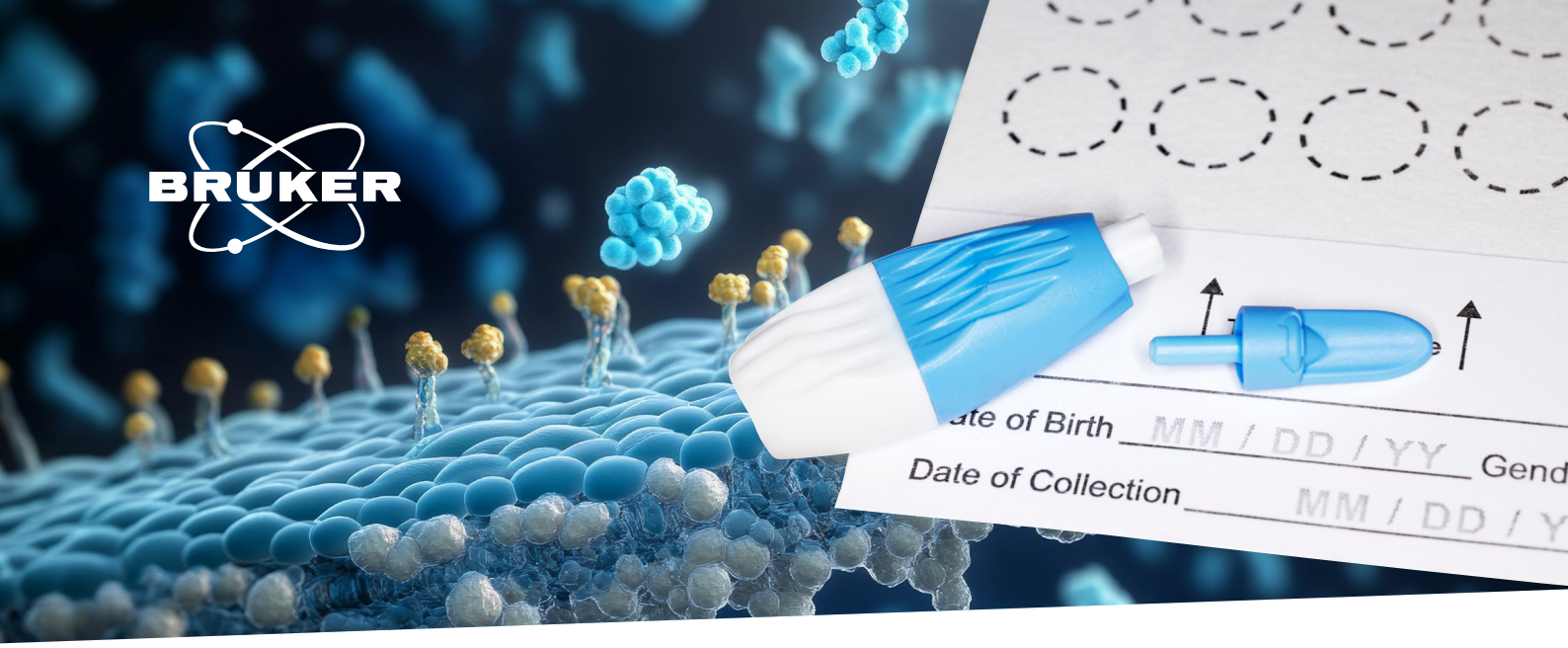

Dried blood spot microsampling: A semi-quantitative 4D-Lipidomics approach utilizing MetaboScape for enhanced lipid profiling.

Lipid profiling from biofluids, such as plasma or serum, derived from phlebotomy has been investigated extensively. The use of dried blood spot (DBS) microsampling has been gaining popularity due to its minimal invasiveness and potential for self-sampling. This enables the possibility of outside the clinic sampling and also high-frequency metabolic profiling which can cater to large-scale population-wide research.

Abstract

Here, we demonstrated the potential use of DBS microsampling for 4D-Lipidomics™ profiling using timsTOF Pro with VIP-HESI. By leveraging real-time data quality monitoring during acquisition, potential issues can be promptly identified and addressed, ensuring optimal data collection. Lipid identification using MetaboScape®'s rule-based annotation complemented by Kendrick mass defect plot enhanced confidence in annotation. Additionally, the incorporation of internal standards allowed for semi-quantification of measured lipids within a single acquisition, providing a deeper understanding of lipid concentration variations across different individuals.

Keywords:
4D-Lipidomics, dried blood spot, microsampling, semi-quantative, timsTOF, MetaboScape, ion mobility

Introduction

Dried blood spot microsampling (DBS) is slowly gaining traction in clinical settings as a minimally invasive alternative to venipuncture, using a simple fingerprick and disposable lancet to deposit blood droplets onto a paper substrate. Compared to traditional venous phlebotomy, which is invasive and requires trained personnel, microsampling is self-administrable, and has since been adapted for analyzing diverse biological matrices, including plasma, saliva,

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urine, and breast milk [1]. While this technique has been employed in routine measurement of targeted metabolites, with the most well-known application being newborn screening for phenylketonuria, DBS could be extended to other biomarkers of metabolic health such as lipids. Lipids are among the most functionally versatile, structurally diverse, and physiologically complex biomolecules. They are increasingly recognized for their role in chronic disease development due to their involvement in energy storage, cell signaling, enzyme regulation, and the maintenance of homeostasis [2].

Mass spectrometry (MS)-based workflows have been extensively employed in the field of lipidomics to characterise lipid profiles and understand biological functions in biofluids. Advancements in high-resolution MS instruments together with Trapped Ion Mobility Spectrometry (TIMS-HRMS) has enabled these in-depth studies to be carried out with minute samples volumes (<10 µL) or even from single cells [3]. The complexity of the lipidome in biological samples is exemplified by a myriad of isobaric lipids, which LC-TIMS-HRMS provides another analytical dimension ("4D") for the elucidation of isobaric compounds based on their structural configuration which is not possible by traditional LC-MS methods.

Herein, we describe a 4D-Lipidomics workflow to evaluate the applicability of in-depth analysis of the whole blood lipidome collected on a DBS substrate, together with MetaboScape 2025 for successful identification and quantitation of these lipids using a microsampling approach.

Methods

Briefly, 10 µL of capillary blood was sampled from healthy control and diabetic individuals (n = 17 participants in each group) using a commercial microsampling device (Capitainer®, Solna, Sweden)[4]. The DBS samples were extracted with 150 µL of 80% IPA containing quantitative labeled internal standards (Avanti EquiSPLASH LIPIDOMIX; 1 µg/mL), before undergoing a 10 min vortex mix, 5 min sonication, 20 min protein precipitation at -20˚C, and 10 min centrifugation at 14,000 x g (4°C). 4D UHPLC-TIMS-HRMS analysis was performed across two ionization modes: positive vacuum insulated probe heated electrospray ionization mode (VIP-HESI+) and negative mode (VIP-HESI–). Pooled quality control (PQC) samples were created by combining the DBS extracts to avoid adsorption issues from extracting multiple DBS.

UHPLC-TIMS-HRMS analyses were conducted as described in Table 1 using a UHPLC connected to a timsTOF Pro system equipped with a VIP-HESI source. Real-time data quality monitoring during acquisition was performed using TASQ® 2025 (Bruker) with real-time quality control (RTQC).

The resulting data was processed with MetaboScape 2025 for non-targeted profiling using T-ReX® 4D algorithm where MetaboScape combines common adducts and isotopes belonging to the same compound into unique features in the feature table. Processed positive and negative mode data were merged into a single feature table for annotation using the rule-based lipid annotation based on the Lipidomics Standards Initiative guidelines [5] and measured CCS of lipid species were matched with the prediction by CCS-Predict Pro. Finally, the annotated lipids were also semi-quantitated with spiked internal standards (ISTD) using MetaboScape 2025.

Results and Discussions

Data quality checks

Data quality in global lipidomic profiling is crucial for biomarker discovery and systems biology studies. However, data quality is frequently checked only after the whole sample set is acquired. This could lead to the loss of precious samples if an issue occurred during the acquisition process.

Herein, we utilise "RealTime QC" (RTQC) to monitor the acquisition process in real-time. Briefly, a processing method using Target Analysis for Screening and Quantitation software (TASQ) is applied to target compounds of interest, such as ISTD or endogenous metabolites. These compounds are then automatically monitored after every run and plotted in RTQC for ease

Table 1. MS acquisition parameters in both positive and negative ionization.

MS	timsTOF Pro	
Source	**VIP-HESI source**	
	End Plate Offset	500 V
	Capillary	4500 V
	Nebulizer	2.0 bar
	Dry Gas	8.0 L/min
	Dry Temp	230°C
	Probe Gas Temp	400°C (+), 450°C (-)
	Probe Gas Flow	4.0 L/min
Acquisition mode	TIMS-PASEF	
Ionization	Positive and Negative	
Tims parameters	Ramp time	100 ms
	Mobility range	0.55 – 1.90 $1/K_0$
	ICC Target Intensity	5.0 M
Transfer parameters	Scan range	100 – 1350 *m/z*
	Funnel 1 RF	250 Vpp
	Funnel 2 RF	200 Vpp
	Multipole RF	200 Vpp
	Collision Energy	10 eV
	Collision RF	450 V
	Quadrupole Low mass	60 *m/z*
	Transfer Time	50 *µs*
	Pre Pulse Storage	3 *µs*
	MS/MS Collision Energy	20 – 50 eV
Calibration	Automatic internal mass calibration using sodium formate and Agilent ESI-L low concentration Tunemix	

Table 2. LC parameters

LC	Waters Acquity		
Column	Waters BEH C18 (100 x 2.1 mm, 1.7 µm)		
Column Oven Temp.	60°C		
Mobile phase	**A**: Water/acetonitrile/isopropanol (50/30/20) + 10 mM ammonium acetate **B**: Water/acetonitrile/isopropanol (1/9/90) + 10 mM ammonium acetate		
Pump	Seal wash	Isopropanol / water 10% / 90%	
Gradient	**Time [min]**	**Flow [ml/min]**	**%B**
	0.0	0.40	10
	2.7	0.40	45
	2.8	0.40	53
	8	0.40	60
	8.1	0.40	80
	11.5	0.40	80
	12	0.40	100
	13	0.40	10
	15	0.40	10
Autosampler	Temperature	8°C	
	Injection volume	5 µl	
	Wash solvent A	IPA / water (10% / 90%)	
	Wash solvent B	Isopropanol (100%)	

Figure 1. **Real-time QC visualization of the whole dataset.**
In this exemplar using ISTD 18:1(d7)-LPC, a sample was highlighted (in red) with the missing ISTD, which was later identified due to issues during the sample extraction process.

of visualization (Figure 1). Plots such as intensity, and differences between measured and exact values—such as mass-to-charge (Δm/z), retention time (ΔRT), and CCS ($\Delta 1/K_0$)—can be customized to display the required information. Outliers within the runs are also automatically highlighted in RTQC (i.e. 18:1(d7)-LPC) to indicate samples or instrument issues for further intervention by the user.

Lipid annotation

Lipid species were annotated with MetaboScape's rule-based annotation using precursor *m/z*, isotopic pattern, characteristic MS/MS spectra and measured CCS as an additional quantifier based on MetaboScape's CCS-Predict Pro. Specifically, the target list spans 4 lipid classes including glycerolipids, glycerophospholipids, sphingolipids, and sterol lipids, across 25 subclasses.

A merged 4D-Kendrick mass defect (KMD) plot was used to visualize the lipid distribution of combined positive and negative ionization datasets. KMD is based on the diagnostic structure feature of the lipid fatty acyl chains (CH_2) to organize structurally related lipid subclasses. Further curation of lipid outliers were also readily identified using the outlier function in MetaboScape where annotated lipid outliers based on CCS or RT were highlighted in the KMD plot. A total of 357 unique lipids were annotated in DBS, shown in Figure 2.

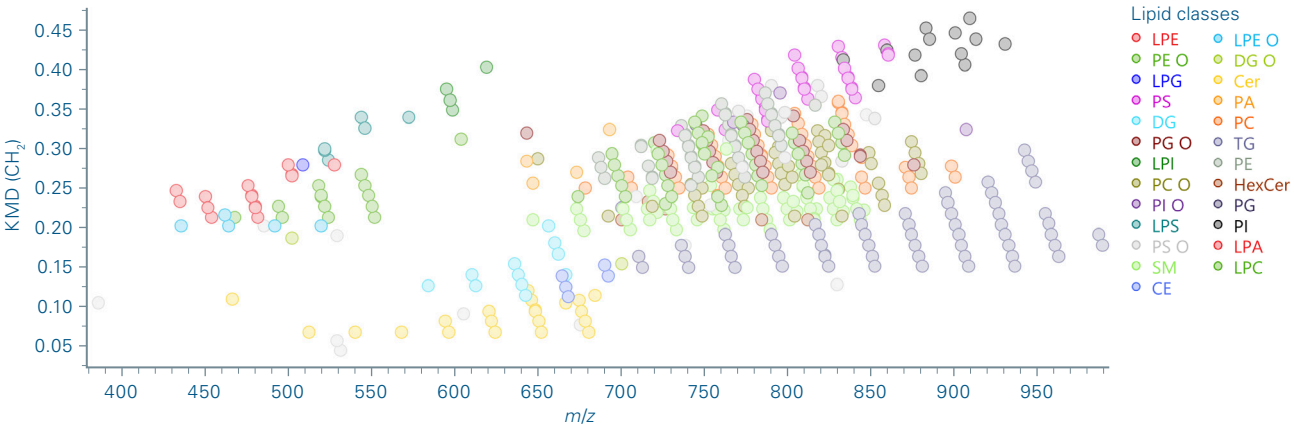

Figure 2. Merged KMD plot of DBS lipids detected in both positive and negative ionization.

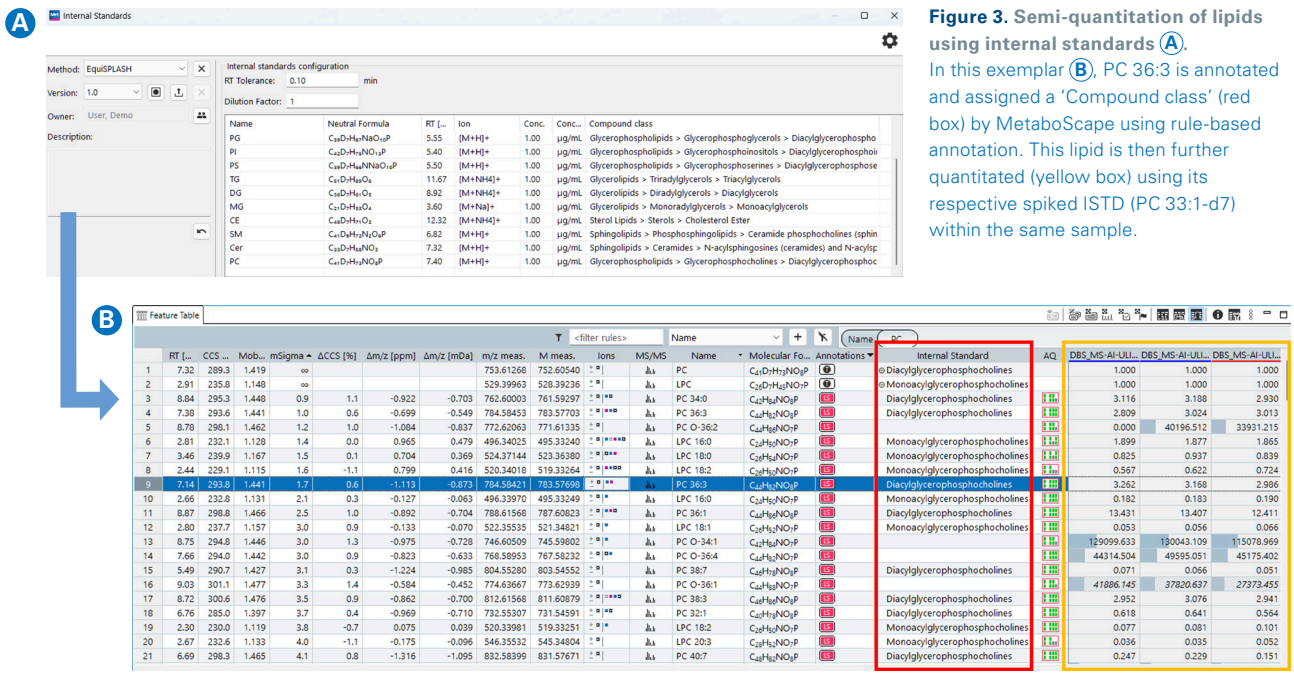

Figure 3. Semi-quantitation of lipids using internal standards (A). In this exemplar (B), PC 36:3 is annotated and assigned a 'Compound class' (red box) by MetaboScape using rule-based annotation. This lipid is then further quantitated (yellow box) using its respective spiked ISTD (PC 33:1-d7) within the same sample.

Semi-quantitation of lipids in advanced microsamples

The concentrations of measured lipids can be calculated in MetaboScape using a single-point internal standard (ISTD) as a surrogate based on their lipid subclasses as shown in Figure 3. A target list containing the ISTD information such as retention time, molecular formula, working concentration and the compound class was created (Figure 3A). This target list was then used to annotate the Feature Table and the annotated ISTDs are shown with a "T" symbol in the 'Annotation' column (Figure 3B). When an annotated lipid (i.e. PC 36:3) has the same compound class as the ISTD (PC 33:1-d7; Figure 3B - red box), the concentration will be calculated based on the following formula: (peak area (measured)/ peak area (ISTD)) x concentration (ISTD). For lipids that do not have a corresponding ISTD, no concentration is reported (Figure 3B – yellow box).

Here in Figure 4, a t-test was employed and visualized using a volcano plot to identify lipid features that are different (p-value < 0.05, fold change >1.5) between the two groups. A series plot within MetaboScape can also be used to quickly visualize the trajectory of lipid species across sample cohorts. Figure 4B demonstrates how some of the annotated lipid species measured in DBS are changing between non-diabetic and diabetic patient controls.

A lower level of lysophosphatidylcholine (LPC) species were observed in DBS from diabetic patients compared to control subjects. Meanwhile, a higher amount of triacylglycerols (TG) are observed in diabetics compared to controls. These lipid concentrations were further quantitated using its respective ISTD, shown in Table 3. These observations are also similarly reported in another paper suggesting that LPC and TG changes are correlated to obesity-related factors such as diet and adiposity [6] as many of these lipids play important signaling roles with diverse biological function and are involved in regulating cellular proliferation, tumor cell invasion and inflammation.

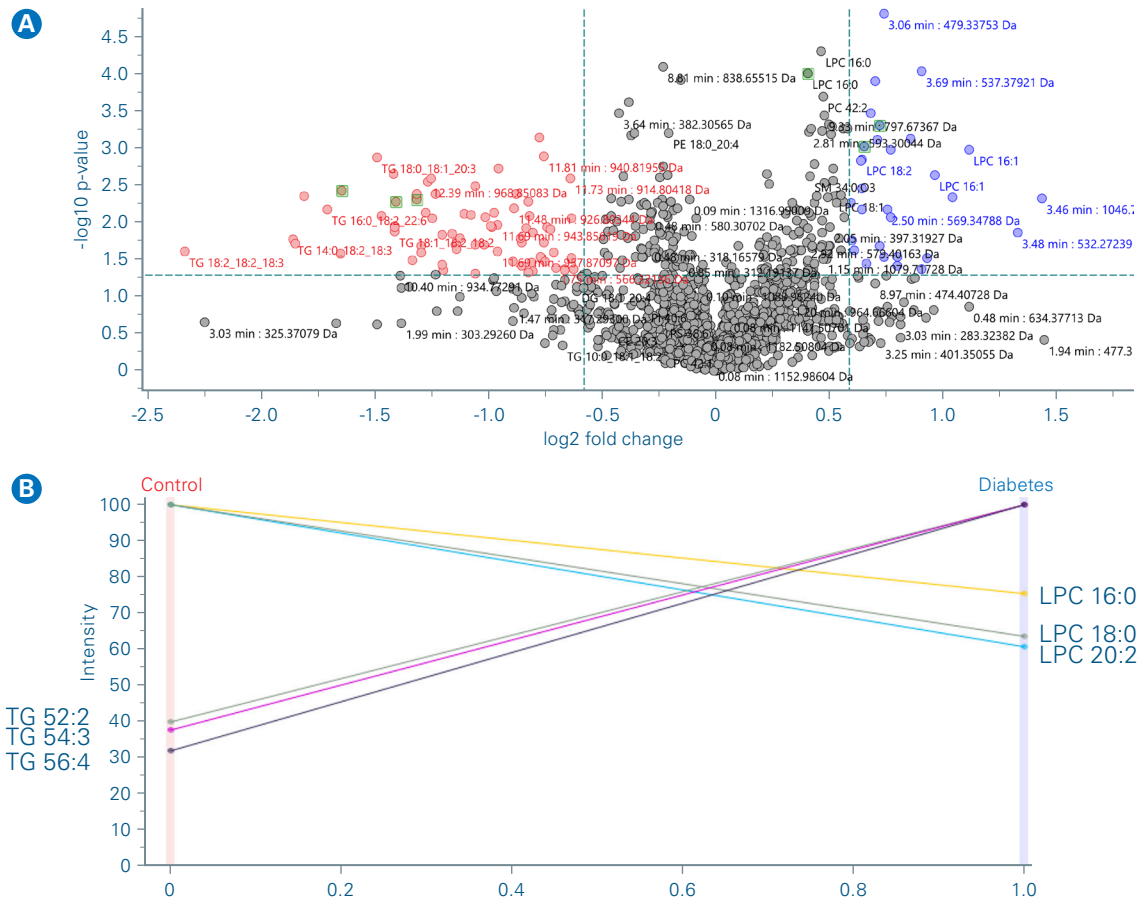

Figure 4. Statistical analysis in MetaboScape showing lipid variations between control and diabetic individuals. (A) A volcano plot illustrating the lipid abundance between two groups. Lipids that are less abundant in the control group are shown on the left (red), while those less abundant in diabetics are displayed on the right (blue). (B) Series plot showing increases of TG and decreases of LPC, based on relative intensities, between controls and diabetic individuals.

Table 3. DBS lipid concentrations in control and diabetic individuals (n=17). Lipids were quantified using its respective spiked ISTD. Lipids shown have a p-value <0.05.

Lipid Species	Control (µM)	Diabetic (µM)
LPC 14:0	0.99 ± 0.35	0.77 ± 0.24
LPC 16:0	58.15 ± 8.65	43.65 ± 9.13
LPC 18:0	25.84 ± 8.37	17.07 ± 4.39
LPC 18:1	15.43 ± 4.34	11.02 ± 3.74
LPC 18:2	18.84 ± 5.98	13.28 ± 4.97
LPC 20:0	0.20 ± 0.05	0.15 ± 0.04
LPC 20:1	0.27 ± 0.09	0.20 ± 0.07
LPC 20:2	0.18 ± 0.06	0.11 ± 0.04
LPC 20:3	1.21 ± 0.43	0.87 ± 0.32
TG 52:2	57.09 ± 56.29	142.39 ± 95.64
TG 54:3	16.21 ± 14.12	43.09 ± 31.47
TG 56:3	1.14 ± 1.08	3.58 ± 2.75

Conclusions

- In-depth analysis of lipids from dried blood spots using a complete 4D-Lipidomics workflow with timsTOF Pro, RealTime QC and MetaboScape 2025 from sample acquisition, quality checks during data acquisition to lipids identification and quantification respectively.
- Real-time monitoring of data acquisition using RealTime QC provides at-a-glance data visualization of selected analytes. Outliers are automatically highlighted for informed decision-making during the experiment.
- 4D-Kendrick mass defect plots, together with MetaboScape RT and CCS outlier detection, offers quick visualization and curation of lipid species for higher-confidence annotation.
- Out of the 357 rule-based annotated lipids, 222 lipids were quantitated in DBS based on internal standard normalization of identical lipid class in MetaboScape 2025.

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