

● Investigating the increased lifespan in *C. elegans* *daf-2* mutants by 4D-Lipidomics

The small nematode *Caenorhabditis elegans* is one of the premier biomedical model organisms and employed in many aspects of basic and applied science

Introduction

Typical application areas for *C. elegans* are aging and longevity research, host-pathogen interactions, neurobiology and others. Its genetic tractability and the ease of cultivation of these mostly self-fertilizing hermaphrodites make

it possible to raise a large population of genetically identical individuals in a short time. The *C. elegans daf-2* gene investigated in this study encodes for the insulin-like growth factor 1 (IGF-1) receptor. *daf-2* mutants were one of the first mutations in *C. elegans* shown to extend

lifespan. The mutant worms exhibit extreme changes in their phenotype compared to wild type worms, including increased adult size and an increased lifespan. Furthermore, changes in the lipid content were reported in *daf-2* mutants [1].

4D-Lipidomics, PASEF, timsTOF Pro, *C. elegans*

Recently, lipidomics, the systematic analysis of all lipids of an organism, joined the *C. elegans* toolbox. Different methods have been used to study the lipid metabolism in this model system and are reviewed elsewhere [2]. Due to the variation in headgroups and fatty acids that can be incorporated into lipids, the lipidome in general is very complex and requires dedicated analysis techniques like UPLC-UHR-ToF-MS [3]. Several specific characteristics were identified in comparison to mammalian systems, e.g. the occurrence of a C17iso branched sphingoid base instead of the typical C18 base in mammals [4].

A high coverage of detected lipids with a corresponding MS/MS spectrum is required for a deep profiling of the lipidome. Using the timsTOF Pro system, this is realized by the unique PASEF (Parallel Accumulation Serial Fragmentation) acquisition mode [5]. PASEF offers the possibility to generate high-quality MS/MS spectra at unmatched acquisition speeds. It can generate clean MS/MS spectra by separating isobaric lipid species co-eluting in the LC domain [6]. Additionally, Trapped Ion Mobility Separation (TIMS) provides highly reproducible Collisional Cross Section (CCS) values for increased confidence in lipid identification.

Here we present a fully integrated workflow for evaluating 4D-Lipidomics data in a single software solution: MetaboScape®. A comparison of the lipid extracts from *C. elegans* wild type and *daf-2* mutants revealed several regulated lipids. Using the 4D-Lipidomics workflow, they were confidently identified. In this process, positive and negative data from PASEF MS/MS measurements provided complementary information on lipid headgroups and fatty

acid side chains. Matching measured CCS values to predicted values substantiated lipid assignments. This prediction is enabled by the machine learning based tool CCSPredict in MetaboScape.

Experimental

C. elegans cultivation

C. elegans strains N2 Bristol and *daf-2(e1370)* were cultivated on nematode growth medium (NGM) using *Escherichia coli* OP50 as sole food source. After age synchronization

by bleaching, the worms were grown until the first day of adulthood, harvested and washed twice with M9 buffer. Each biological replicate contained 5000 adult worms. Samples were snap-frozen in liquid nitrogen and stored at -80°C until extraction.

Lipid extraction

Lipids were extracted by a modified version of a methyl-tertiary-butyl ether (MTBE) extraction originally developed by Matyash *et al.* [3, 7]. Briefly, worms were suspended in 500 μL methanol and homogenized

Table 1: UHPLC MS equipment and setup for 4D-Lipidomics profiling

MS	timsTOF Pro
Source	Apollo II ESI source
Ionization	ESI(+), 4500 V Capillary Voltage ESI(-), 4200 V Capillary Voltage
Scan range	100–1500 m/z
Calibration	Internal mass calibration through automation, sodium formate, Mobility calibration before sequence using Agilent Tunemix
PASEF	Positive mode precursors were fragmented from 300-1500 m/z. Negative mode precursors were fragmented from 100-1500 m/z.
LC	Elute UHPLC
Column	Bruker intensity C18 column (100 x 2.1 mm, 1.9 μm)
Column Oven Temp.	55°C
Flow Rate	0.4 mL/min
Mobile phase	A: Acetonitrile / water (60:40, 10 mM NH_4 Formate, 0.1% FA) B: Isopropanol / acetonitrile (90:10, 10 mM NH_4 Formate, 0.1% FA)
Wash solvents	Strong wash: Isopropanol / acetonitrile (90:10) Weak wash: Acetonitrile / isopropanol / water (40:30:30)
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 and evaluation	MetaboScape® 5.0 (preliminary Version)

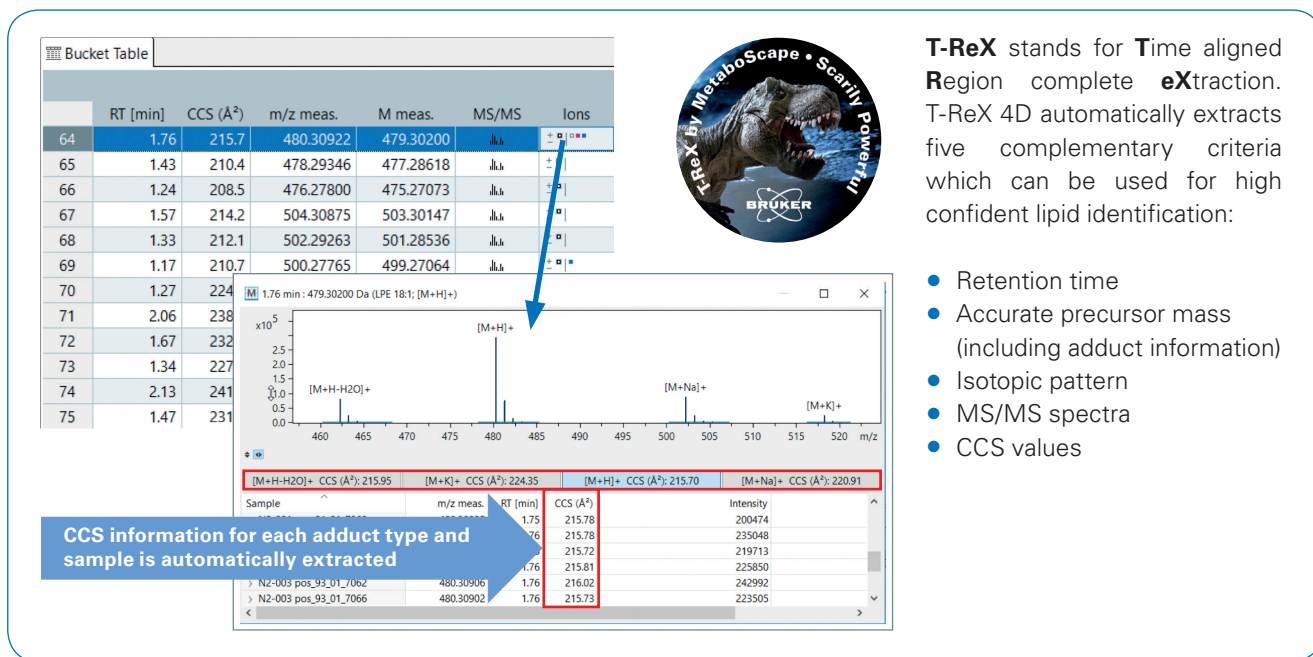


Figure 1: 4D-Lipidomics feature extraction technology: T-ReX 4D

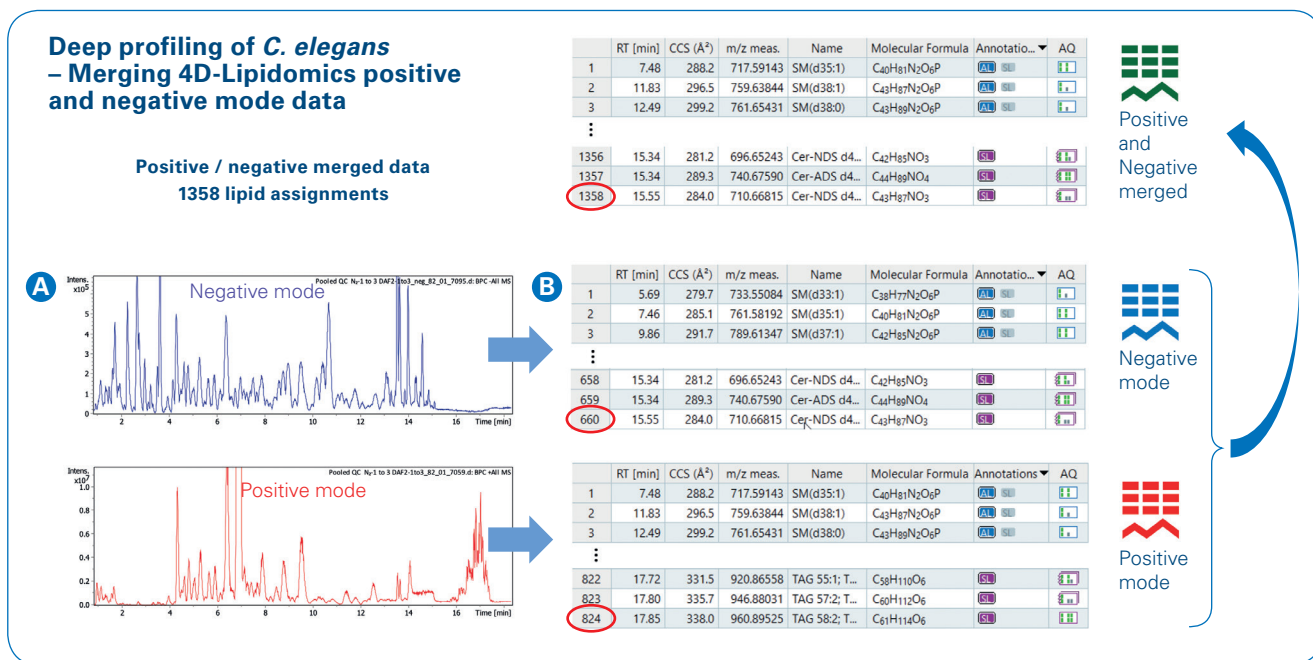


Figure 2: 4D-Lipidomics: **A** Base peak chromatograms of selected pooled Quality Control sample from *C. elegans* lipid extracts measured in positive mode (bottom, red) and negative mode (top, blue) by LC-PASEF. **B** Automatic lipid assignment of T-ReX 4D extracted lipids by LipidBlast [9-12] and custom Analyte list. 824 lipid assignments in ESI positive mode (bottom), 660 lipid assignments in ESI negative mode (middle), and 1358 lipid assignments in positive / negative mode merged data (top).

in a Precellys Bead Beating system. Subsequently, the samples were transferred to 4 mL glass vials. After addition of 1.7 mL MTBE the samples were vortexed and incubated for 60 minutes at room temperature. 420 μ L water were added to induce phase separation. Samples were centrifuged at 4°C. The upper organic phase was transferred to fresh 4 mL glass vials and the lower phase was re-extracted with additional 650 μ L MTBE. After centrifugation, the organic phases were combined and evaporated. The residue was reconstituted in 500 μ L acetonitrile / isopropanol / water (65/30/5, v/v/v)

and stored in 125 μ L aliquots at -80°C until analysis.

Three biological replicates each of wild type and *daf-2* mutant lipid extract were analyzed. A pooled quality control sample was generated by combining equal amounts from all six samples. All samples were analyzed as three technical replicates. For ESI positive and negative mode measurements 2 μ L and 10 μ L were injected, respectively.

Data acquisition

see Table 1

Results

T-ReX 4D feature extraction

The 4D-Lipidomics data generated by LC-PASEF on the timsTOF Pro system provides complementary retention time, accurate precursor mass, true isotopic pattern, mobility ($1/k_0$), and MS/MS information. By default, the **T**ime aligned **R**egion complete **eX**traction algorithm

T-ReX 4D performs a retention time alignment (see Figure 1). Mass and mobility calibration of the raw data are applied optionally as well.

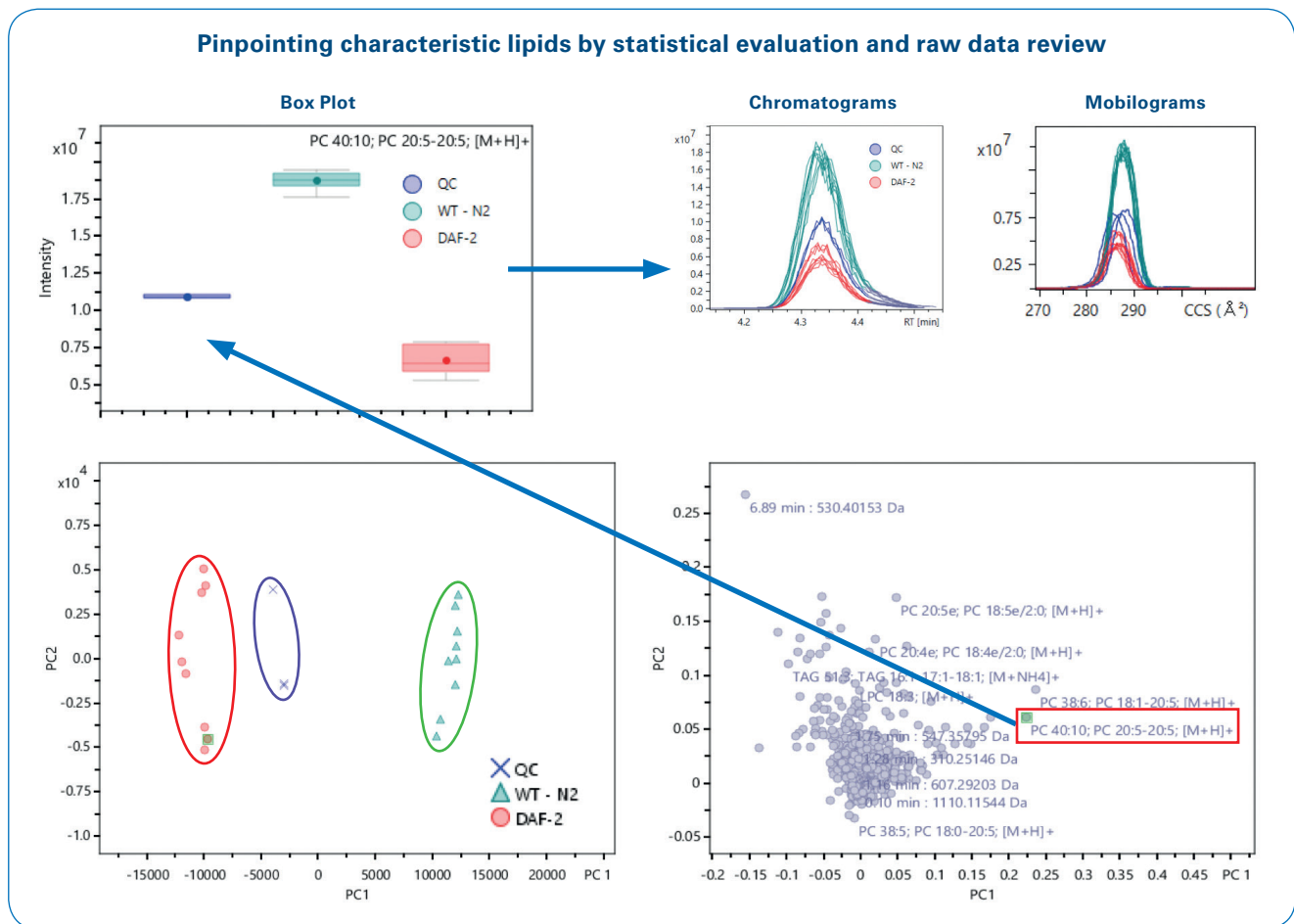


Figure 3: Principal Component Analysis (PCA) of 4D-Lipidomics data. The scores plot (bottom left) shows clear separation of wild type and *daf-2* mutant lipid extracts. Pooled QC samples cluster in the middle between wild type and *daf-2* mutants. One loading contributing to wild type and mutant sample separation is highlighted (bottom right). Interlinked displays and access to raw data in MetaboScape enables quick visual validation of statistical changes observed in PCA. The Box plot, chromatogram and mobilogram view for the lipid assigned as PC 40:10 are shown in the top of the figure.

Ions belonging to the same lipid are automatically combined into so called features which are collected for all samples in the Bucket Table. These buckets or features include isotopic peaks, adducts, as well as neutral losses. Furthermore, the acquired MS/MS spectra are assigned to the different ion types of a feature. The region complete extraction routine also ensures that for small peaks which were missed in the first pass extraction, the intensity values are provided for robust statistics. This happens in a targeted second pass extraction triggered automatically by the T-ReX algorithm (= recursive extraction). Finally, the ion mobility information is automatically converted from $1/k_0$ values to collisional cross section values (CCS) for all extracted features.

Deep profiling by 4D-Lipidomics:
> 1000 lipids assigned

Figure 2 highlights the deep profiling of the *C. elegans* lipidome by 4D-Lipidomics. Figure 2 A shows the base peak chromatograms of a selected QC sample analysed in negative (top) and positive (bottom) mode.

In the 21 data files from wild type, *daf-2* and pooled QC sample measured in positive mode, 824 lipid features could be tentatively annotated. The annotation was based on matching precursor masses and MS/MS spectra to the LipidBlast [9-12] *in-silico* library and by matching precursor masses to a custom Analyte List. This Analyte List was generated based on lipids reported to be present in *C. elegans*

[4]. All features annotated by the Analyte List also contained MS/MS spectra which enables a further validation of the annotations (see below). In negative mode, a total of 660 lipids were assigned by automatic LipidBlast MS/MS and Analyte List based annotation. MetaboScape enables the merging of tables generated in positive and negative ion mode. Based on such a merged bucket table from the *C. elegans* 4D-Lipidomics experiment, 1358 lipids could be assigned by the joint LipidBlast and Analyte List based annotation approaches.

Pinpointing characteristic lipids

Following the tentative assignment of lipids, a statistical evaluation readily pointed to lipids with difference in

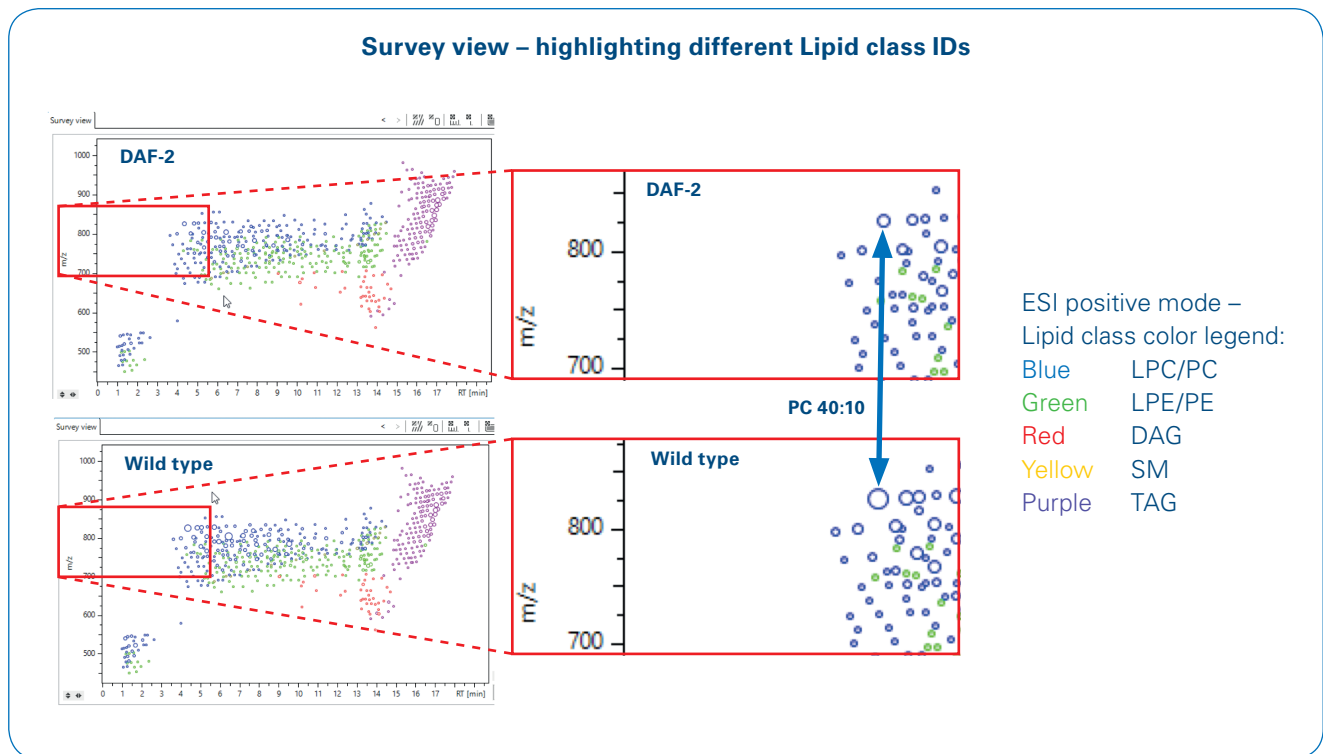


Figure 4: Interactive survey view display in MetaboScape provides further insights to raw data and lipid ID assignment. Here used to plot retention time vs. m/z and highlighting lipid class ID in color coding. The bubble sizes are reflecting peak intensities. Survey view and zoom-in for *daf-2* mutant (top) and wild type (bottom) lipid extract. The highlighted PC 40:10 lipid shows higher intensity (bubble size) in wild type sample.

abundance between groups. The PCA scores and loadings plots in Figure 3 showed a clear separation of the wild type and mutant *C. elegans* worm extracts. One loading responsible for this differentiation is highlighted. The corresponding box plot representation as well as chromatogram and mobilogram views verified the higher abundance in wild type samples. This lipid was tentatively assigned as a phosphocholine PC 40:10.

The tentative assignment of lipid classes in MetaboScape enabled a flagging per class. Visualizing these by false color coding in the survey view of MetaboScape enables the user to readily check for possible wrong assignments. Figure 4 presents the survey views for two selected data files of *daf-2* (top) and wild type (bottom) lipid extracts. The survey view plots m/z versus retention time and the intensity of the extracted features as bubble size. The colour coding shows that the lipid classes are separated as expected by reversed phase chromatography (e.g. lysophospholipids (LPC) eluting early and triacylglycerides (TAGs) eluting last). This view quickly highlights lipids with potentially wrong assignment, e.g. if a LPC would elute later than a TAG. In addition, this view highlights the data points which correspond to the lipid assigned as PC 40:10. The circle sizes correspond to the abundances for this lipid in the wild type sample versus the *daf-2* mutant worms.

Verification of lipid assignment

To confirm the lipid assignment automatically generated by LipidBlast

MS/MS spectral library matching, the data acquired in positive and negative mode were merged in MetaboScape. As shown in Figure 5A, MS/MS spectra from positive and negative data acquisition are merged into the same feature to support the verification of the lipid identity. Figure 5B represents the PASEF spectrum acquired from the $[M+H]^+$ precursor. Evaluation of this MS/MS spectrum using SmartFormula3D in MetaboScape enabled the assignment of molecular formulae for fragment ions and corresponding neutral losses. This helped to confirm the identity of the lipid as phosphocholine based on the characteristic headgroup fragment with 184 m/z . Additionally, characteristic fatty acid and ketene neutral losses in this MS/MS spectrum indicated that the lipid is a PC 40:10, containing two C20 fatty acids with in total five double bounds (20:5). The MS/MS spectrum in negative mode substantiated this lipid identification. The top of Figure 5C shows the MS/MS spectrum acquired from the $[M+HCOOH-H]^-$ precursor. Below, the MS/MS LipidBlast library spectrum is shown. Only the characteristic 20:5 fatty acid side chain fragment is present in the PASEF spectrum.

In summary, the information of characteristic fragment ions from positive and negative mode MS/MS spectra enabled to verify the identity of the target lipid to be PC 40:10; PC 20:5-20:5. Our assignment of PC 20:5-20:5 is substantiated by earlier work from Castro *et al.* [1] who also reported this lipid to be characteristic and higher abundant in wild type vs. *daf-2* mutant *C. elegans* worms.

CCSPredict for higher confidence in lipid IDs

PASEF measurements acquired on the timsTOF Pro not only generate clean spectra and hence characteristic fragment ions for lipids (see also [6]) but at the same time they provide reproducible collisional cross section (CCS) values. MetaboScape automatically extracts these CCS values from the raw data and enables an optional recalibration of the mobility domain. Figure 6A shows the CCS values for the lipid PC 20:5-20:5 extracted from the 21 LC-PASEF runs investigated in this study. The deviation across these measurements was only 0.23%, highlighting a very high reproducibility of CCS values generated by the timsTOF Pro instrument.

CCS values are characteristic for analytes and can provide additional information for increasing the researcher's confidence in compound annotation. MetaboScape enables to predict CCS values for lipid structures using the CCSPredict routine, which is based on a machine learning algorithm from Zhou *et al.* [13]. As shown in Figure 6B for the PC 20:5-20:5, CCSPredict generated for both the $[M+H]^+$ and the $[M+Na]^+$ species CCS values with a low deviation to the measured ones. Thus, CCSPredict provided further evidence for the lipid assignment.

MetaboScape uses the extra mobility dimension in the Annotation Quality scoring (AQ). This score reports up to 5 criteria according to user definable confidence levels (see Figure 5C). A custom Analyte List created using

the information obtained for PC 20:5-20:5 enables a quick de-replication in future studies. All five complementary criteria for lipid identification (precursor mass, retention time, isotopic pattern fidelity, MS/MS similarity, CCS) can be matched automatically and will be used in the AQ score. If this matches perfectly, as shown in Figure 5C, all criteria of the AQ scores will be highlighted with two green bars.

CCS value matching vs. public repositories

The rapidly increasing interest in CCS values as characteristic measure for target compounds has led to the generation of several CCS repositories. One of these is the CCS Compendium, with values generated on drift tube ion mobility systems (DT-IMS) [14]. For 70 lipids in positive mode and 30 lipids in negative mode identified

in *C. elegans* in this study, we found corresponding values in the CCS Compendium list (see Figure 7). The average deviation was <0.95% and <0.8%, respectively. This highlights that CCS values determined on timsTOF Pro instruments match CCS values generated on DT-IMS systems very well.

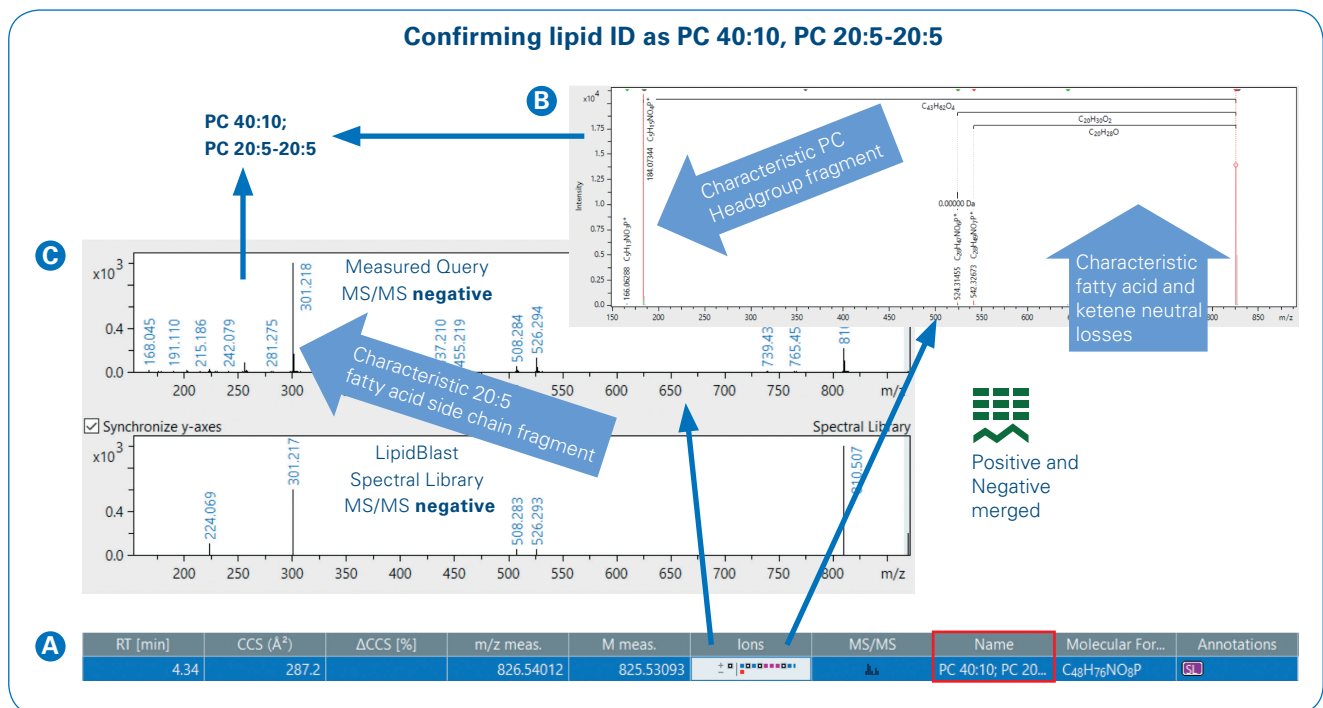


Figure 5: (A) Screenshot of a positive and negative mode merged bucket table in MetaboScape highlighting the extracted lipid PC 40:10. (B) Screenshot of MS/MS spectrum of [M+H]⁺ automatically annotated with molecular formula for fragment ions and neutral losses in SmartFormula3D. The characteristic headgroup fragment 184 m/z validates the automatic ID as phosphocholine. Neutral losses and fragment ions indicate side chains to be 20:5 fatty acids. (C) Measured MS/MS spectrum of [M+HCOOH-H]⁻ (top) and LipidBlast MS/MS library spectrum (bottom). Characteristic 301 m/z side fragment validates lipid ID as PC 20:5_20:5.

CCSPredict for higher confidence in Lipid ID

C

RT [min]	CCS (Å ²)	ΔCCS [%]	m/z meas.	mSigma	MS/MS score	Name	Molecular Formula	Annotations	AQ	Annotation Source
4.34	287.2	0.0	826.54012	11.6	937.6	PC 40:10; PC 20:5-20:5; [M+H] ⁺	C ₄₈ H ₇₆ NO ₈ P	AL SF SL CP		AnalyteList C. elegans pos

A CCS values for PC 20:5_20:5

CCS (Å ²)	delta CCS (%)*
287.76	0.21
285.67	0.52
286.85	0.10
288.11	0.33
285.96	0.41
285.96	0.41
287.23	0.03
287.22	0.02
287.15	0.00
286.85	0.10
286.92	0.08
287.47	0.11
288.11	0.33
287.76	0.21
288.11	0.33
286.85	0.10
285.67	0.52
286.86	0.10
285.96	0.41
288.05	0.31
287.75	0.21
Average	0.23 %

AQ Scoring



- A the precursor mass accuracy
- B retention time fit
- C isotopic pattern quality
- D MS/MS spectra matching
- E CCS values

B

Name	Molecular For...	[M+H] ⁺ Prediction	Ion Notation	Measured	Prediction	Δ CCS [%]
PC 40:10; PC 20:5-20:5; [M+H] ⁺	C ₄₈ H ₇₆ NO ₈ P	291.2	[M+Na] ⁺	288.2	291.7	-1.2
			[M+H] ⁺	287.2	291.2	-1.4

CCSPredict

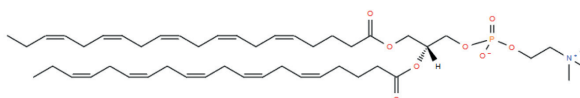
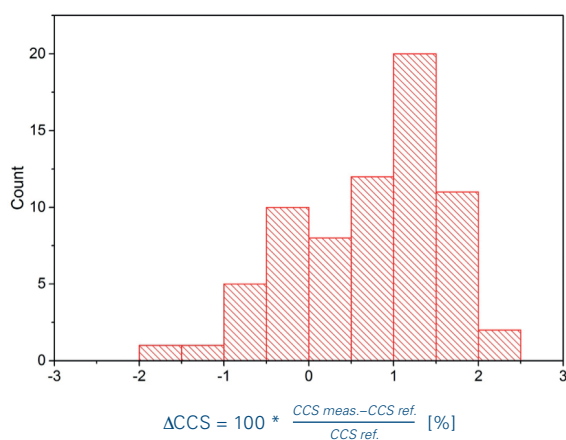


Figure 6: **A** Highly reproducible CCS values automatically extracted by T-ReX 4D: For PC 20:5_20:5 the average delta CCS across 21 analysis is 0.23%. (*|(CCS measured - mean CCS) / mean CCS x 100|). **B** CCSPredict machine learning based prediction of CCS value in MetaboScape increases confidence in lipid ID: low deviation of measured vs. predicted CCS value for both [M+H]⁺ and [M+Na]⁺. **C** Analyte List based annotation enables quick de-replication and Annotation Quality scoring (AQ Scoring). Five complementary measures in AQ scoring provide users with a quick graphical feedback on confidence in identification.

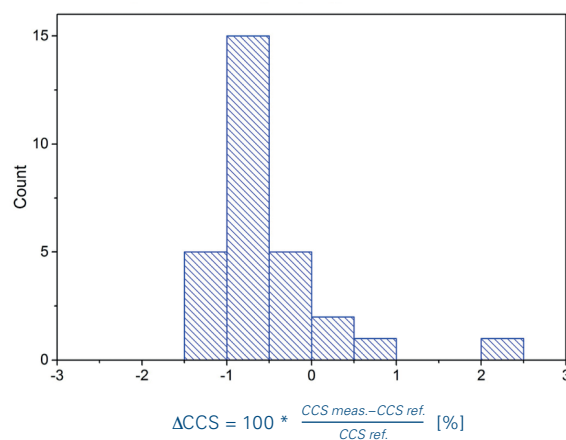
timsTOF Pro CCS values matching vs. DT-IMS-MS: <1% average deviation from CCS Compendium

A Delta CCS [%] - Positive mode



Positive mode:
Average |ΔCCS| = 0.95%

B Delta CCS [%] - Negative mode



Negative mode:
Average |ΔCCS| = 0.8%

Figure 7: Deviation of CCS values measured in timsTOF Pro versus CCS values from public CCS Compendium repository [14]. **A** CCS values of 70 measured lipids in C. elegans vs. CCS compendium. **B** CCS values of 30 measured lipids in C. elegans vs. CCS compendium.

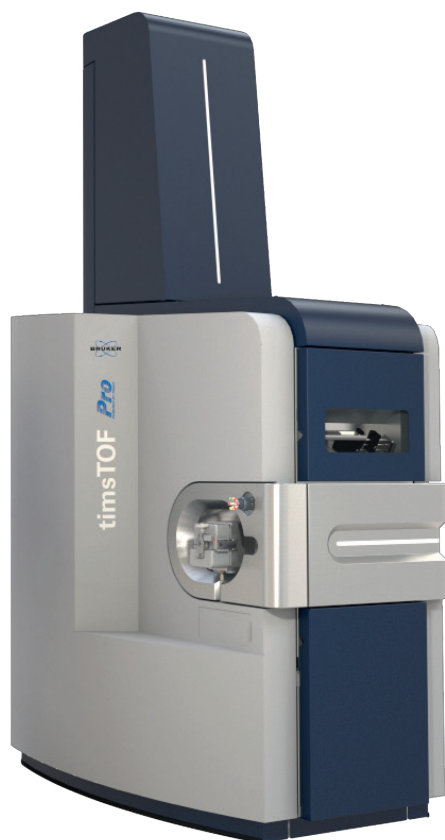
Conclusions

- The 4D-Lipidomics workflow was presented as a powerful tool for the deep profiling of the *C. elegans* lipidome. This is the basis for an investigation of characteristic changes induced by the *daf-2* mutation and a first step for a better understanding of how this mutation relates to an increased lifespan. The automatic identification of lipids by MS/MS library queries and targeted Analyte List based annotations in MetaboScape enabled the assignment of >1300 lipids in a merged positive / negative mode PASEF data set.
- Characteristic lipids can be detected readily by complementary statistical tools, such as PCA, in the same software solution. This was presented for one example, together with the verification of the lipid assignment based on characteristic fragments in negative and positive mode MS/MS spectra. PASEF was shown to not only generate clean MS/MS spectra for confident lipid ID but also to provide highly reproducible CCS values.
- CCS values generated on a timsTOF Pro can be matched to public CCS repositories, such as the CCS Compendium. The average deviation was <1% for 100 lipid species in this study. In combination with CCSPredict, this becomes a powerful workflow solution for researchers, providing additional confidence in lipid identifications.

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

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Phone +1 (978) 663-3660

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