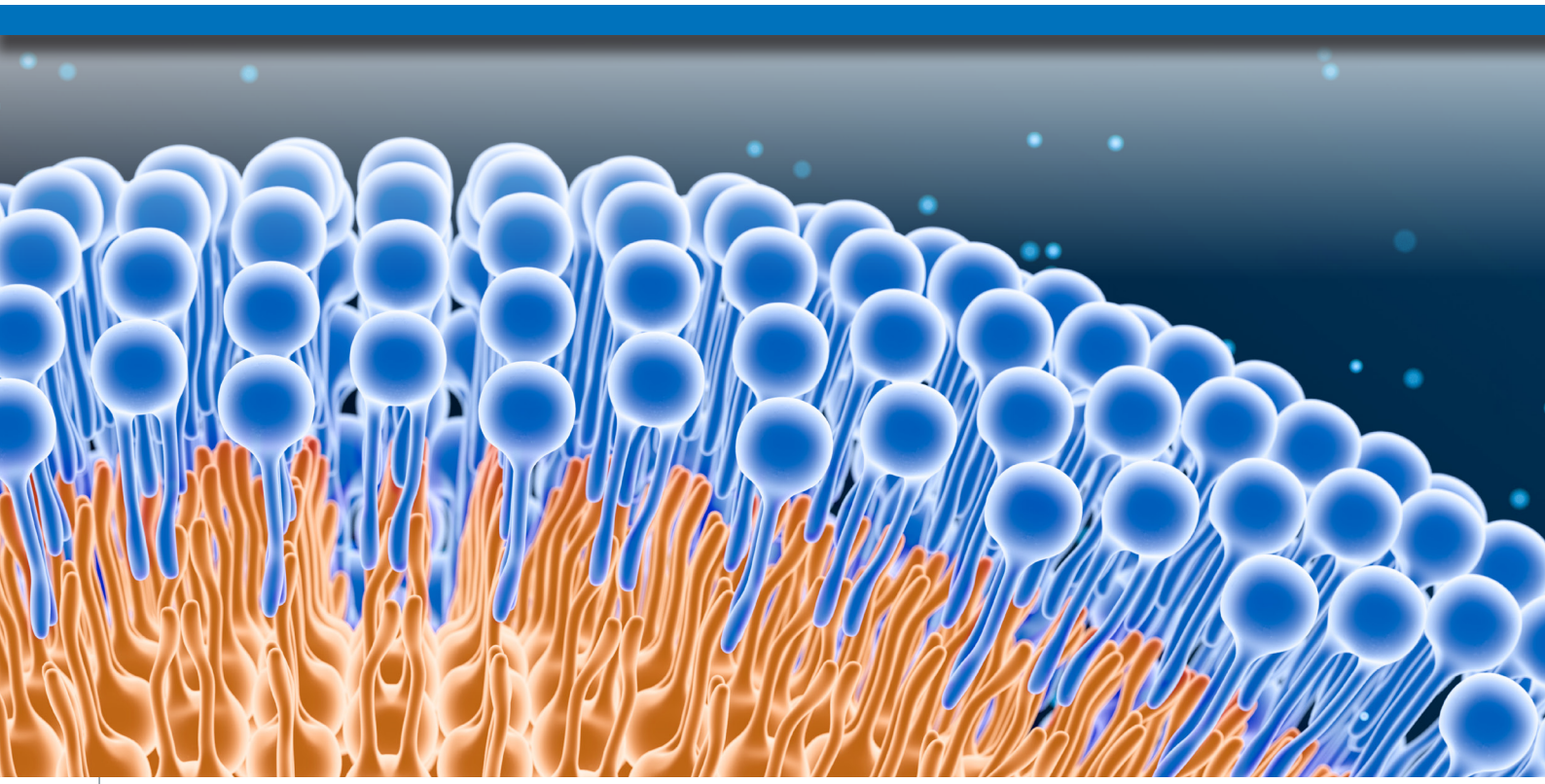


# Using Parallel Accumulation Serial Fragmentation (PASEF) to speed up untargeted 4D lipidomics LC-MS/MS workflows

The search for new and validated biomarkers is of particular interest in clinical areas like oncology [1, 2] or neurology [3]. As lipids play an important role in many diseases, the area of lipidomics has become central for clinical research.

## Introduction

While commonly an in-depth oriented approach to ID as many lipids as possible is applied, clinically-oriented projects demand a high throughput for large sample cohorts. Therefore, a short cycle time per sample is necessary to realize research projects with hundreds or even thousands of samples in a reasonable time frame. To enable this, the analytical instrumentation needs to deliver an uncompromised high data quality at high acquisition speeds. This is realized by the PASEF (Parallel Accumulation Serial Fragmentation) [4] acquisition mode on the timsTOF Pro system.

*Keywords:*
*PASEF, TIMS, lipidomics, SRM 1950, lipids, profiling*

*Authors: Sven. W. Meyer, Aiko Barsch, Ulrike Schweiger-Hufnagel; Bruker Daltonik GmbH, Bremen, Germany*

## Methods

Lipids from NIST SRM 1950 reference plasma were extracted based on a protocol published by Shevchenko *et al.* [5]. Extracts were dissolved in Methanol:Dichloromethane (9:1). The total amount of lipids injected on column equaled 0.5 µL extracted standard (5 µL injections with 5 replicates, each).

The reversed phase based LC separation was performed using an Elute UHPLC system and a Bruker intensity C18 column (100 x 2.1 mm, 1.9 µm). Run times were 6, 11 and 20 minutes, respectively. The MS data was acquired in positive ESI mode using a timsTOF Pro instrument in PASEF MS/MS mode. The transfer parameters were optimized for 100-1500 m/z, precursors were fragmented from 300-1500 m/z.

The resulting data were processed considering all four dimensions (m/z, RT, mobility and intensity) using MetaboScape 5.0. In the specified range of 300-1200 m/z, the T-ReX 4D algorithm combined all adducts and isotopes belonging to the same lipid into features in the so-called bucket

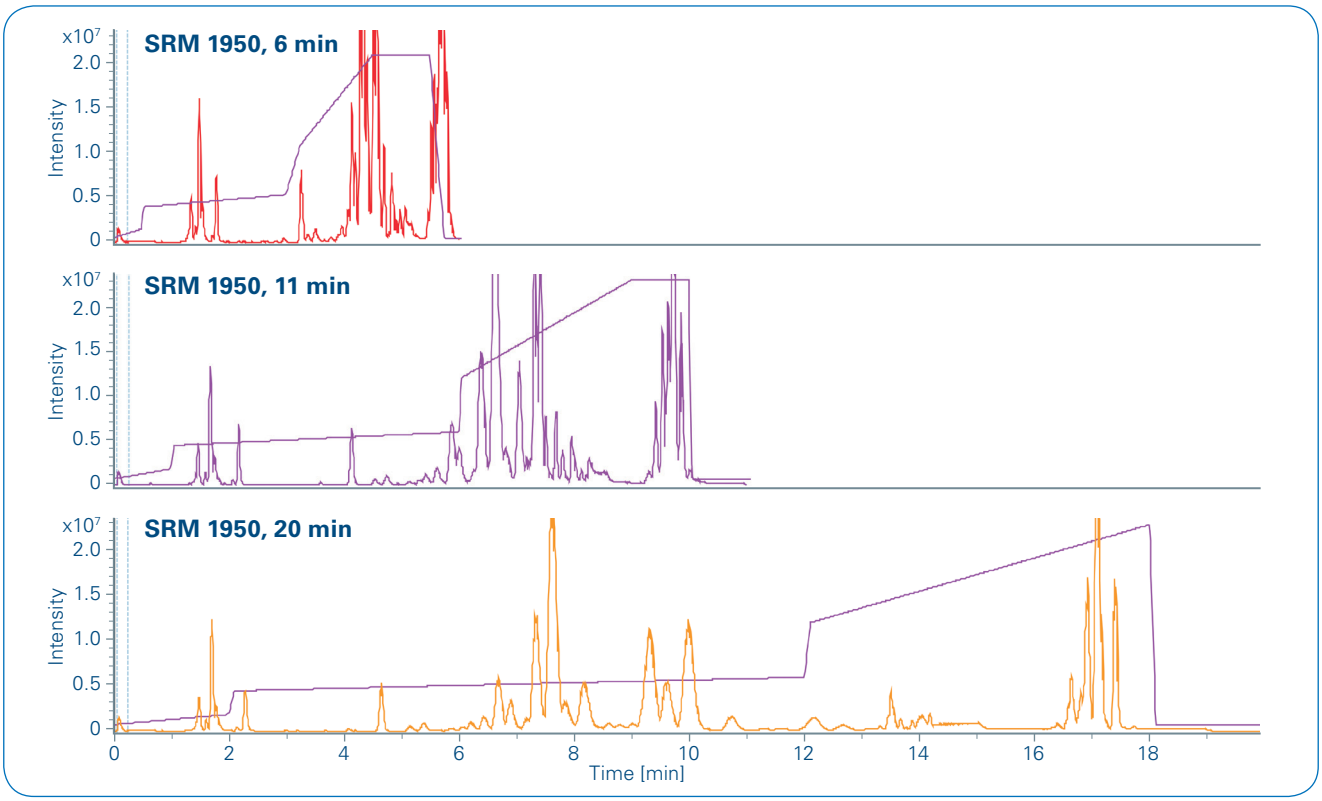



*Figure 1: Different gradient lengths applied to SRM 1950 lipid extract*

Bucket Table

<filter rules>

| | RT [min] | m/z meas. | MS/MS | CCS (Å²) | Name | Molecular Formula | AQ | Δm/z [mDa] | mSigma | MS/MS score | Annotations | SRM1950_20min_88_01_6951 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.29 | 524.37207 | | 238.3 | LPC 18:0; [M+H]+ | $C_{26}H_{54}NO_7P$ | | 0.910 | 24.7 | 988.0 | | 5149326 |
| 2 | 7.86 | 784.58709 | | 289.2 | PC 36:3; PC 18:1-18:2; [M+H]+ | $C_{44}H_{82}NO_8P$ | | 2.003 | 25.3 | 995.4 | | 3739426 |
| 3 | 9.63 | 786.60147 | | 291.3 | PC 36:2; PC 18:1-18:1; [M+H]+ | $C_{44}H_{84}NO_8P$ | | 0.737 | 3.4 | 995.0 | | 987101 |
| 4 | 16.94 | 690.61931 | | 293.2 | CE 20:4; [M+NH4]+ | $C_{47}H_{76}O_2$ | | 0.951 | 34.2 | 913.4 | | 850903 |
| 5 | 6.20 | 806.57050 | | 289.5 | PC 38:6; PC 18:2-20:4; [M+H]+ | $C_{46}H_{80}NO_8P$ | | 1.070 | 15.7 | 990.9 | | 554671 |
| 6 | 2.15 | 524.37232 | | 239.3 | PC 18:0e; PC 16:0e/2:0; [M+H]+ | $C_{26}H_{54}NO_7P$ | | 1.254 | 16.9 | 998.3 | | 506231 |
| 7 | 16.33 | 844.74010 | | 310.4 | TAG 50:4; TAG 16:1-16:1-18:2; [M+NH4]+ | $C_{53}H_{94}O_6$ | | 1.232 | 9.2 | 866.2 | | 457948 |
| 8 | 16.81 | 924.80126 | | 326.8 | TAG 56:6; TAG 16:0-18:2-22:4; [M+NH4]+ | $C_{59}H_{102}O_6$ | | -0.206 | 33.3 | 670.5 | | 433032 |
| 9 | 1.61 | 546.35558 | | 231.2 | LPC 20:3; [M+H]+ | $C_{28}H_{52}NO_7P$ | | 0.082 | 9.3 | 981.3 | | 358744 |
| 10 | 17.40 | 668.63501 | | 288.9 | CE 18:1; [M+NH4]+ | $C_{45}H_{78}O_2$ | | 1.008 | 11.3 | 915.7 | | 393085 |
| 11 | 8.44 | 810.60145 | | 293.7 | PC 38:4; PC 19:2-19:2; [M+H]+ | $C_{46}H_{84}NO_8P$ | | 0.714 | 2.5 | 990.6 | | 348388 |

*Figure 2: Screenshot of MetaboScape 5.0 showing a bucket table of the 20 minute LC-PASEF analyses. The AQ score gives feedback on the quality of IDs*

table. Features occurring in blank samples with an intensity > 2 fold vs. samples were subtracted. The remaining features were matched against the open source *in-silico* MS/MS library LipidBlast [6] and identified based on the fitting of precursor m/z, isotopic pattern and MS/MS spectra. In order to compare the numbers of assigned lipid classes on sum composition level, only the most abundant species of each class were considered (i.e. isomers eluting with different retention times were removed). CCS prediction was done using CCSPredict, a tool implemented in MetaboScape. It is based on a machine learning approach first described by Zhou *et al.* [7]. For the spike-in experiment, a SRM 1950 plasma extract was spiked with lipid standards.

*Table 1: UHPLC MS equipment and setup for lipid profiling*

| **MS** | **timsTOF Pro** |
|---|---|
| Source | Apollo II ESI source |
| Ionization | ESI(+), 4500 V Capillary Voltage |
| Scan range | 100–1500 m/z |
| Acquisition mode | PASEF MS/MS, 100 ms ramp time, 2 PASEF MS/MS cycles |
| Calibration | Internal mass calibration through automation, Sodium Formate, Mobility calibration before sequence using Agilent Tunemix |

| **UHPLC** | **Bruker Elute** |
|---|---|
| Column | Bruker intensity C18 column (100 x 2.1 mm, 1.9 µm) |
| Column Oven Temp. | 55°C (20 and 10 minutes); 65°C (6 minutes) |
| Flow Rate | 0.4 mL/min |
| Mobile phase | A: ACN/$H_2O$ (60:40, 10 mM $NH_4Ac$, 0.1% FA)<br>B: iPrOH/ACN (90:10, 10 mM $NH_4Ac$, 0.1% FA) |
| Gradient | 0 min 40% B |
| | 2 min 43% B |
| | 2.1 min 50% B |
| | 12 min 54% B |
| | 12.1 min 70% B |
| | 18 min 99% B |
| | 18.1 min 40% B |
| | 20 min 40% B |
| Data processing | MetaboScape 5.0 & DataAnalysis 5.2 |

## Results

The aim of this study was to demonstrate the power of PASEF for 4D lipid profiling. The setup of the experiments was chosen to prove first the performance for an in-depth "ID as many lipids as possible" approach in hyphenation with an analytical Elute UHPLC and gradient lengths of 20 minutes. Second, the LC run times were reduced in order to evaluate the feasibility to enable increased sample throughput which is required for large cohort profiling, e.g. in clinical research studies (Figure 1). Here, in particular the benefit of the super-fast MS/MS acquisition speed of PASEF was investigated (Figure 3).

For an in-depth 20 min runtime analysis, the number of identified lipids was 392 (Figure 2, Table 2). On a sum composition level, this corresponds to 286 lipid classes (Figure 5), in comparison to 217 classes described in an inter-laboratory study by Bowden *et al.* [7]. A high overlap of 158 classes was observed, confirming the validity of the 4D-lipidomics profiling workflow. The number of lipids assigned in the present study was higher for several lipid classes, especially the number of PCs increased by a factor of > 2. The total numbers of buckets lowered to 87% and 82% from the initial amount when reducing the run time from 20 minutes to 11 and 6 minutes, respectively. Still more than 200 lipids could be identified even in 6 minute analyses.

The power of the high speed PASEF technique is presented in Figure 3: within 0.1 minutes, 102 precursors were picked for fragmentation, some of them multiple times. Per 100 ms ramp, PASEF picked up to 9 precursors. The trapped ion mobility provides the resolving power

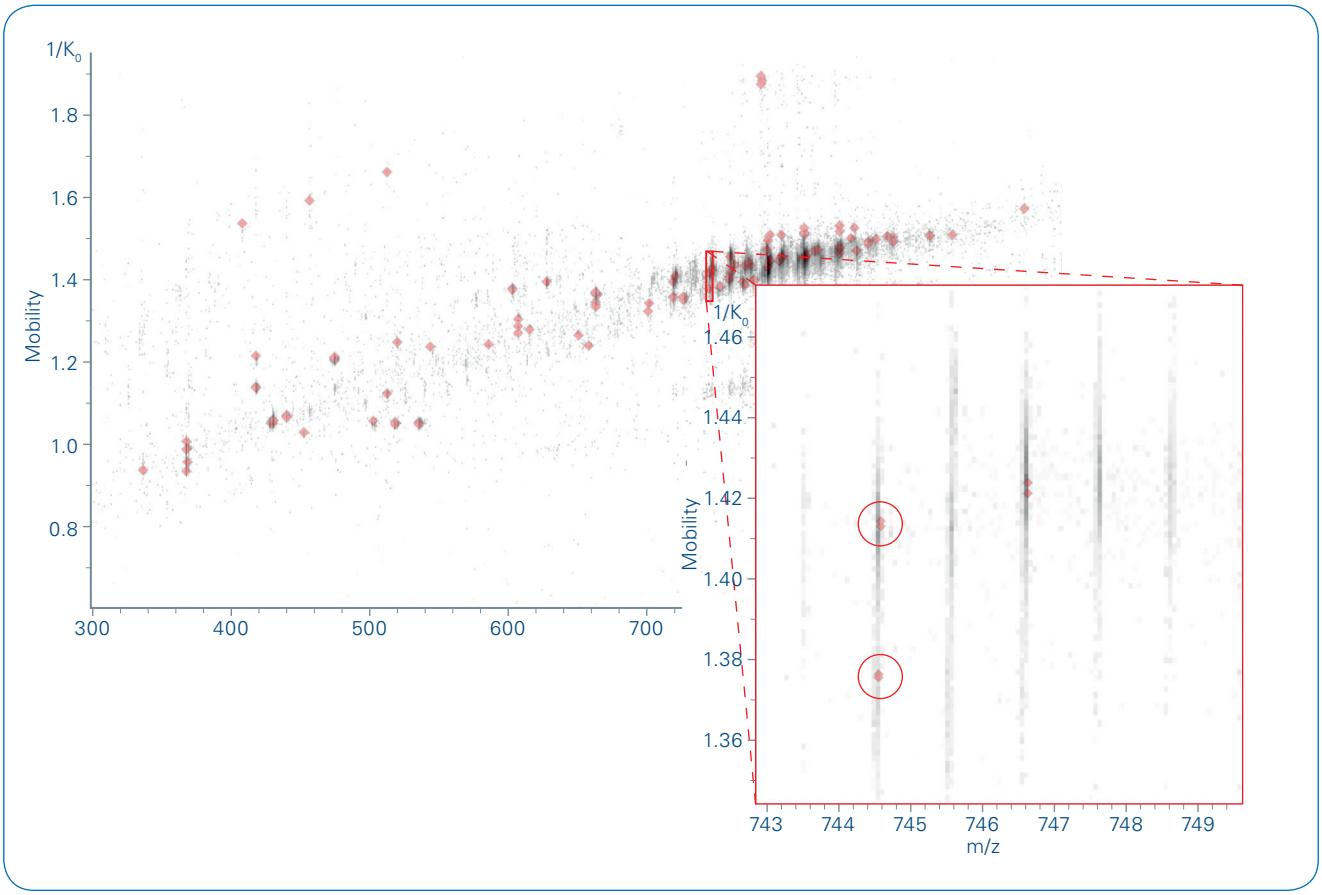



*Figure 3: In the time range of 4.6 to 4.7 minutes, PASEF picked 102 precursors, some of them several times. Multiply charged ions were excluded from fragmentation. The cutout shows two co-eluting isobaric lipids that were fragmented separately by PASEF (see also Figure 4)*

*Table 2: Lipids identified in SRM 1950 at different LC run times*

| Runtime [min] | Buckets | IDs |
|---|---|---|
| 6 | 2123 | 213 |
| 11 | 2260 | 283 |
| 20 | 2591 | 392 |

to separate critical pairs. This is presented in the cutout in Figure 3 on two isobaric PC 34:2e and PE 36:2 lipid species. They co-elute on the LC domain but are separated by TIMS and fragmented subsequently. Figure 4a displays the respective extracted ion chromatogram traces. The mass difference of the precursors was only 36 mDa (Figure 4b). No standard QTOF system would be able to isolate these masses and thus acquire mixed MS/MS spectra (Figure 4c). The mobilogram traces of both compounds show a mobility separation close to baseline level. Additionally, the benefit of the automatically extracted CCS values is presented (Figure 4d). These can be compared with values predicted by CCSPredict in MetaboScape. The matching of values for both lipids was < 0.5%. The additional CCS values and the clean MS/MS spectra increased the confidence in the lipid assignment. Figure 4e highlights the mobility separated PASEF MS/MS spectra for both lipids. The fragment spectrum of the PC contains only the characteristic PC head group fragment (184 m/z) while the PE MS/MS spectrum shows a neutral loss of 141 m/z which is characteristic for PEs.

Finally, in order to evaluate the performance of PASEF for high-throughput lipidomics applications using short gradients, an experiment using lipid standards spiked into the SRM 1950 plasma sample was performed.

The PCA and the *t*-Test statistics calculated using the 6 minute data were able to reliably differentiate between the two different sample groups (spiked and non-spiked SRM 1950, Figures 6a and b). This proves the performance of PASEF for high throughput lipidomics applications, e.g. for large cohort or clinical profiling studies.

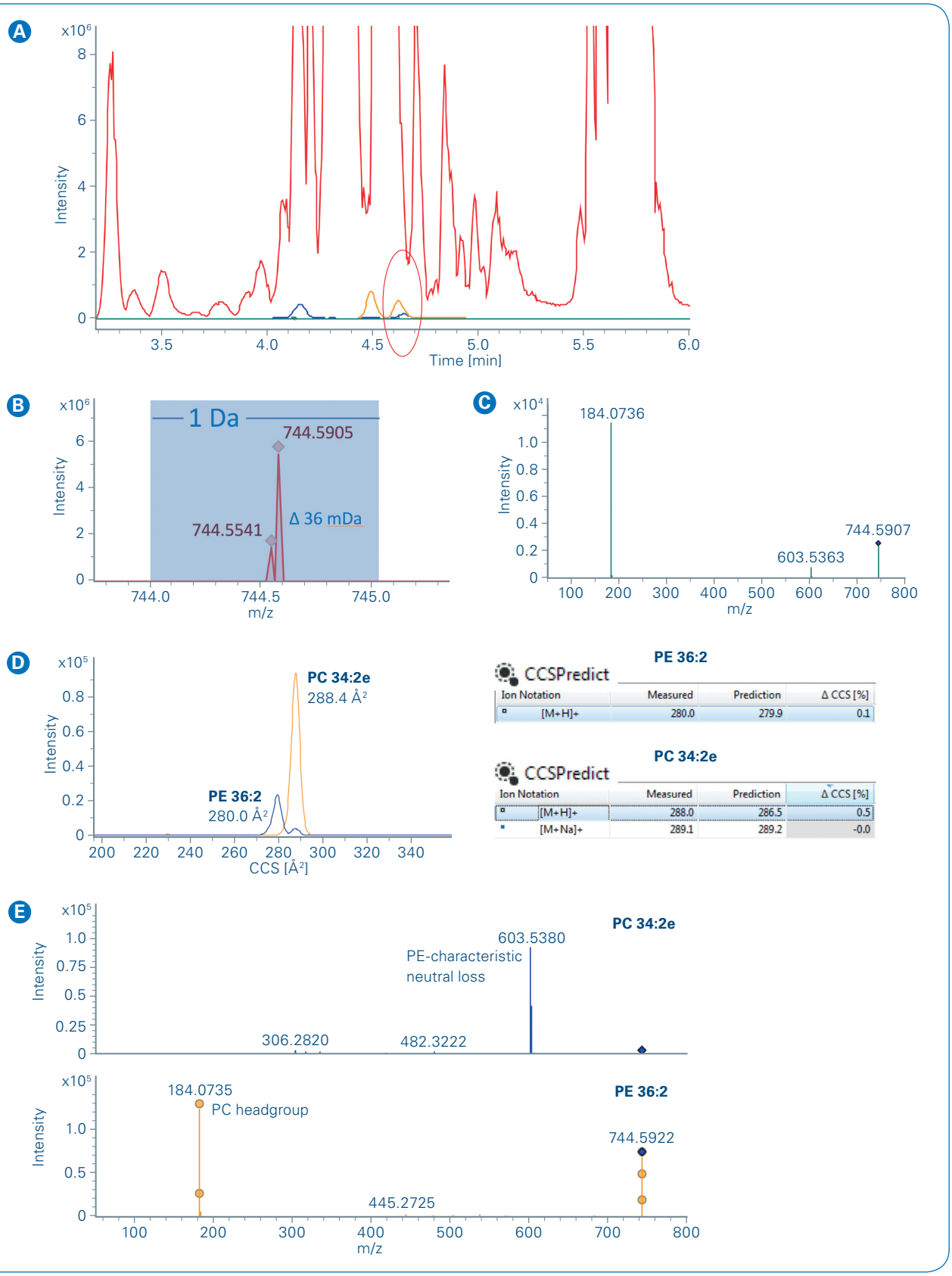



*Figure 4:* **A** *Base peak chromatogram (red), extracted ion chromatogram traces of an isobaric PC 34:2e (744.5911 m/z, orange) and PE 36:2 (744.5575 m/z, blue).* **B** *MS spectrum showing the acquired precursor masses. The difference is 36 mDa only.* **C** *The mixed MS/MS spectrum was acquired without additional mobility separation.* **D** *Extracted ion mobilograms showing a near-baseline separation of the two lipids and as well the matching of the measured vs. predicted CCS values.* **E** *Clean MS/MS spectra achieved by the mobility separation in PASEF mode.*

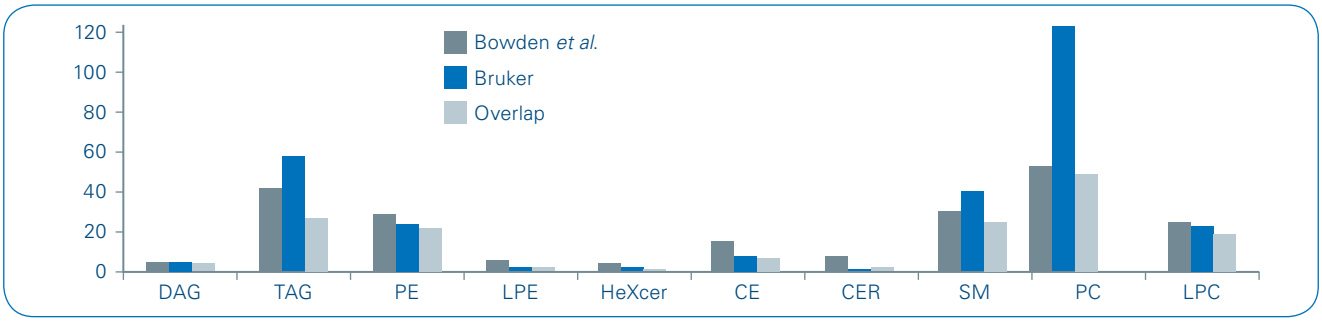
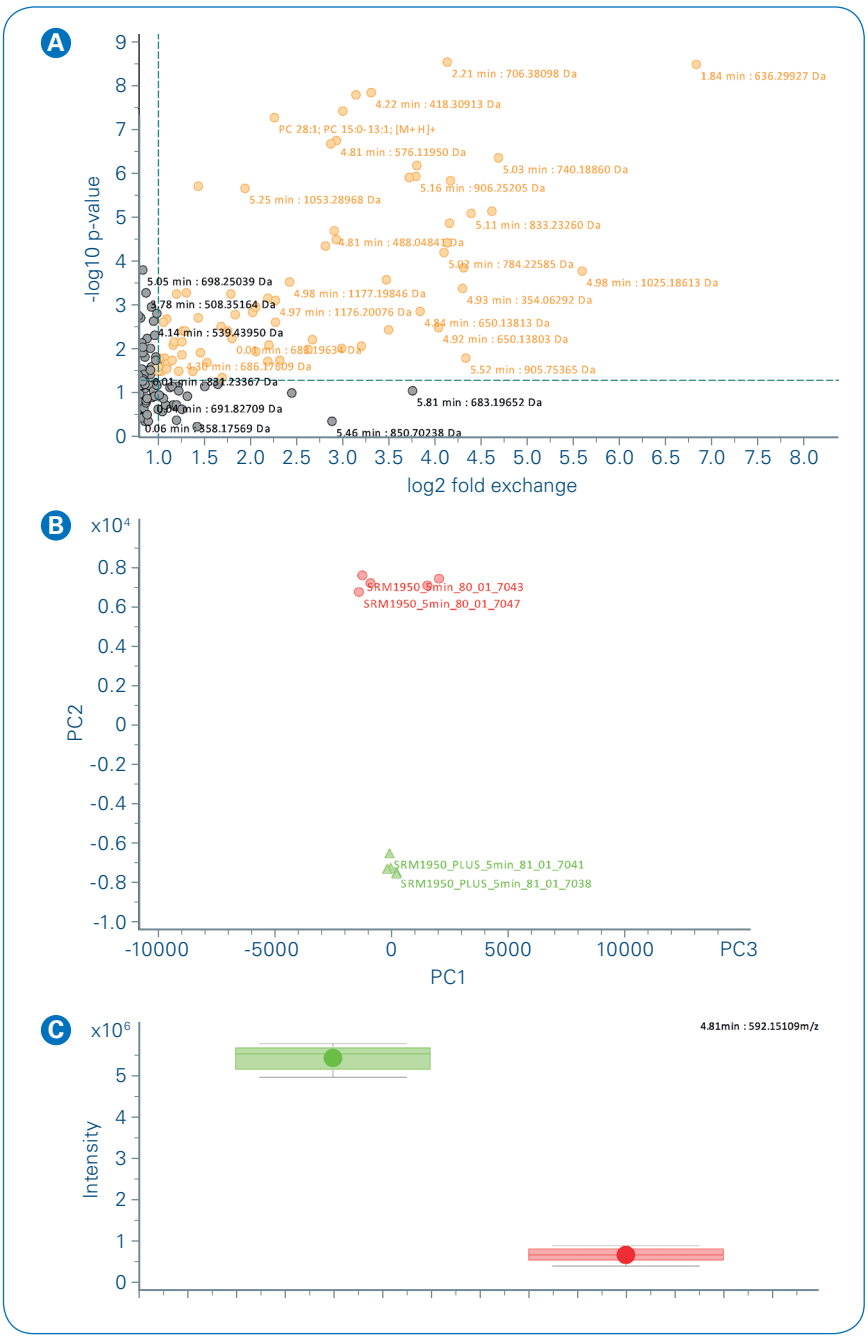



*Figure 5: Comparing the identified lipid classes with data from an interlaboratory study. Especially for the Phosphatidylcholines (PCs), the presented approach shows improved performance*

*Figure 6:* **A** *Differences can be reliably detected via t-Test, even with 6 minute run times (Volcano plot, the dotted lines show the limits for p-value (0.05) and fold change (2)).* **B** *PCA plot showing the grouping of spiked vs. non-spiked samples (PC1 plotted vs. PC2).* **C** *Box plot of one of the spiked compounds*



## The *perfect* solution for high-throughput 4D Lipidomics


+


Elute UHPLC timsTOF Pro featuring PASEF

MetaboScape featuring CCSPredict

PASEF

timsTOF flex

timsTOF Pro

## Conclusions

The potential of PASEF to increase the sample throughput by 4D lipidomics profiling was demonstrated. The crucial ability to separate co-eluting isobaric compounds and to identify differences between sample groups was maintained. With this, PASEF proves to be an optimal acquisition mode for deep profiling as well as for projects with high turnover needs, e.g. in clinical lipidomics research. Additionally, in combination with CCS prediction by CCSPredict the automatically extracted, accurate and reproducible CCS values add confidence for lipid identification.

- PASEF enables to increase the sample throughput using 4D lipid profiling by a factor of almost four
- Even at reduced LC run times, trapped ion mobility separates co-eluting isobaric or isomeric compounds and provides accurate and reproducible CCS values for high confident lipid ID. These CCS values can be used to confirm structures using CCSPredict
- Complementary to an in-depth "ID as many as possible" approach, PASEF enables a very fast lipid profiling based on clean MS/MS spectra

# Learn More

You are looking for further Information?
Check out the link or scan the QR code for more details.

**www.bruker.com/metabolomics**


**References**

[1] Röhrig F & Schulze A (2016), Nat. Rev. Cancer **16**, 732–749

[2] Vriens K et al. (2019), Nature **566**, 403–406

[3] Yang Q, Vijayakumar A & Kahn BB (2018), Nat. Rev. Mol. Cell Biol. **19**, 654–672

[4] Meier F et al. (2015), J. Proteome Res. **14**, 5378–5387

[5] Shevchenko A et al. (2008), J. Lipid Res., **49**, 1137-1146

[6] https://fiehnlab.ucdavis.edu/projects/LipidBlast

[7] Bowden JA et al. (2017), J. Lipid Res. **58**, 2275–2288


For Research Use Only. Not for Use in Clinical Diagnostic Procedures.


**Bruker Daltonik GmbH**

Bremen · Germany
Phone +49 (0)421-2205-0

**Bruker Scientific LLC**

Billerica, MA · USA
Phone +1 (978) 663-3660

**ms.sales.bdal@bruker.com – www.bruker.com**