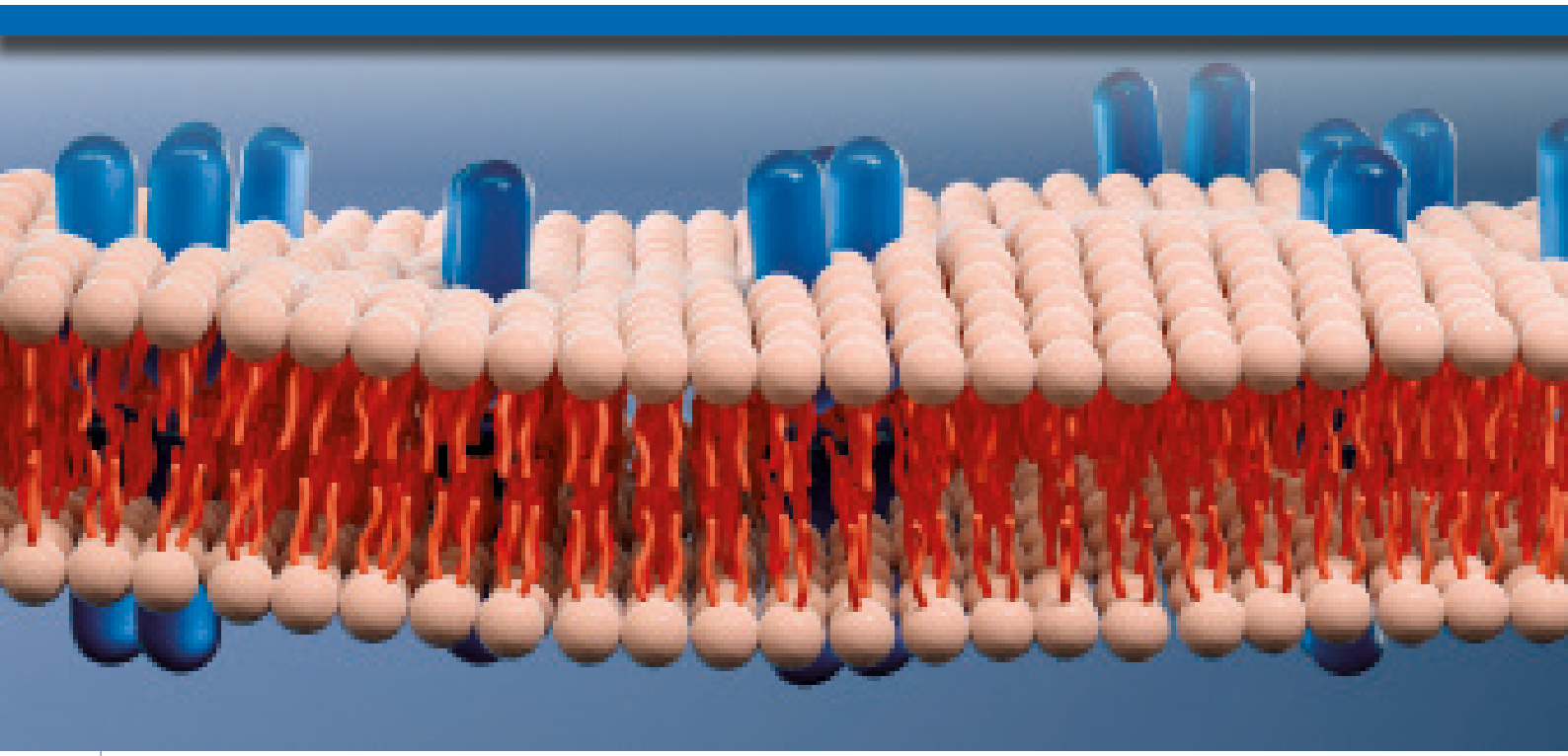

4D-Lipidomics™ workflow for increased throughput

Lipid profiling from complex lipid extracts can be a challenging and time consuming task. The high complexity of samples and co-elution of isobaric or isomeric compounds complicate the confident annotation of lipids. The presented 4D-Lipidomics workflow simplifies and streamlines the annotation and validation process using mobility-enhanced MS data.

Introduction

Lipids represent an important compound class in biological systems as they play key roles in cell membranes, in the energy storage or as signaling molecules. This makes them interesting markers for studying biological systems regarding clinical research [1]. At the same time, lipids depict a highly complex class of compounds. Both, the structural diversity as well as the high dynamic range in concentrations makes the analysis of lipid extracts challenging. A major bottleneck in lipid profiling is the annotation and subsequent validation of lipid assignments. Additionally, a high throughput is often necessary, e.g. in phenomics related large cohort studies.

Using the timsTOF Pro or timsTOF fleX systems, a high MS/MS coverage can be achieved by the PASEF® [2] acquisition mode, even with single injections and when using fast LC

Keywords: PASEF, TIMS, lipidomics, 4D-Lipidomics, KMD plot, SRM 1950, lipids, profiling, MetaboScape

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gradients. Furthermore, the trapped ion mobility separation cleans up the MS/MS spectra and improves the matching of fragmentation rules or spectral libraries. As a third factor of improvement, the automatically acquired CCS values can be directly used to increase the confidence in annotations. This 4D-Lipidomics workflow is demonstrated in detail in the following.

Methods

Lipids from NIST SRM 1950 reference plasma (Sigma-Aldrich, Germany) were extracted using methyl-tert-butyl ether (MTBE) based on a protocol published by Matyash *et al.* [3]. Extracts were dissolved in Methanol : Dichloromethane (9:1). The total amount of lipids injected on column equaled 0.5 µL and 5 µL extracted standard for positive and negative polarity, respectively. To assess the reproducibility of the system, 5 replicates were analyzed in each polarity (5 µL each).

The RP-LC separation was performed using an Elute UHPLC system (Bruker, Germany) and a Triart C18 column (100 x 2.1 mm, 1.9 µm) (YMC, Japan). Run times were 5, 10 and 20 minutes, respectively. The MS- and MS/MS data was acquired in positive and negative ESI mode using a timsTOF Pro (Bruker Daltonik, Germany) instrument in PASEF MS/MS mode. The transfer parameters were optimized for 100-1500 *m/z*.

The resulting four-dimensional data (*m/z*, RT, mobility, and intensity) was processed using MetaboScape® 2021b (Bruker, Germany). The T-ReX® 4D algorithm combined common adducts and isotopes belonging to the same compound into features in the bucket or feature table. Features detected in less than three of five samples were discarded. Furthermore, features with a Coefficient of Variance (CV) of >20% were excluded. The remaining features were annotated using a rule-based annotation tool in MetaboScape 2021b. It makes use of published fragmentation rules for >40 lipid subclasses and it annotates based on the precursor *m/z*, isotopic pattern, and characteristic fragments in the MS/MS spectra. To compare the number of unique lipid annotations on species level, only the most abundant species of isomers were considered. All isomers eluting with different retention times were removed.

Data of positive and negative ionization was processed separately and merged based on retention time and neutral mass in MetaboScape.

Table 1. Acquisition parameters

MS	**timsTOF Pro**
Source	Apollo II ESI source
Ionization	ESI (+ and -)
Acquisition mode	PASEF MS/MS, 100 ms ramp time, 2 PASEF MS/MS ramps per cycle
Calibration	Automatic internal mass calibration using Sodium Formate Mobility calibration before sequence using Agilent Tunemix
LC	**Elute UHPLC**
Column	YMC Triart C18 column (100 x 2.1 mm, 1.9 µm)
Column Oven Temp.	55°C (20 min gradient), 60°C (10 min gradient), 65°C (5 min gradient)
Flow rate	0.4 mL/min (20 min gradient), 0.5 mL/min (10 min gradient), 0.55 mL/min (5 min gradient)
Mobile phase	A: MeCN/H_2O (60:40 10 mM ammonium formate, 0.1% FA) B: Isopropanol/MeCN/H_2O (90:8:2, 10 mM ammonium formate, 0.1% FA)
20 min Gradient	0 min 40% B 2 min 43% B 2.1 min 50% B 12 min 54% B 12.1 min 70% B 18.0 min 99% B 18.1 min 40% B 20 min 40% B
10 min Gradient	0 min 50% B 0.6 min 50% B 2.0 min 55% B 6.5 min 80% B 6.6 min 95% B 8.6 min 99% B 8.7 min 50% B 10 min 50% B
5 min Gradient	0 min 50% B 0.3 min 50% B 0.75 min 60% B 0.85 min 85% B 3.0 min 80% B 3.05 min 95% B 4.33 min 99% B 4.35 min 50% B 5.0 min 50% B

Results

The aim of this study was to investigate the benefits of the timsTOF Pro / fleX-based 4D-Lipidomics worflow to profile the lipid content of a SRM 1950 extract. The scheme of the workflow is outlined in Figure 1. Each step will be discussed in more detail in the following.

4D-data acquisition

The timsTOF Pro and timsTOF fleX instruments are trapped ion mobility-enhanced QTOF mass spectrometers that provide an advanced acquisition mode (PASEF) for high-speed and high-quality MS/MS data [2,4,5]. Since confident lipid identification is dependent on high-quality fragment spectra, both a comprehensive coverage as well as sensitivity are of key importance for downstream analysis. In addition to generating cleaner MS/MS spectra, the trapped ion mobility separation automatically acquires accurate and precise CCS values that can be directly used in CCS-aware processing softwares to increase the quality and

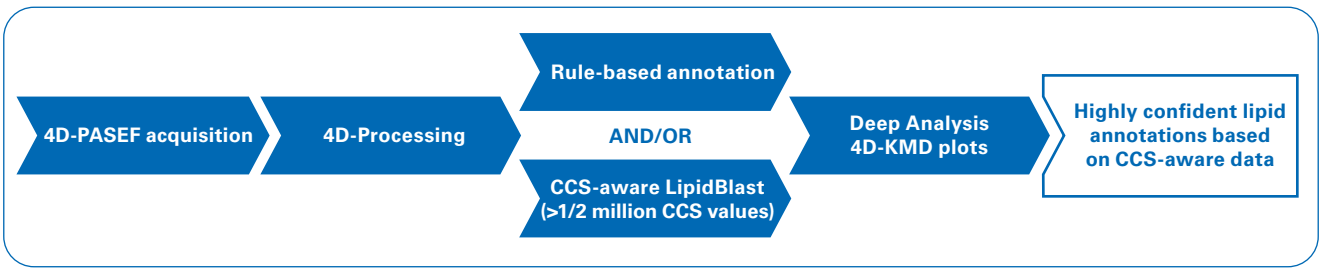

Figure 1. Fully integrated 4D-Lipidomics workflow for confident lipid annotations in MetaboScape 2021b.

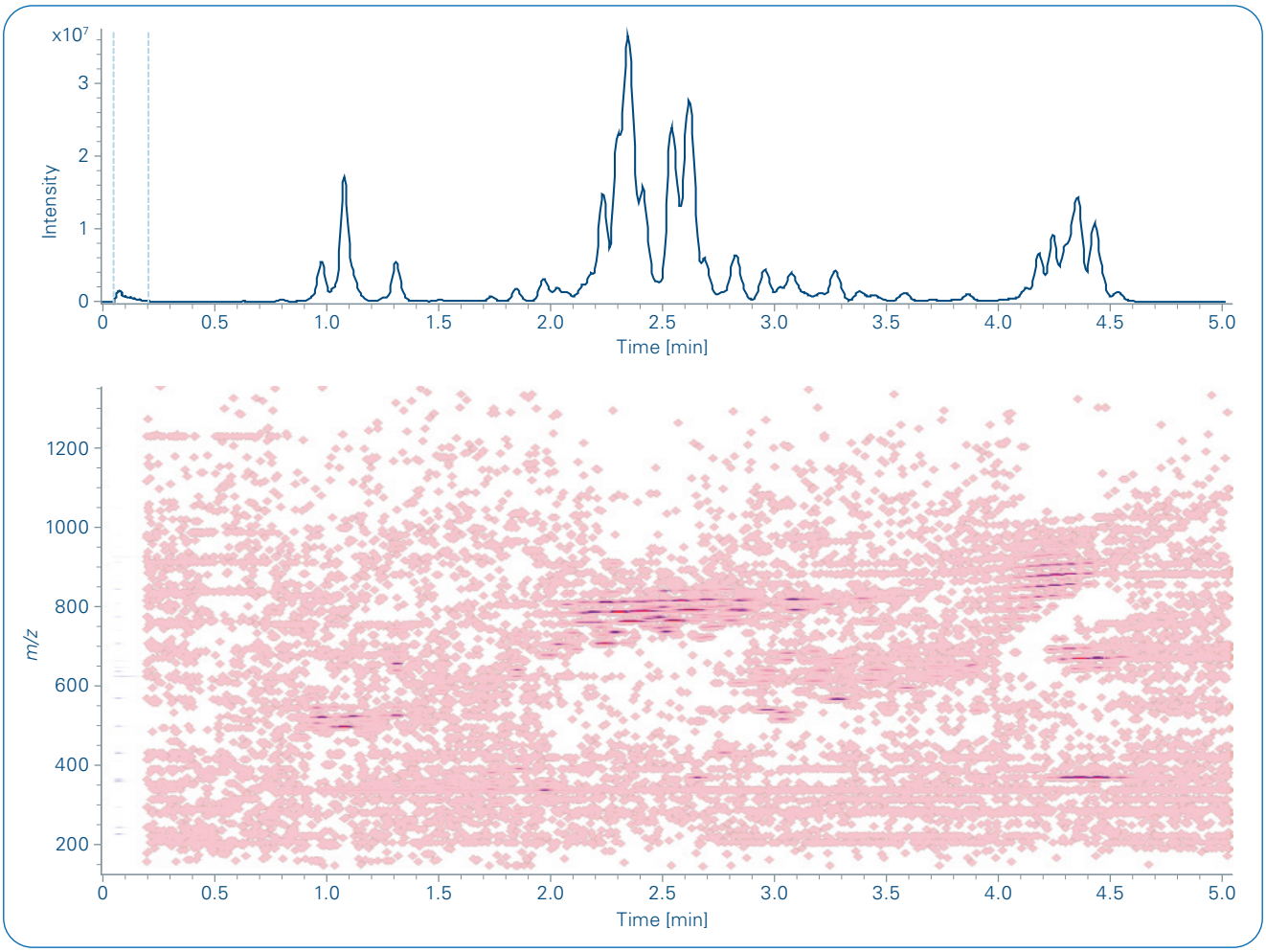

Figure 2. The heatmap shows the coverage of fragmented precursors (red squares) across the whole retention time and mass range. The first time segment was excluded from the selection. Typical data has an MS/MS coverage of > 65% from single injections.

hence also the user's confidence in results.

The coverage of MS/MS spectra acquired with PASEF is shown in Figure 2. Multiple replicate injections are not required to improve the precursor fragment coverage but were acquired to assess the Coefficient of Variance (CV) for filtering reproducible ions. This is not necessary if biological replicates are available.

4D-processing

MetaboScape 2021b is a server-client based software allowing multiple users to process and evaluate their data at the same time. It enables processing of 4D-timsTOF data as well as MALDI-TIMS-Imaging data, high-end MRMS data or standard QTOF data. The central processing algorithm, T-ReX (**T**ime aligned **R**egion complete **eX**traction) extracts all relevant feature information and creates a retention time aligned 4D compound or feature table. Automatically applied charge deconvolution, deisotoping and recursive feature extraction grant robust results as a basis for subsequent statistical experiments, dereplication or de-novo annotation workflows that are also implemented in the software solution. Figure 3 shows a screenshot of a compound table with example annotations from the presented data.

CCS-aware lipid annotation

To annotate lipids, MetaboScape offers two complementary workflows that can be used independently from each other. The rule-based lipid annotation is fully implemented in MetaboScape 2021b and aims at providing high-quality, confident lipid annotations according to the latest developments in the lipidomics community [6]. It features fragmentation rules for a wide range (> 40) of common lipid sub classes. By applying rule-based annotation, over-annotation of lipids can be avoided which can occur when matching lipid spectra against MS/MS libraries. An example of how the rule-based annotation is applied is illustrated in Figure 4 for the lipid PC 22:6_16:0.

Alternatively or additionally, the CCS containing open-source MS/MS library LipidBlast can be used. It was compiled by Tsugawa and Fiehn *et al.* [7] and contains > 1/2 million of CCS values for lipids, together with their *in-silico* generated MS/MS spectra. The CCS values of the library were predicted based on CCS values acquired on a timsTOF Pro instrument.

	RT [min]	CCS (Å²)	m/z meas.	Δm/z [ppm]	mSigma	MS/MS	MS/MS score	Ions	Name	Molecular For...	Annotations	AQ
1	10.50	295.8	758.57109	2.091	17.9		936.9		PC 16:0_18:2	$C_{42}H_{80}NO_8P$	LC	
2	13.34	302.5	786.60228	1.967	4.3		930.1		PC 18:2_18:0	$C_{44}H_{84}NO_8P$	LC	
3	2.03	239.6	496.33983	0.150	21.7		713.9		LPC 16:0	$C_{24}H_{50}NO_7P$	LC	
4	10.15	299.1	782.57098	1.981	3.2		923.5		PC 16:0_20:4	$C_{44}H_{80}NO_8P$	LC	
5	13.24	305.2	810.60186	1.386	28.9		909.5		PC 18:0_20:4	$C_{46}H_{84}NO_8P$	LC	
6	12.96	299.8	760.58645	1.777	11.5		937.7		PC 18:1_16:0	$C_{42}H_{82}NO_8P$	LC	
7	17.54	329.6	874.78667	0.973	31.8		891.9		TG 16:0_18:2_18:1	$C_{55}H_{100}O_6$	LC	
8	1.74	236.3	520.33994	0.338	9.6		705.2		LPC 18:2	$C_{26}H_{50}NO_7P$	LC	
9	9.00	295.6	703.57579	1.339	18.3		949.6		SM 34:1;O2	$C_{39}H_{79}N_2O_6P$	LC	
10	13.86	305.4	788.61729	1.149	20.2		915.5		PC 18:0_18:1	$C_{44}H_{86}NO_8P$	LC	
11	2.86	247.5	524.37114	0.131	28.1		682.8		LPC 18:0	$C_{26}H_{54}NO_7P$	LC	
12	13.57	306.4	812.61732	1.151	27.0		911.8		PC 20:3_18:0	$C_{46}H_{86}NO_8P$	LC	
13	17.80	331.3	876.80255	1.172	11.9		899.8		TG 16:0_18:1_18:1	$C_{55}H_{102}O_6$	LC	
14	17.30	327.3	872.77113	1.104	49.7		740.1		TG 18:2_18:2_16:0	$C_{55}H_{98}O_6$	LC	
15	11.40	300.8	784.58641	1.694	17.0		927.1		PC 20:3_16:0	$C_{44}H_{82}NO_8P$	LC	
16	17.50	326.3	848.77098	0.953	2.9		867.9		TG 18:1_16:1_16:0	$C_{53}H_{98}O_6$	LC	
17	14.37	315.6	813.68513	0.893	22.8		941.5		SM 42:2;O2	$C_{47}H_{93}N_2O_6P$	LC	
18	2.16	242.2	522.35583	0.788	40.6		705.0		LPC 18:1	$C_{26}H_{52}NO_7P$	LC	
19	9.56	301.8	806.57052	1.344	34.9		930.6		PC 16:0_22:6	$C_{46}H_{80}NO_8P$	LC	
20	10.90	299.6	784.58619	1.418	30.4		925.4		PC 18:1_18:2	$C_{44}H_{82}NO_8P$	LC	
21	14.40	312.8	787.66946	0.896	10.7		955.2		SM 40:1;O2	$C_{45}H_{91}N_2O_6P$	LC	
22	13.21	303.1	786.60112	0.582	7.6		931.1		PC 18:1/18:1	$C_{44}H_{84}NO_8P$	LC	
23	17.77	330.3	850.78659	0.911	7.5		885.5		TG 18:1_16:0_16:0	$C_{53}H_{100}O_6$	LC	
24	17.25	324.0	846.75535	0.980	7.6		628.9		TG 18:2_16:0_16:1	$C_{53}H_{98}O_6$	LC	
25	1.69	239.0	544.33975	-0.025	13.1		728.2		LPC 20:4	$C_{28}H_{50}NO_7P$	LC	
26	17.49	323.6	822.75528	0.924	5.2		918.8		TG 18:1_14:0_16:0	$C_{51}H_{96}O_6$	LC	
27	13.86	312.1	811.66893	0.271	24.4		905.7		SM 18:1;O2/24:2	$C_{47}H_{93}N_2O_6P$	LC	

Figure 3. Screenshot of a feature table in MetaboScape. The Name *column lists the annotated lipids on species or molecular species level. The icon in the Annotation Quality* (AQ) *column gives a quick visual feedback on the quality of the annotation for the key parameters Rt,* m/z*, isotopic pattern quality and MS/MS matching.*

Imported into MetaboScape, the library can directly be used to annotate the feature table. This complementary annotation can increase the coverage of lipid classes. Additionally, it enables to gain a higher confidence in the results by confirming previous annotations and matching MS/MS spectra as well as CCS values. Figure 5 shows an example of the data.

Both annotation strategies use the Annotation Quality Scoring (AQ column in Figures 4 and 5) to give a visual feedback on the key qualifiers (rt, *m/z*, isotopic pattern, MS/MS matching, CCS for LipidBlast). The ranges for this rating are user-defined.

In addition, the acquired CCS values can be used to verify potential lipid

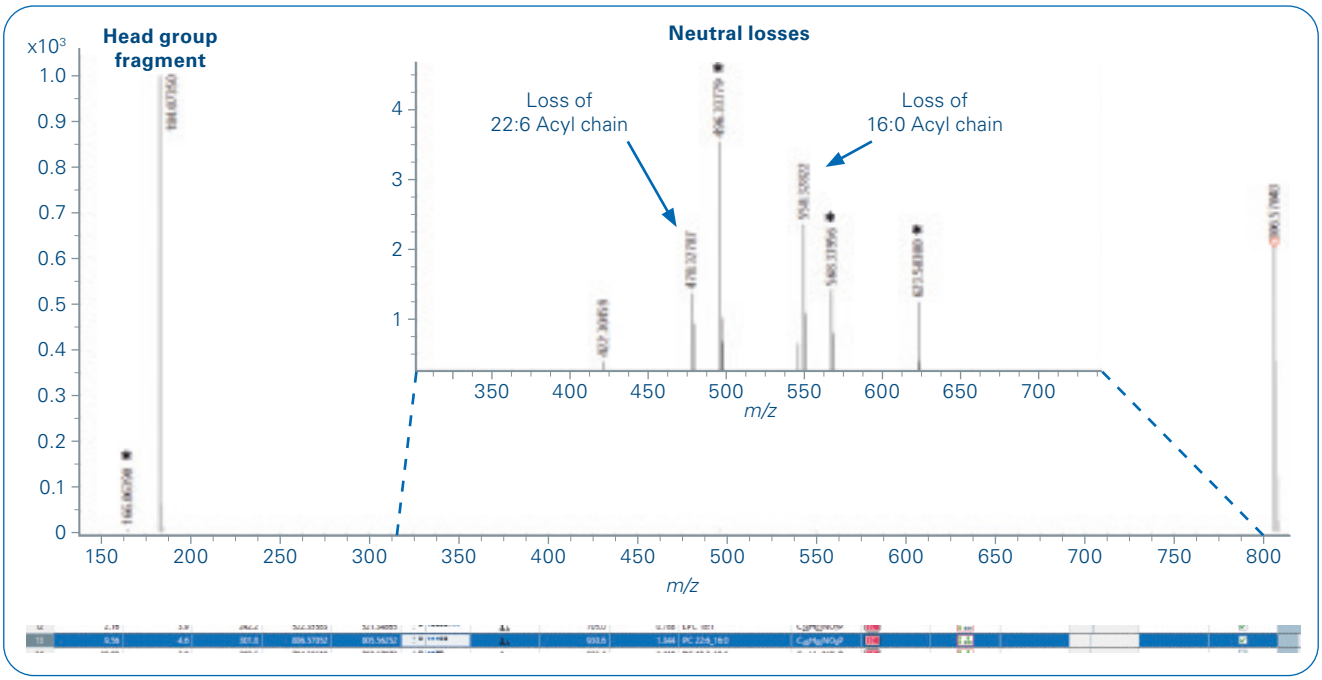

Figure 4. Annotated MS/MS spectrum of PC 22:6_16:0. The annotation was based on the characteristic head group fragment (m/z 184). In combination with the precursor's accurate mass and isotopic pattern, this lipid can be identified on species level as PC 38:6. Since additional neutral losses of acyl chains are present in the spectrum, the annotation is performed on the molecular species level, resulting in a confident annotation as PC 22:6_16:0. Fragments marked with an asterisk are further fragments from the lipid that were not used for annotations.

	RT [min]	CCS (Å²)	ΔCCS [%]	m/z meas.	Δm/z [ppm]	mSigma	MS/MS	Ions	Name	Molecular For...	Annotations	AQ
1	1.50	226.3	0.2	518.32564	3.505	106.1			LPC 18:3-SN2	$C_{26}H_{48}NO_7P$		
2	1.50	225.7	0.6	468.30877	0.613	21.1			LPC 14:0-SN2	$C_{22}H_{46}NO_7P$		
3	1.69	239.0	2.0	544.33971	-0.095	12.2			PC O-20:4	$C_{28}H_{50}NO_7P$		
4	1.81	219.6	0.3	478.29316	0.716	11.5			PE O-16:2_2:0	$C_{23}H_{44}NO_7P$		
5	1.81	235.5	0.2	546.35801	4.753	53.7			PC O-20:3	$C_{28}H_{52}NO_7P$		
6	2.14	222.9	1.8	454.29263	-0.704	24.2			PE O-12:0_4:0	$C_{21}H_{44}NO_7P$		
7	2.18	233.1	0.3	496.34117	2.818	16.4			PC O-16:0	$C_{24}H_{50}NO_7P$		
8	5.36	276.6	1.1	673.52696	-1.391	72.8			SM 18:2;2O/14:0	$C_{37}H_{73}N_2O_6P$		
9	7.88	281.5	1.0	730.53887	0.971	2.3			PC 16:1_16:1	$C_{40}H_{76}NO_8P$		
10	8.19	284.8	0.5	756.55387	0.260	12.4			PC 16:1_18:2	$C_{42}H_{78}NO_8P$		
11	8.99	286.9	0.6	703.57513	0.297	37.4			SM 18:1;2O/16:0	$C_{39}H_{79}N_2O_6P$		
12	9.03	285.4	0.6	756.55444	0.669	27.8			PC 14:1_20:2	$C_{42}H_{78}NO_8P$		
13	10.22	275.0	2.3	732.55507	1.876	190.0			PC 18:0_14:1	$C_{40}H_{78}NO_8P$		
14	10.95	291.4	0.0	808.58622	1.835	81.4			PC 18:1_20:4	$C_{46}H_{82}NO_8P$		
15	11.56	294.2	0.3	818.60531	-1.907	129.8			PC O-40:7	$C_{48}H_{84}NO_7P$		
16	11.84	292.7	0.5	792.59148	0.999	55.4			PC O-38:6	$C_{46}H_{82}NO_7P$		
17	12.84	288.3	1.1	757.62208	0.904	94.4			SM 16:0;2O/22:2	$C_{43}H_{85}N_2O_6P$		
18	12.97	305.4	2.3	783.63830	1.078	119.4			SM 22:1;2O/18:2	$C_{45}H_{87}N_2O_6P$		
19	13.20	296.0	1.4	810.60110	0.460	50.2			PC 20:1_18:3	$C_{46}H_{82}NO_8P$		

Figure 5. Screenshot of a feature table that was annotated with the CCS-containing LipidBlast library in addition to the rule-based annotation. The AQ scoring also features the matching of CCS values.

structures by comparison with predicted values. This is possible in MetaboScape using an implemented tool called CCSPredict. The CCS values for several adducts can be compared to increase the user's confidence in the automatic annotations.

Deep analysis of annotation results

To simplify the process of validation of the annotations, MetaboScape features a 4-dimensional Kendrick mass defect (KMD) plot that enables a deep analysis of the annotation results [8]. Annotated lipids can be validated and outliers (possible false positives) as well as missing lipids (possible false negatives) can be readily identified. In Figure 6, a KMD plot for the PC lipid class is shown, demonstrating the power of this plot to validate annotations based on trend lines that can be observed for the homologous series.

Highly confident lipid annotations based on CCS-aware results

To investigate the increased MS/MS quality and peak capacity provided by PASEF, we analyzed a lipid extract of NIST SRM 1950 reference plasma with different chromatographic run times of 20, 10 and 5 minutes. As shown in Figure 7a, the maximum number of unique identified lipids (362) was observed with 20 min gradient times. Even when reducing the runtime to 25% (5 min), still 75% (271) of the annotated lipids with a CV of less than 20% where detected.

A comparison of the measured CCS values from the 20 min and 5 min gradients shows a very good correlation (Figure 7b). This demonstrates that in contrast to retention times, CCS values are independent of chromatography and can be reliably used for compound identification (Figure 7C). The results highlight the exceptional usefulness of CCS values for compound identification. Different gas conditions during the ionization of compounds did not influence the collisional cross sections acquired in TIMS. These results set the stage for deep lipidomics profiling at high throughput to enable studies that require a high turnover, for example clinical research studies.

In addition, the 4D-Lipidomics workflow can be leveraged by using the annotated compounds in combination with MALDI Imaging experiments. The so-called SpatialOMx® workflow matches accurate mass and CCS values to correlate the annotations to MALDI Imaging experiments. This enables to assess e.g. the spatial distribution of identified lipids in tissue [9,10].

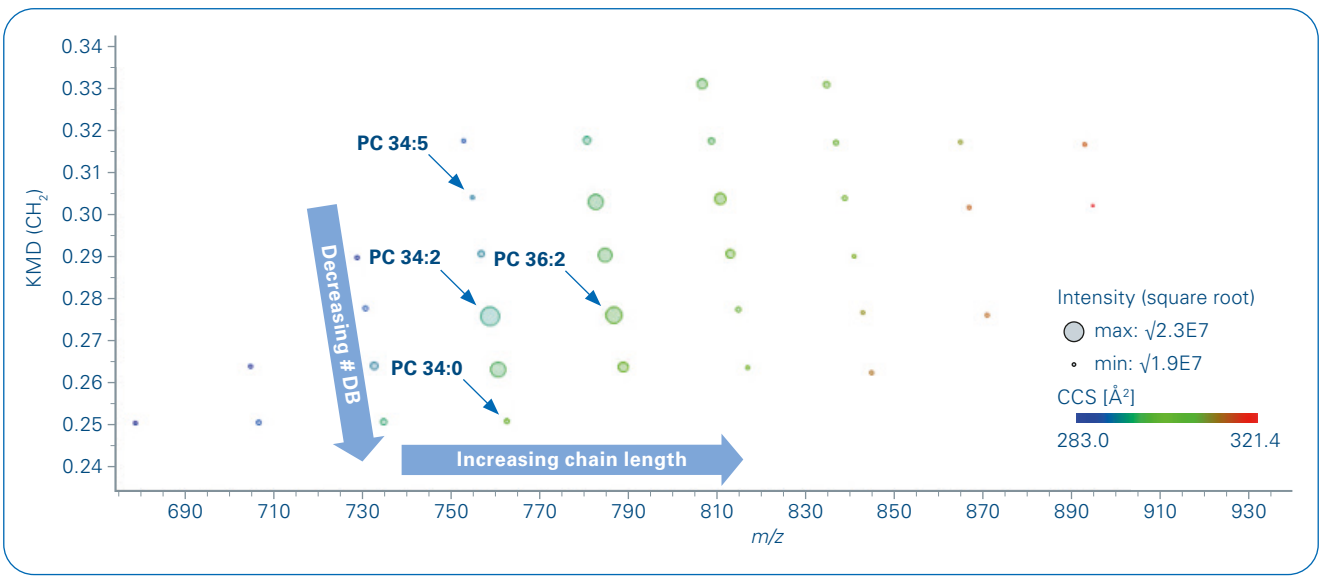

Figure 6. 4D Kendrick mass defect (KMD) plot filtered to display unique PC lipids. This plot shows m/z *on the x-axis vs. KMD (CH_2) on the y-axis. CCS values are represented by a color gradient and intensities by bubble size. Trend lines can be used to assess potential wrong annotations or missing ones.*

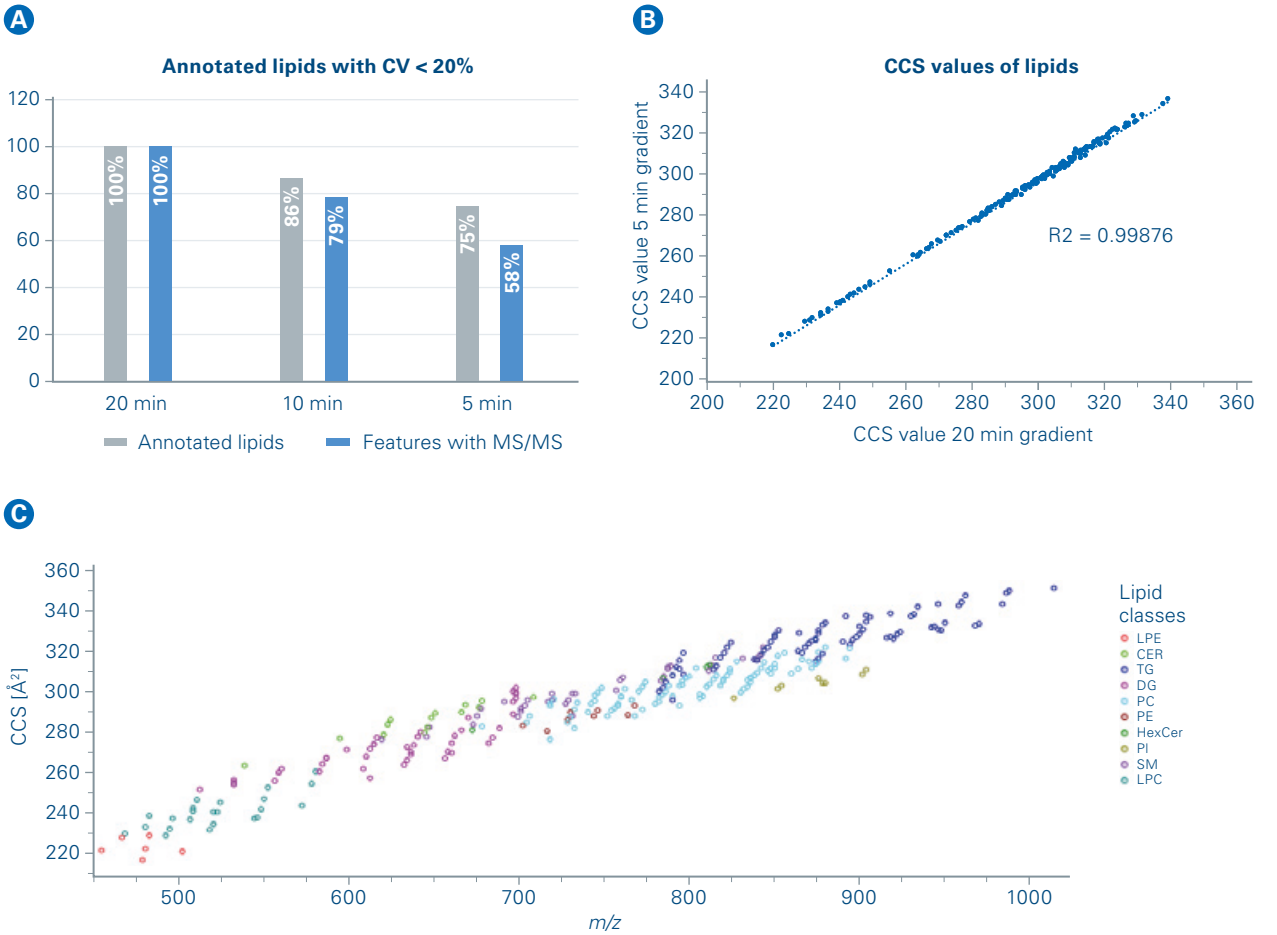

Figure 7. Ⓐ *Overview of the number of annotated unique lipids at different LC runtimes from merged data of both polarities. Reducing the LC runtime by a factor of 4, the number of annotated lipids decreases to 75%. The relative values refer to 20 min (362 lipids, 7592 features with MS/MS).* Ⓑ *Comparison of observed lipid CCS values from 5- and 20-min gradient run. The plot demonstrates the power of CCS values as a qualifier for compound identification as the CCS values are independent of the chromatographic setup.* Ⓒ *Distribution of the detected lipids in the* m/z *and CCS space (5 min runs, ESI-(+)). For different lipid classes, clear trends can be observed that enable validation of the annotations.*

Conclusion

The presented 4D-Lipidomics workflow features highly confident CCS-aware lipid annotation by state-of-the-art rule-based annotation. It simplifies the inspection of the annotations by 4D Kendrick Mass Defect plots. The timsTOF Pro or fleX instruments build the basis of this workflow delivering high-quality data at short runtimes.

- PASEF enables high throughput lipid profiling.
- The 4D-Lipidomics workflow ensures a confident annotation of a wide range of lipids, even from single injections.
- The lipid annotation tool in MetaboScape provides rule-based annotation for >40 sub-classes and reports according to most recent guidelines.
- Increasing the chromatographic speed four-fold, the presented workflow still yielded 75% of the maximum annotations observed. This underlines the suitability for high-throughput studies.
- By matching the CCS values and accurate *m/z* of annotated lipids, the spatial distribution e.g. in tissue can be assessed by MALDI Imaging data using the SpatialOMx workflow.

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