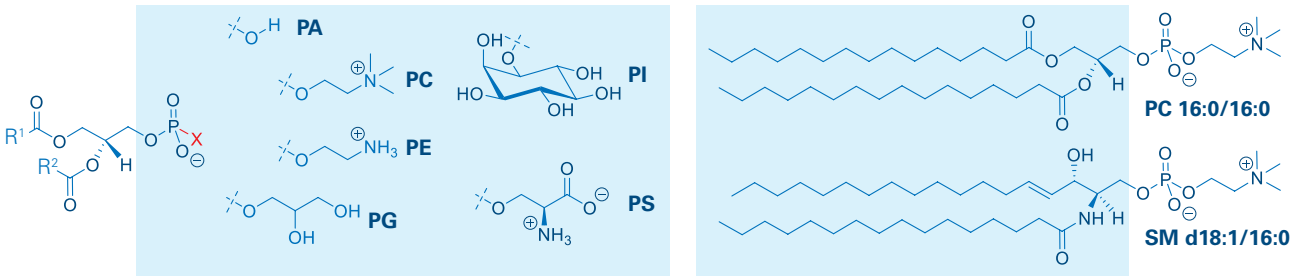

Figure 1.
Schematic overview of the glycerophospholipid classes phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), as well as comparison of the phospholipid classes sphingomyelin (SM) and phosphatidylcholine (PC) based on selected lipid species.

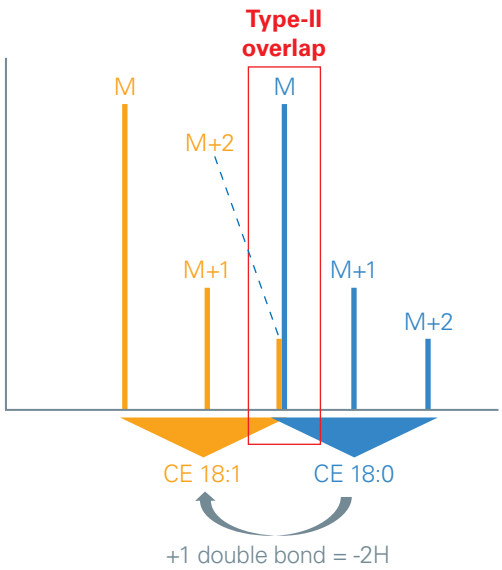

Figure 2. Schematic illustration of a type-II overlap.

Here, trapped ion mobility spectrometry (TIMS) provides further opportunity for lipid species resolution, benefitting quantitative lipidomics. Furthermore, when utilizing parallel accumulation serial fragmentation (PASEF®) data acquisition, MS/MS spectra obtained from lipids are also mobility-resolved, leading to cleaner fragmentation spectra for enhanced lipid identification. PASEF data acquisition can be deployed in various forms supporting untargeted (DDA or DIA PASEF) and targeted (via prm-PASEF) workflows based on the nature of the desired analysis [7]. However, especially in the case of clinically translational discovery lipidomics, practitioners are likely to utilize both approaches: untargeted to drive lipidomics discovery, and targeted quantitative lipidomics to perform study validation and enable reliable and accurate phospholipid quantitation in downstream populations. Here we introduce a seamless software-enabled workflow for traversing the discovery-to-targeted quantitation lipidomics pipeline using a HILIC-based 4D-Lipidomics approach. The study aims to demonstrate the usage of the enhanced selectivity offered by TIMS for accurate lipid quantitation and the potential of prm-PASEF to reliably quantify lipids at the species or molecular species level in large batches of samples.

Methods

For proof-of-principle, NIST SRM 1950 human plasma and SPLASH LIPIDOMIX IS mixture were obtained from Sigma-Aldrich (Steinheim, Germany). Plasma extraction was performed based on Matyash *et al.* [8]. Briefly, 5 µL plasma (0.25 µL plasma equivalent on column) as well as 1 µL SPLASH LIPIDOMIX IS mixture were mixed, extracted, and reconstituted in 100 µL IPA/ACN/H_2O (72.75/24.25/3, v/v/v) owing to the high solubility of phospholipids in this mixture [9].

HILIC-based 4D-Lipidomics experiments (Table 1 and 2) were performed on a timsTOF fleX mass spectrometer (Bruker) hyphenated to an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Dreieich, Germany). For lipid class separation, an iHILIC® Fusion(+) column (HILICON AB, Umeå, Sweden) was utilized. Screening experiments of plasma phospholipids were conducted in dda-PASEF mode, while prm-PASEF was used for reliable MS quantitation on species or molecular species level.

MS quantitation was performed on MS^1 level based on a single-point internal calibration with one IS per lipid class. To compensate shifts in concentration resulting from the species-specific MS response, a type-I isotopic pattern correction during data processing was performed. Furthermore, type-II correction of isotopomer-based overlaps was conducted either in a conventional way during data processing or using TIMS during acquisition.

MetaboScape® 2024b (Bruker) was utilized to evaluate lipid screening experiments by dda-PASEF. Data was processed, calibrated, and lipid annotation was performed using the implemented rule-based lipid annotation as well as predefined target lists for the SPLASH LIPIDOMIX internal standards.

TASQ® 2024b (Bruker) was utilized to generate and evaluate prm-PASEF methods for high-confidence lipid quantitation. Annotated lipids including MS2 transitions were transferred from MetaboScape.

Table 1. MS acquisition parameters

MS	timsTOF fleX	
Source	Apollo II ESI source	
Ionization	Negative ion mode	
Acquisition mode	TIMS-MS/MS in prm-PASEF mode	
	Ramp time	500 ms
	Mobility range	0.80 – 1.65 $1/K_0$
dda-PASEF	2 PASEF scans per cycle (1.52 s total cycle time)	
	Collision energy	40 eV
	Isolation width	1.80 *m/z*
	Target intensity	4000
	Intensity threshold	100
	Active exclusion	0.2 min
prm-PASEF	Target list based on dda-PASEF sceening	
	Collision energy	40 eV
	Isolation width	1.80 *m/z*
	Retention time window	30 s
	Mobility window	0.03 $1/K_0$
	Cycle time	1.5 s
Calibration	Automatic internal mass calibration using sodium formate	
	Automatic internal mobility calibration using Agilent Tunemix	

Table 2. LC parameters

LC	UltiMate 3000 UHPLC system		
Column	iHILIC® Fusion(+) column (20 x 2.1 mm; 5.0 µm) (HILICON AB, Umeå, Sweden)		
Mobile phase	**A**: Ammonium formate (35 mM; pH 3.5; 5% ACN) **B**: Acetonitrile		
18 min Gradient	**Time [min]**	**Flow [mL/min]**	**%B**
	0	0.3	97
	0.2	0.3	97
	8.2	0.3	75
	8.5	0.3	60
	11.5	0.3	60
	12	0.3	97
	18	0.3	97
Injection volume	5 µL		
Column oven temp.	40°C		

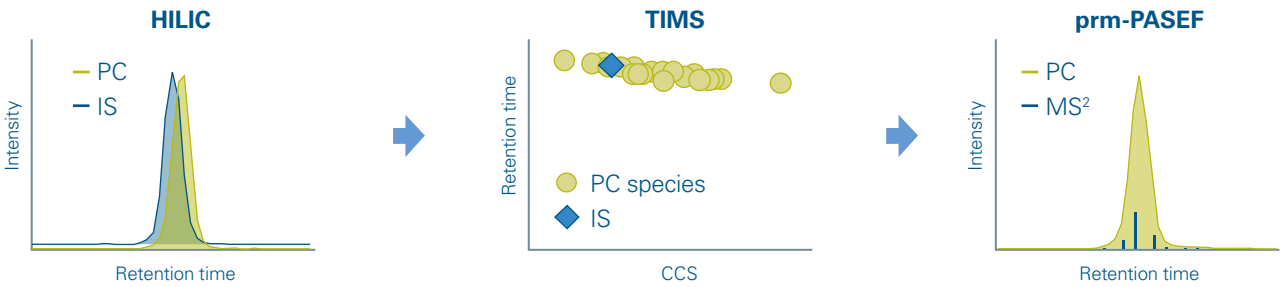

Figure 3. Schematic overview of the utilized HILIC-TIMS-MS/MS workflow for targeted and quantitative 4D-Lipidomics.
With this setup, a coelution of the IS and the whole lipid class is achieved by HILIC, while TIMS serves for additional selectivity on species level and prm-PASEF for reproducible and robust fragmentation patterns in targeted analysis.

Results

HILIC-TIMS-MS/MS in prm-PASEF mode for lipidomics

Recently, the analytical benefits of HILIC-TIMS-MS/MS in lipidome characterization were discussed (Figure 3) [7]. Lipid class-based chromatographic separation is well paired with TIMS, increasing peak capacity and selectivity (Figure 4A). Moreover, the HILIC-based 4D-Lipidomics approach advantages lipid quantitation owing to the coelution of lipid class-specific IS with the entire lipid class. This approach minimizes matrix effect discrepancies that can affect MS quantitation results [10].

The PASEF mode comparison study also emphasized the advantages of prm-PASEF for multiplexed fragmentation in lipidomics, including speed, reproducibility, and sensitivity in generating clean MS/MS spectra (Figure 4B) [7]. Here, the mobility dimension provides a more precise definition of the precursor based on time/mobility windows than conventional targeted LC-MS/MS. These windows can be tightly defined around each target, striking a balance between achieving high TIMS resolution at high speed. Moreover, the PASEF approach utilizes mobility-based ion sorting which allows for greater MS/MS coverage per cycle than conventional MS/MS.

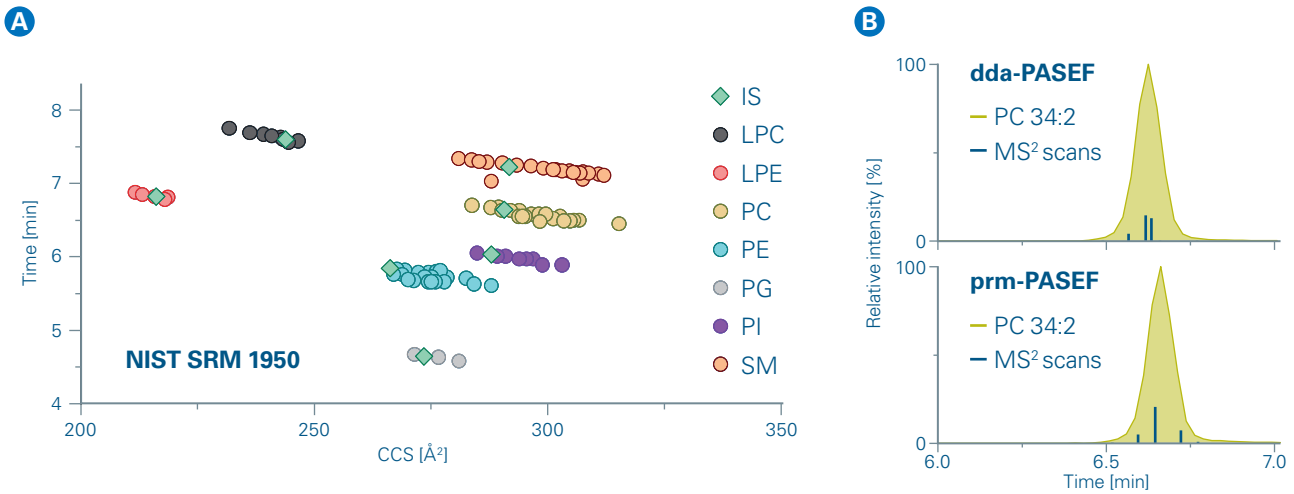

Figure 4.
(A) Overview of the identified NIST SRM 1950 plasma phospholipids using HILIC-TIMS-PASEF. (B) MS² scheduling of dda-PASEF vs. prm-PASEF, exemplified for PC 34:2.

Workflow for streamlined translation from discovery to targeted analysis

The dda-PASEF-to-prm-PASEF workflow joins MetaboScape (untargeted analysis) with TASQ (quantitative evaluation) software for a seamless discovery-to-targeted quantitation workflow (Figure 5). The workflow includes a strategy for user-friendly implementation of lipid MS/MS transitions to enhance the reliability in lipid species quantitation based on rule-based lipid annotations, and all data-associated processing steps.

1) Discovery workflow for lipid screening

In a first step, HILIC-TIMS-MS/MS was applied in dda-PASEF mode for phospholipid screening, serving as basis for targeted acquisition. The multidimensional dataset was used in the exploratory MetaboScape software to perform high-confidence annotations based on the implemented rule-based lipid annotation tool, resulting in deep lipidome coverage. This tool considers several parameters, including accurate mass, isotope pattern (mSigma), CCS, and fragmentation behavior. A total of 87 phospholipid species were annotated in negative ionization mode from seven phospholipid classes (Figure 6A: survey plot). The negative ionization mode benefits from the formation of diagnostic fragment ions for species-specific lipid annotation. These transitions are highlighted by the rule-based lipid annotation (Figure 6B: MS/MS plot) and may be used to monitor reliability in quantitation on species or molecular species level.

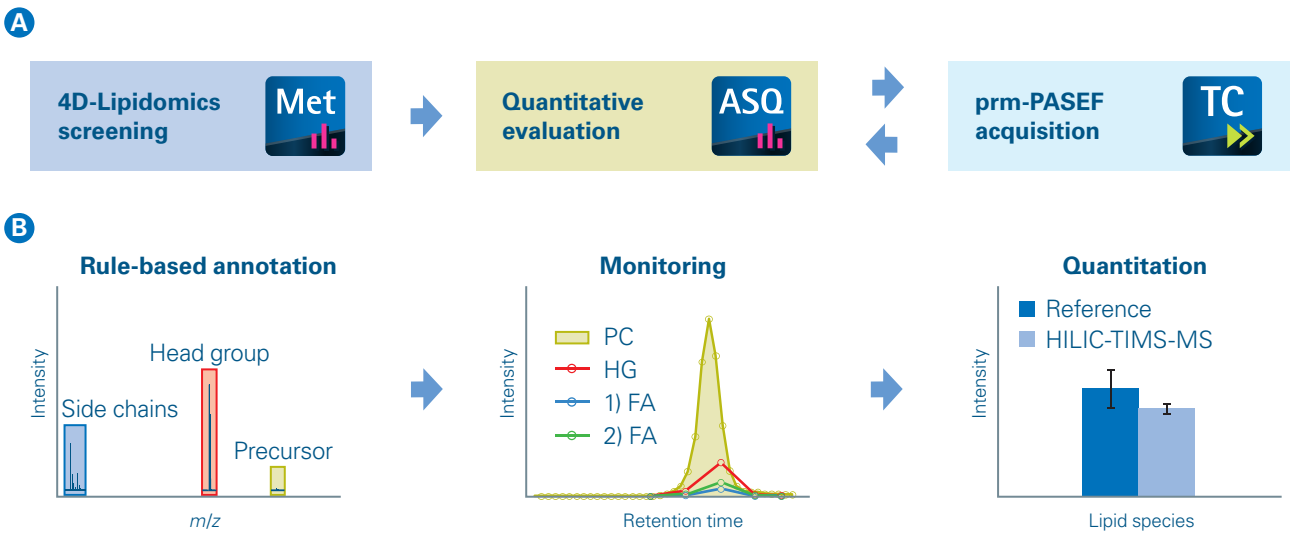

Figure 5. Streamlined workflow for quantitative lipidomics.
Combination of MetaboScape and TASQ software solutions as well as timsControl for acquisition to translate discovery results to targeted analysis (A). In this context, the rule-based lipid annotation tool from MetaboScape was used to transfer and monitor lipid MS/MS transitions in TASQ for enhanced specificity. Moreover, the accuracy in lipid quantitation was evaluated based on consensus values (B).

HILIC-TIMS-MS/MS screening in MetaboScape

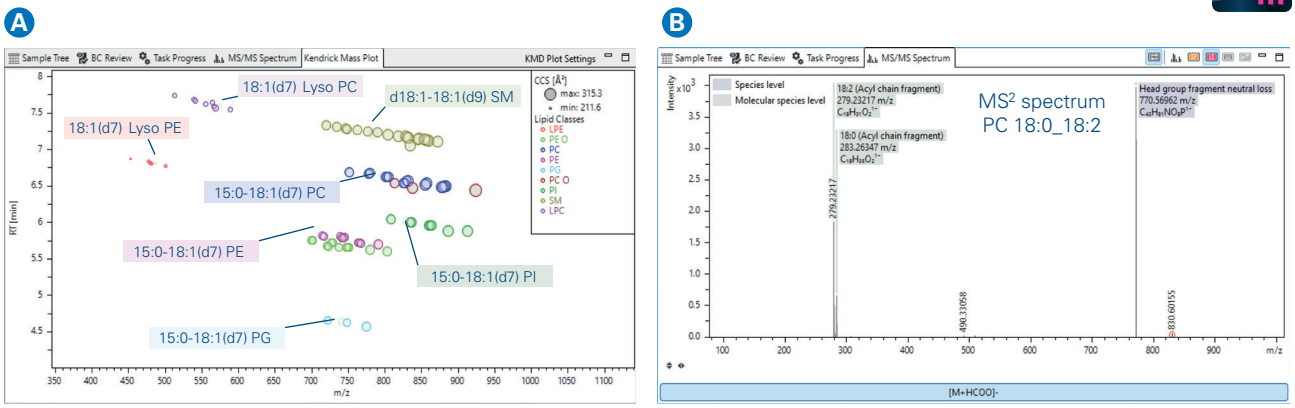

Figure 6.
(A) Survey plot of the annotated lipid species in MetaboScape including IS and (B) MS^2 spectrum of PC 18:0_18:2 with highlighted transitions generated by the rule-based lipid annotation tool.

Quantitative data processing in TASQ

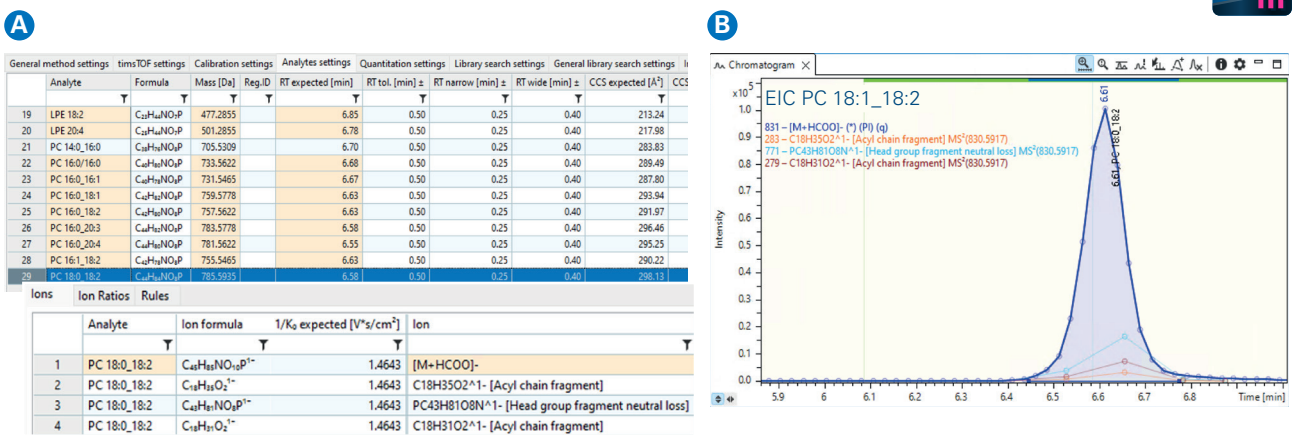

Figure 7.
(A) Overview of the transferred lipid species including transitions from MetaboScape to TASQ exemplified for PC 18:0_18:2, as well as (B) EIC of PC 18:0_18:2 and its transitions of targeted data by prm-PASEF for reliable quantitation using the developed workflow.

Targeted prm-PASEF acquisition in timsControl

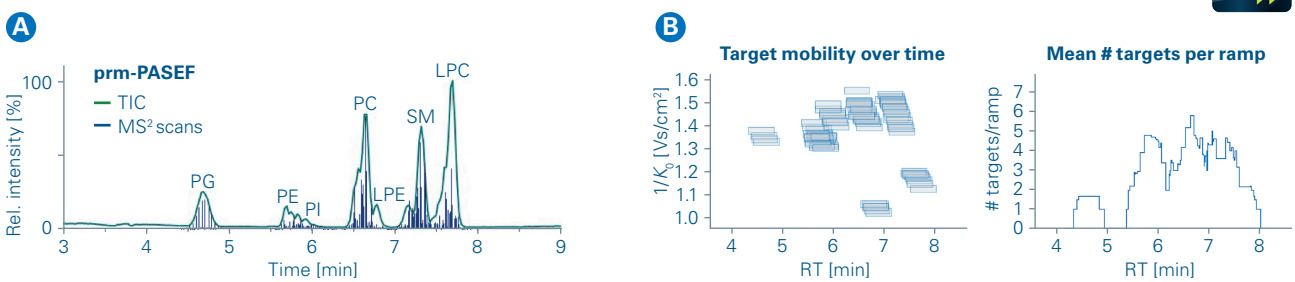

Figure 8.
(A) Overview of the time/mobility windows for each target and the number of mobility-resolved targets for each prm-PASEF scan as well as (B) TIC of the NIST plasma extract with highlighted MS^2 scans focusing on lipids of interest using prm-PASEF.

2) Method management to generate and process targeted methods

Based on the 4D-Lipidomics screening in MetaboScape, species-specific parameters including lipid MS/MS transitions were transferred to the TASQ software, where they served as a reference list (Figure 7A: analyte list). This transfer enables the seamless generation of prm-PASEF methods for targeted acquisition, which is integrated in the TASQ software.

Following prm-PASEF acquisition, this targeted dataseta can be processed directly in the TASQ software to evaluate quantitative results. Based on the aforementioned reference list, data processing also included lipid MS/MS transitions on species or molecular species level, which is useful for MS^1-based lipid quantitation by monitoring the reliability of correct species quantitation (Figure 7B: EIC monitoring). The monitoring of lipid MS/MS transitions is particularly essential for the analysis of large batches of varying samples, where coelution of interfering lipid species might occur that lead to falsified results in species or molecular species quantitation. Otherwise, the creation of lipid MS/MS transition lists in quantitation software solutions is a time-consuming process, due to the considerable number of individual lipid species.

3) Targeted analysis by prm-PASEF

Robust fragmentation is essential to achieve high-confidence lipid annotation. By focusing on lipids of interest, targeted acquisition based on prm-PASEF allows for highly reproducible fragmentation of specific lipids with high sensitivity in generating MS^2 spectra (Figure 8A: TIC). In timsControl, survey plots are generated of the imported prm-PASEF method from TASQ. These survey plots highlight both the multidimensional character of the time/mobility windows for precise precursor definition and the multiplexing character of prm-PASEF (Figure 8B: survey plots). The high speed of mobility-resolved fragmentation by PASEF is utilized in prm-PASEF to schedule multiple precursor selections simultaneously based on differences in ion mobility. For the HILIC-TIMS-MS/MS setup scheduling of 87 phospholipid species, up to six precursors were isolated and fragmented in the same TIMS frame using prm-PASEF, highlighting the potential for targeted analysis of large number of lipids.

In summary, screening and quantitation software solutions were combined to create a straightforward workflow for HILIC-TIMS-MS/MS-based quantitative plasma lipidomics using prm-PASEF. This workflow facilitated both method development and data evaluation.

Application for quantitative plasma lipidomics

In HILIC-based lipid quantitation, isobaric overlaps must be considered. Specifically, the type-II overlap caused by natural isotopic pattern of homologous lipid species (e.g., PC 36:1 vs. PC 36:2-$^{13}C_2$; $\Delta m/z$ = 0.009) can result in overestimated quantitation results (Figure 9). To resolve type-II overlaps, ultrahigh-resolution mass spectrometry (UHRMS) is occasionally used; however, type-II correction factors are more commonly applied during data processing [6]. An alternative approach in this context may be high-resolution IMS-MS. Therefore, the developed HILIC-TIMS-MS/MS approach was used to investigate the resolution of type-II overlaps in the NIST SRM 1950 plasma.

The time-of-flight-MS resolving power is insufficient for the resolution of isobaric type-II overlaps. For instance, in the case of low concentrated PC 36:1, an interference caused by the isotope pattern of the highly abundant PC 36:2 (PC 36:2-$^{13}C_2$, also known as M+2) is likely (resolving power $R > 92.500$ at m/z 832.60 required). In contrast, with increasing mobility resolution (PC 36:4: $R = 95$, 124, and 142; PC 36:3: $R = 90$, 126, and 136; PC 36:2: $R = 98$, 132, and 152 for 100 ms, 300 ms, and 500 ms ramp times, respectively), the mobilogram of PC 36:1 can be partially resolved. The two peaks obtained can be assigned according to their mobility values. Therefore, a straightforward identification of type-II overlaps can be achieved using TIMS with high ramp times. However, since no baseline separation was achieved, the transferability of this approach for lipid quantitation has to be taken into account.

In NIST SRM 1950 plasma, two major type-II overlaps were identified, where the homologous species with an additional double bond showed significantly higher concentrations. These included the interferences for PC 36:1, as previously discussed, and for SM d34:0. As a consequence of the missing baseline resolution, the integration limits must be adjusted. This process can be extremely laborious when applied to large batches of samples. Therefore, tailored and automated software solutions are required. The TASQ software solution enables the dynamic adaptation of chromatograms and mobilograms based on the specified integration limits. Accordingly, the shape of the mobilogram reflects the area of the integrated chromatogram (Figure 10A). For instance, saturated SM species such as SM d34:0

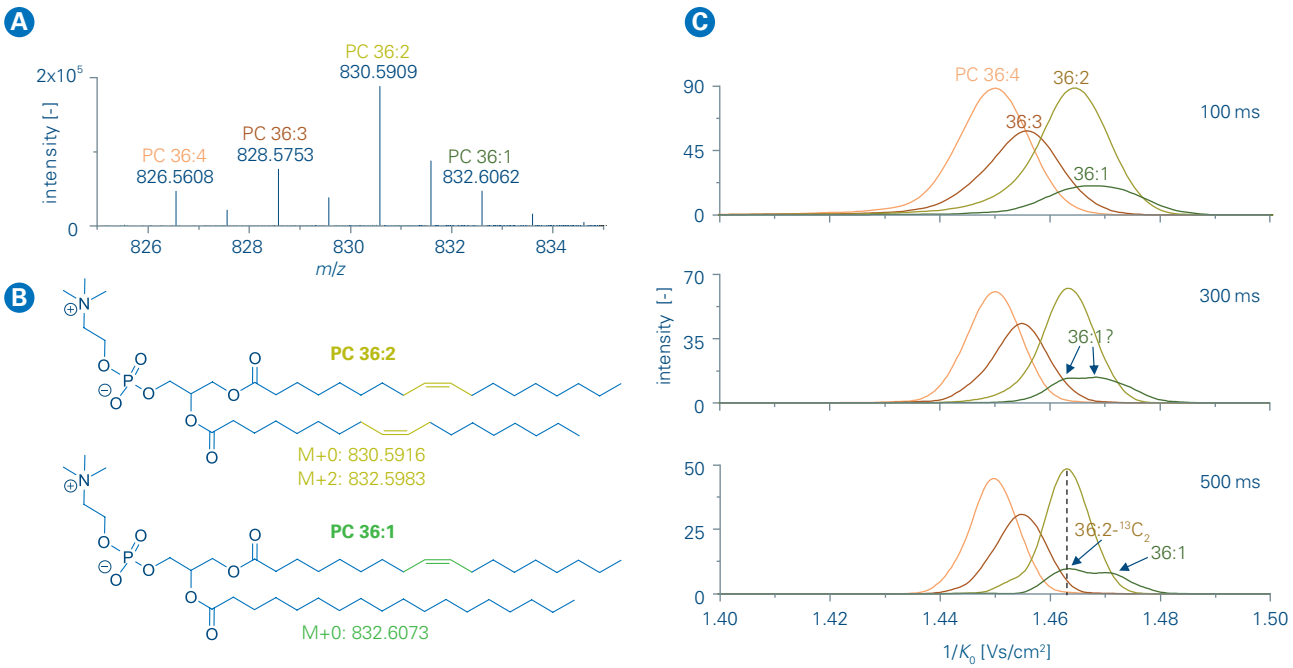

Figure 9.
Ⓐ Isobaric type-II overlaps of homologous PC lipid species with varying double bond number based on the natural isotopic pattern of lipids, as exemplified for Ⓑ PC 36:1 by accurate mass and structural formula. Ⓒ TIMS separation of these homologous PC species with varying resolution (100 ms, 300 ms, 500 ms ramp times).

exhibit different retention times in comparison to unsaturated species, resulting in a partial chromatographic separation of the type-II overlap caused by SM d34:1-$^{13}C_2$. Adjusting the chromatographic integration limits improves the mobility resolution for this type-II overlap in this multidimensional approach.

The global transfer of integration limit adjustments is particularly advantageous in high-throughput lipidomics of large sample batches. Furthermore, the adjusted integration limits are automatically applied to the single isotope signal (M+1), as isotopic compositions have no impact on HILIC and TIMS separation. Using the M+1 signal, the mobility resolution of type-II overlaps is enhanced according to the natural isotopic abundances. Therefore, the MS quantitation of PC 36:1 and SM d34:0 was performed using both the M+0 and M+1 signals. For comparison of the quantitative accuracy, the consensus values provided for NIST SRM 1950 were considered. However, the consensus values are not defined as accurate reference values and should therefore be used with caution, particularly regarding the notable standard uncertainties obtained for the type-II overlap suffering species PC 36:1 and SM d34:0 (PC 36:1: 26.0 ± 4.6 μM (17.7% standard uncertainty); SM d34:0: 5.8 ± 1.3 μM (22.4% standard uncertainty)) [11]. In addition to the TIMS type-II corrected M+0 and M+1 quantities, MS quantitation was further performed using the chromatographic signal without or with conventional type-II correction (Figure 10B).

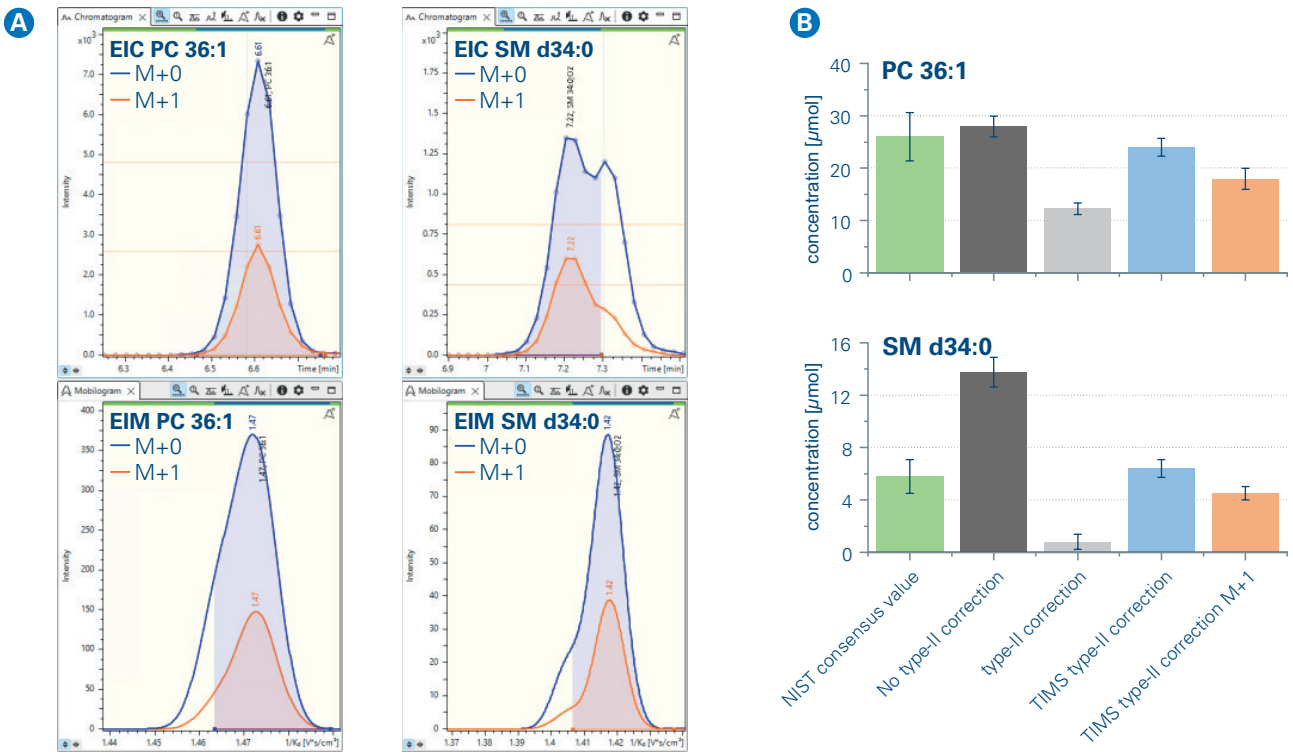

Figure 10.
(A) EIC and EIM traces of PC 36:1 and SM d34:0 in TASQ 2024b, including integration limits, highlighting the (HILIC and) TIMS separation of isobaric type-II interferences in TASQ software. (B) Quantitation accuracies for PC 36:1 and SM d34:0 in the NIST reference plasma depending on the used correction factors.

As expected, non-corrected type-II overlaps yielded overestimated absolute concentration results, particularly for SM d34:0 (PC 36:1: +2.0 μM (+7.5%); SM d34:0: +8.0 μM (+137.2%)). It is recommended that conventional type-II correction factors should be employed to compensate for the overestimation. However, an overcompensation was observed, resulting in underestimated absolute concentration results (PC 36:1: -13.8 μM (52.9%); SM d34:0: 5.0 μM (-86.1%)). Such overcompensation may be attributed to fluctuations in the observed isotope pattern obtained during acquisition. For major isobaric overlaps, even minor alterations in the isotope pattern can result in considerable deviations when using theoretical correction factors. In contrast, the application of the TIMS type-II correction yielded more accurate concentration values for both M+0 (PC 36:1: -2.0 μM (-7.6%); SM d34:0: +0.6 μM (+10.5%)) and M+1 quantitation (PC 36:1: -8.0 μM (-31.0%); SM d34:0: -1.3 μM (-22.1%)), while the M+0 TIMS type-II correction yielded the most accurate results. However, the use of the M+1 signal for quantitation of major type-II overlaps is still recommended, as the enhanced mobility resolution facilitates appropriate integration. Also, the consensus values should be treated with caution, as they may be subject to potential bias based on the employed analytical methods, as mentioned before [11]. For minor type-II overlaps, the use of M+0 TIMS type-II correction is sufficient. Nevertheless, M+1 TIMS type-II correction has been demonstrated to enhance the quantitation accuracy by 42% and 74% in comparison to conventional type-II correction factors for PC 36:1 and SM d34:0, respectively. Using the developed workflow, 81 phospholipid species were quantified at species or molecular species level with both high accuracy and reliability, which is pivotal for clinical lipidomics.

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Conclusions

- Accurate and reliable lipid quantitation using HILIC-based 4D-Lipidomics in prm-PASEF mode
- User-friendly, streamlined workflow for quantitative lipidomics by combination of MetaboScape 2024b and TASQ 2024b software solutions
- TIMS-enhanced quantitative accuracy by resolving critical type-II overlaps in lipidomics at high ramp times of 500 ms (up to 74% improvement compared to conventional type-II correction factors)
- prm-PASEF-based monitoring of lipid MS/MS transitions for advanced reliability in quantitation of >80 phospholipids on species or molecular species level

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