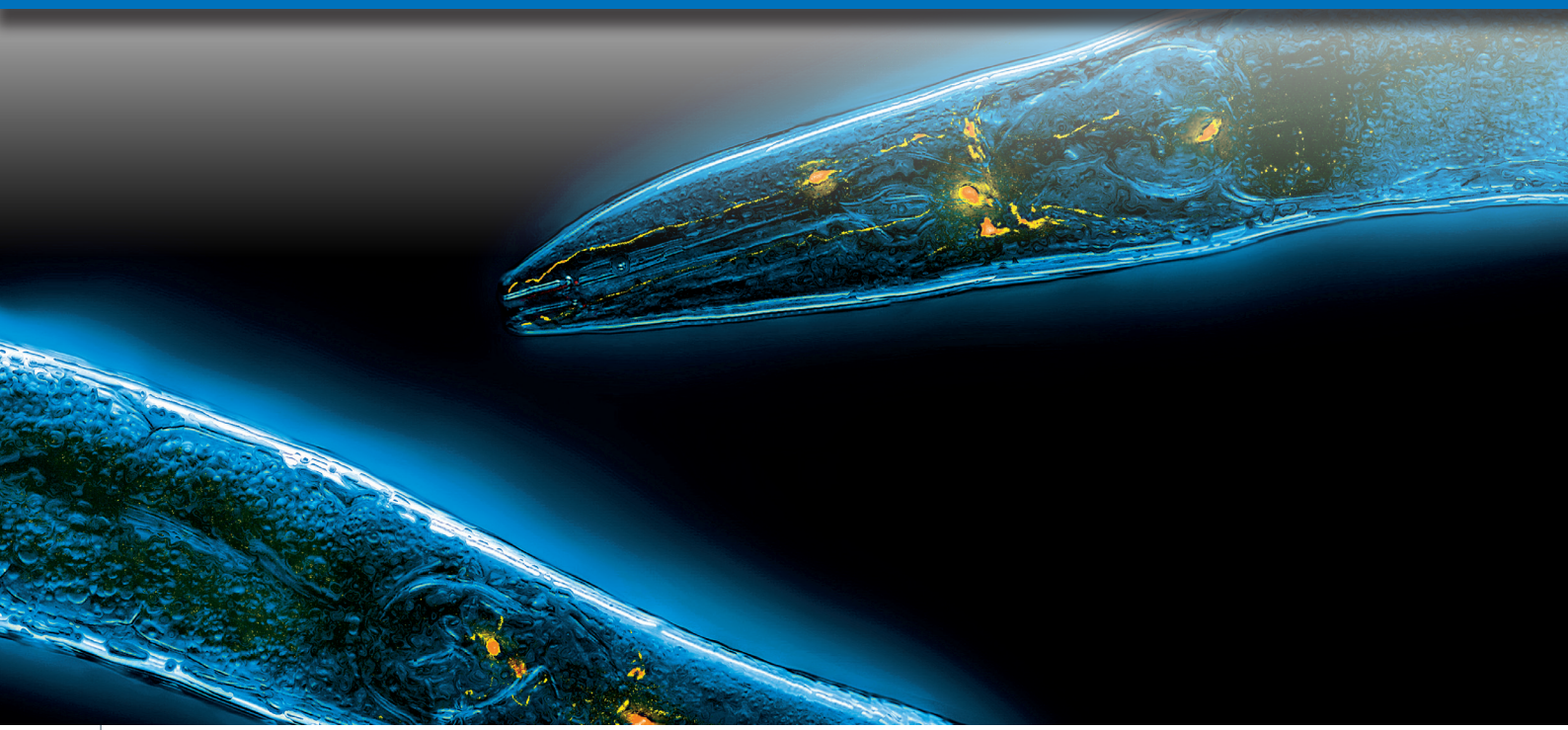

Investigating the increased lifespan in *C. elegans daf-2* mutants by 4D-Lipidomics™

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].

Keywords:
4D-Lipidomics, PASEF, timsTOF Pro, C. elegans

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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 and matching to LipidBlast [8-14] in MetaboScape.

Experimental

C. elegans cultivation

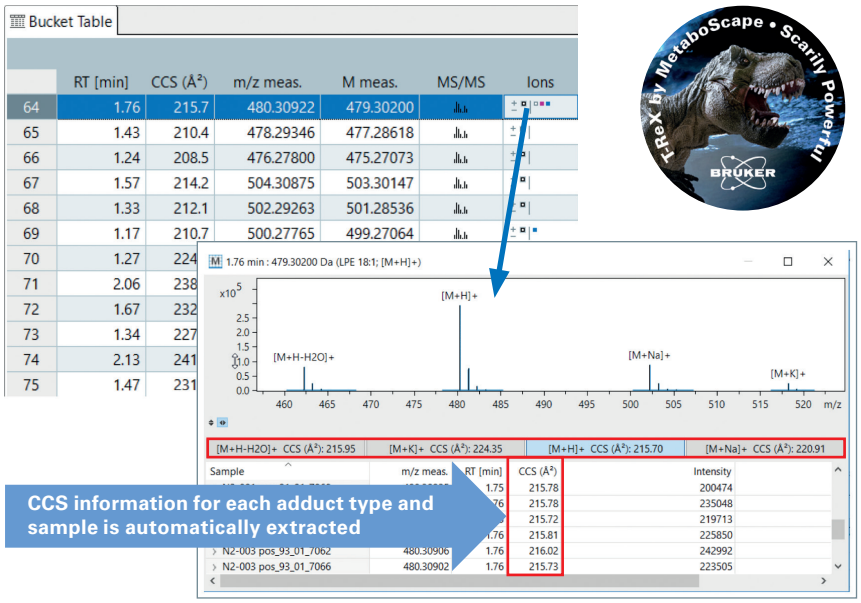
C. elegans strains N2 Bristol and *daf-2*(e1370) were cultivated on nematode growth medium (NGM) using *Escherichia coli* OP50 as sole food source. After age synchronization by bleaching, the worms were grown until the first day of adulthood, harvested and washed twice with M9 buffer. Each biological 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

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® 2021



T-ReX stands for **T**ime aligned **R**egion complete **eX**traction. T-ReX 4D automatically extracts five complementary criteria which can be used for high confident lipid identification:

- Retention time
- Accurate precursor mass (including adduct information)
- Isotopic pattern
- MS/MS spectra
- CCS values

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

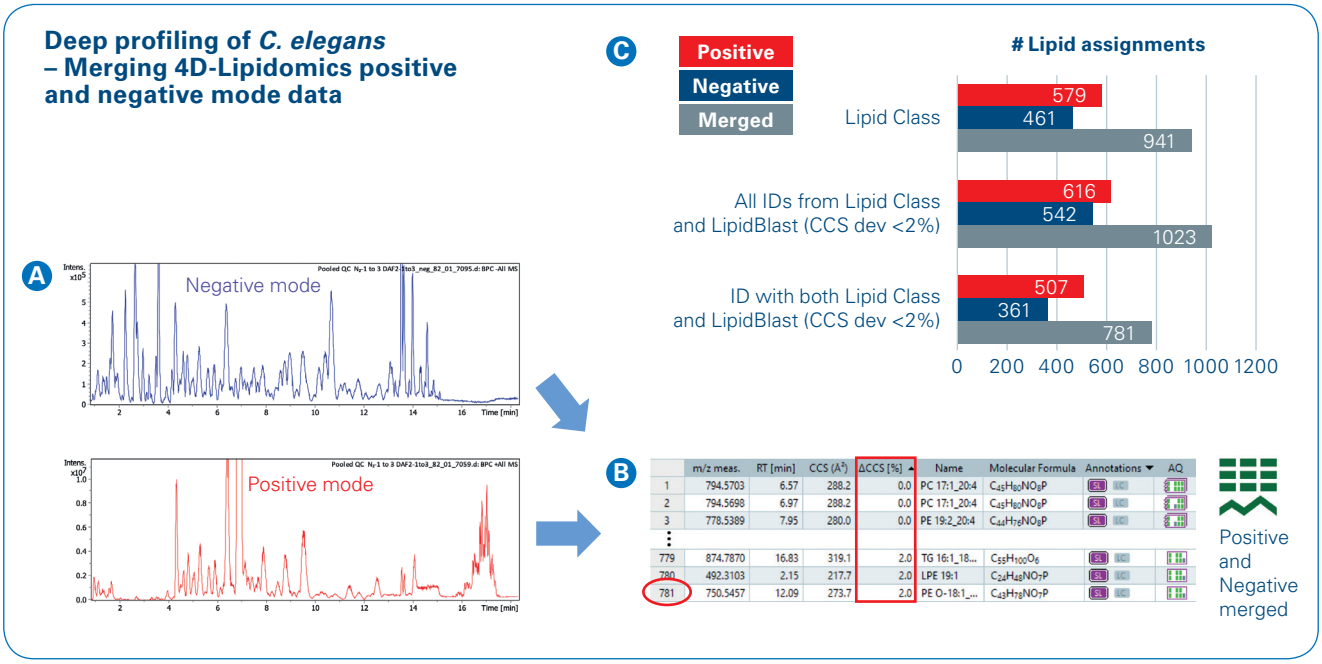


Figure 2: 4D-Lipidomics: (A) Base peak chromatograms of selected pooled Quality Control sample from C. elegans *lipid extracts measured in positive mode (bottom, red) and negative mode (top, blue) by LC-PASEF. (B) Automatic lipid assignment of T-ReX 4D extracted lipids in merged positive / negative data. Hits are shown that were annotated with both, combinatorial rule based lipid annotation and LipidBlast in-silico MS/MS matching [8-13]. Results were additionally filtered for matches with <2% CCS deviation (measured vs. predicted CCS values contained in LipidBlast). (C) Lipid profiling: Comparison of complementary lipid assigment and filtering strategies as basis for subsequent manual ID validation using 4D Kendrick Mass Defect Plots (See Figure 4). Top: Lipid assignment with rule based Lipid Class annotation. Middle: All annotations generated by rule based annotation and by LipidBlast. Hits from LipidBlast were additioanlly filtered for assignments with CCS deviation <2%. BOTTOM Shows only annotations with assignments from both rule based annotation and by LipidBlast. Additionaly, CCS filtering for <2% deviation vs. LipidBlast was applied.*

500 µL methanol and homogenized 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 µL 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 µL MTBE. After centrifugation, the organic phases were combined and evaporated. The residue was reconstituted in 500 µL acetonitrile / isopropanol / water (65/30/5, v/v/v) and stored in 125 µL 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 µl and 10 µl 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. Ions belonging to the same lipid are automatically combined into so called

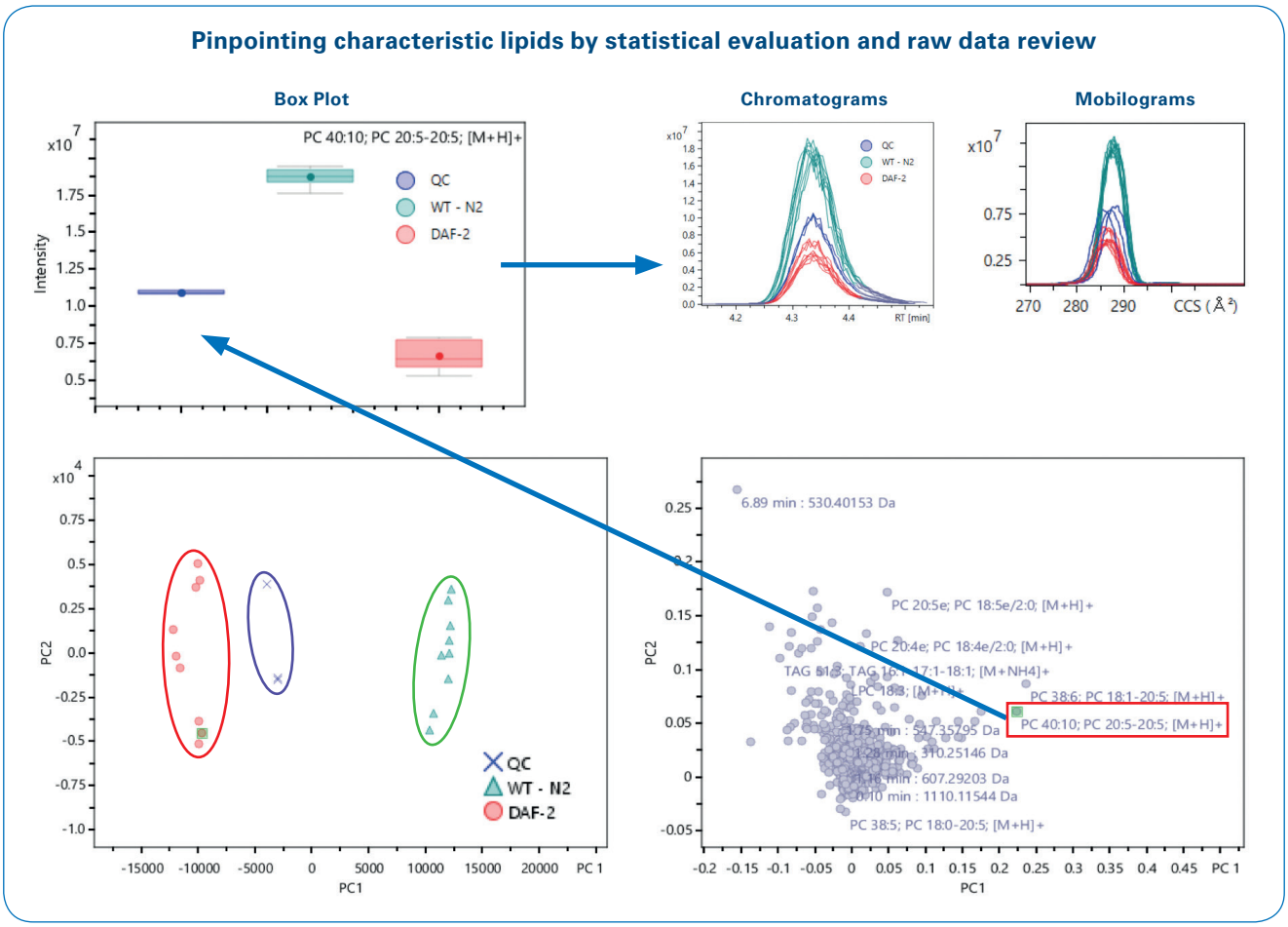


Figure 3: Principal Component Analysis (PCA) of 4D-Lipidomics data. The scores plot (bottom left) shows clear separation of wild type and daf-2 *mutant lipid extracts. Pooled QC samples cluster in the middle between wild type and* daf-2 *mutants. One loading contributing to wild type and mutant sample 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.*

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

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, 507 lipid features could be tentatively annotated. The annotation was based on rule based lipid class annotation implemented in MetaboScape and complementary assignment by LipidBlast [8-14] *in silico* MS/MS spectral library and CCS value matching. The tentatively assigned lipids are those that recieved an annotation by rule based annotation and LipidBlast (Version 68) assignment, both within 5 ppm precursor mass matching and <200 mSigma isotopic fidelity. Additionally, assignments were filtered for matching with lower than 2% deviation of measured CCS vs. the predicted CCS values contained in LipidBlast.

In negative mode, a total of 361 lipids were assigned by automatic LipidBlast and rule based annotations. 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, 781 lipids could be assigned by the joint rule based and LipidBlast annotation approaches and filtering for <2% deviation in CCS.

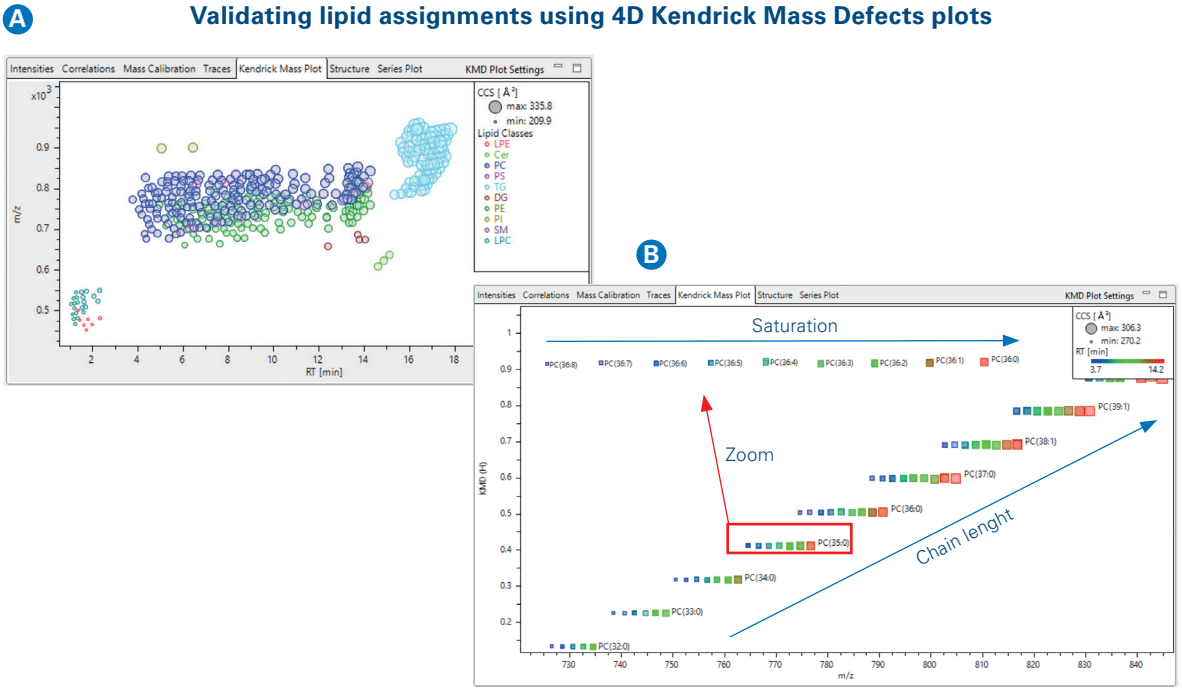


Figure 4: Intuitive lipid profile investigation by customizable 4D Kendrick Mass Defects plots. (A) 4D plot: retention time vs. m/z plot using different colors for different lipid classes and bubble sizes for the CCS values. This plot provides a quick overview on the lipid profile and enables to easily spot obvious deviations in lipid assignments. (B) 4D plot: m/z vs. KMD with H specified as repeating unit using different colors for different lipid classes and bubble sizes for the CCS values. This visualization allows to quickly investigate lipid species of a selected class for saturation and chain length consistency as well as matching of expected retention time and CCS value.

Pinpointing characteristic lipids

Following the tentative assignment of lipids, a statistical evaluation readily pointed to lipids with difference in 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 20:5-20:5.

Verification of lipid assignment

Rule based Lipid Class assignment in MetaboScape enables to calculate and visualize Kendrick Mass Defects (KMD), turning complex mass spectral information into a compositional map with informative clustering of points based on lipid specific homologous repeating units. The customizable 4D KMD plot highlighted in Figure 4 allows for intuitive lipid ID validation. Various characteristics of the extracted features can be plotted in 4 dimensions (x-axis, y-Axis, color scale, and bubble size), allowing versatile applications.

Plotting retention time vs. *m/z* plot, using different colors for different lipid classes and bubble size for the CCS values provides a quick overview on the lipid profile (See Figure 4A). Possible false annotations, for example a lipid not following the expected retention time like for other lipids of the same assigned class, can be spotted.

Figure 4B shows the plot for *m/z* vs. KMD with H specified as repeating unit; bubble size for CCS; color for retention time. This visualization allows to quickly investigate lipid species of a selected class for saturation and chain length consistency. The highlighted phosphocholine (PC) lipids also reveal the benefit to represent the retention time and CCS value. The color coding for retention time (blue to red) for lipids with the same number of carbons follow the expected increase in reversed phase chromatography retention time with increasing saturation level. The CCS value (bubble size) increases with saturation level.

To confirm the lipid assignment PC 20:5-20:5, 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 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) [15]. 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.

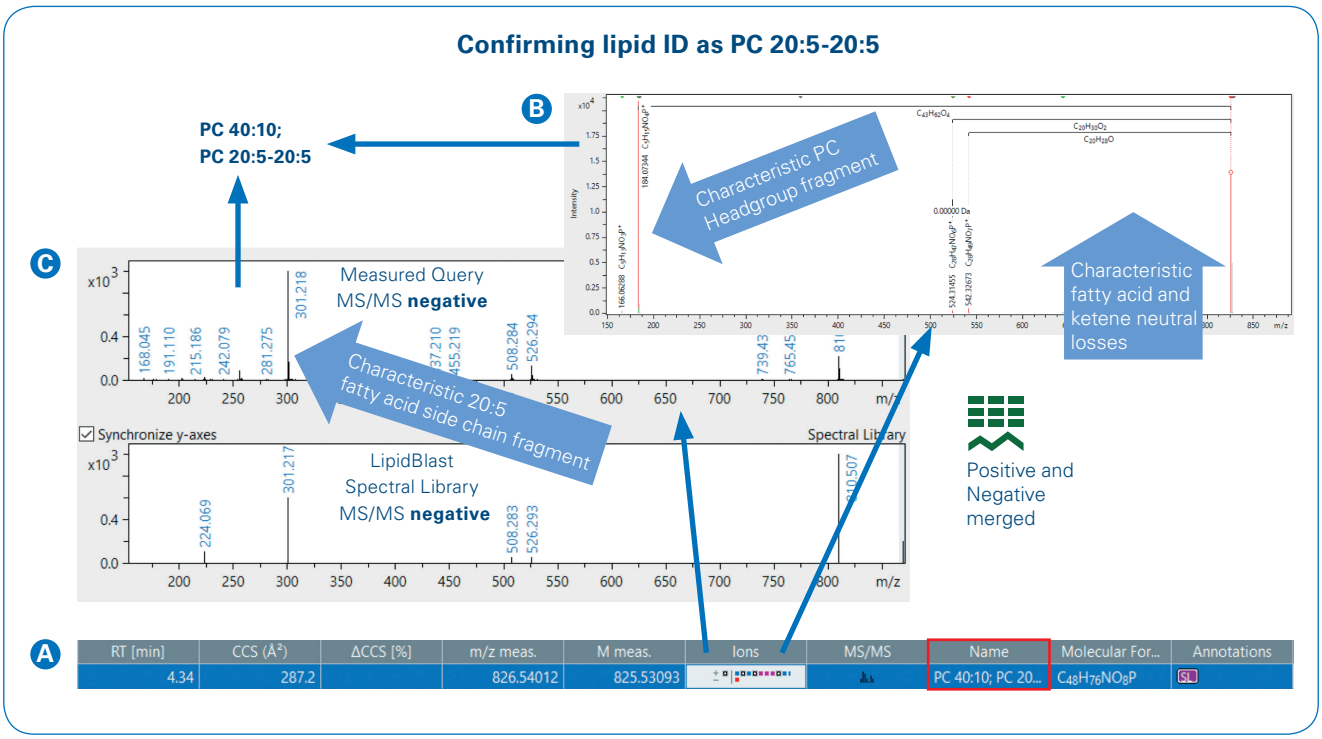


Figure 5: (A) Screenshot of a positive and negative mode merged bucket table in MetaboScape highlighting the extracted lipid PC 40:10. (B) Screenshot of MS/MS spectrum of $[M+H]^+$ automatically annotated with molecular formula for fragment ions and neutral losses in SmartFormula3D. The characteristic 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.

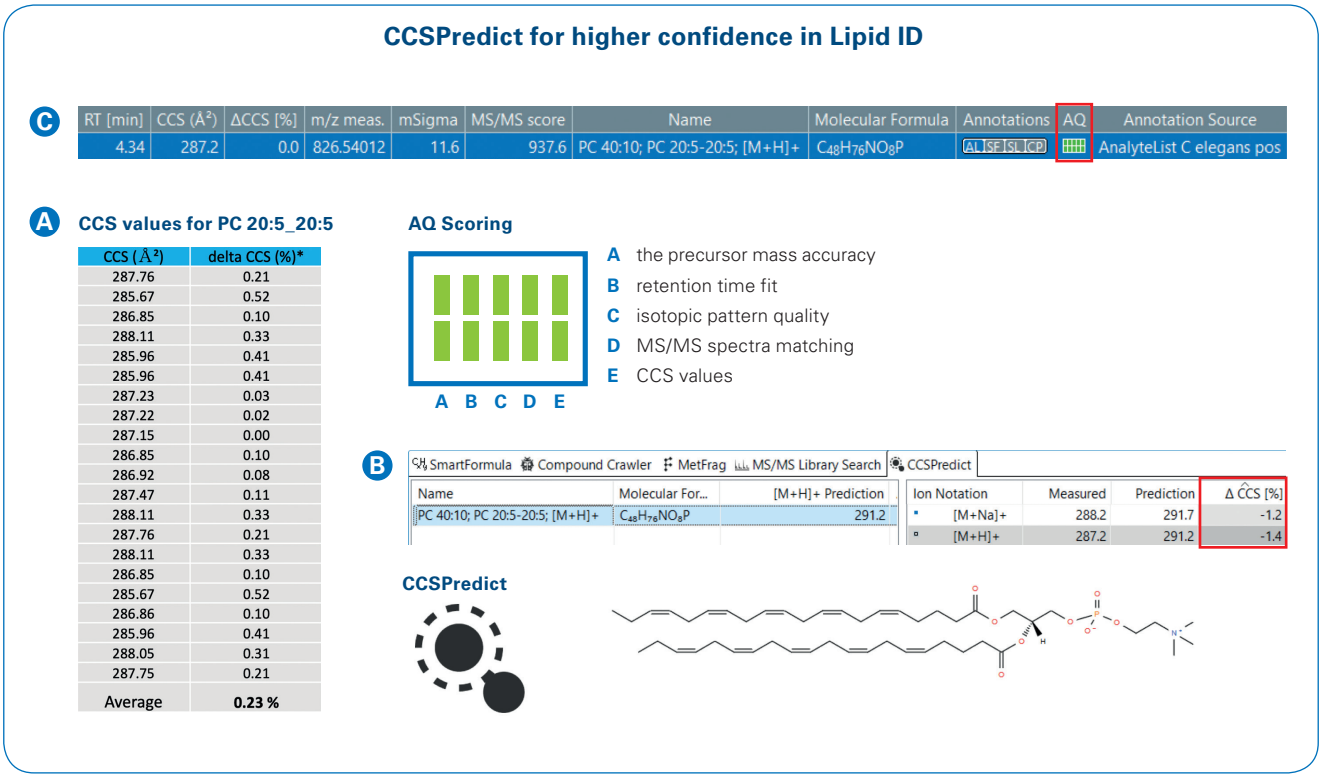


Figure 6: (A) Highly reproducible CCS values automatically extracted by T-ReX 4D: For PC 20:5_20:5 the average delta CCS across 21 analysis is 0.23%. (|(CCS measured - mean CCS) / mean CCS x 100|). (B) CCSPredict machine learning based prediction of CCS value in MetaboScape increases confidence in lipid 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.*

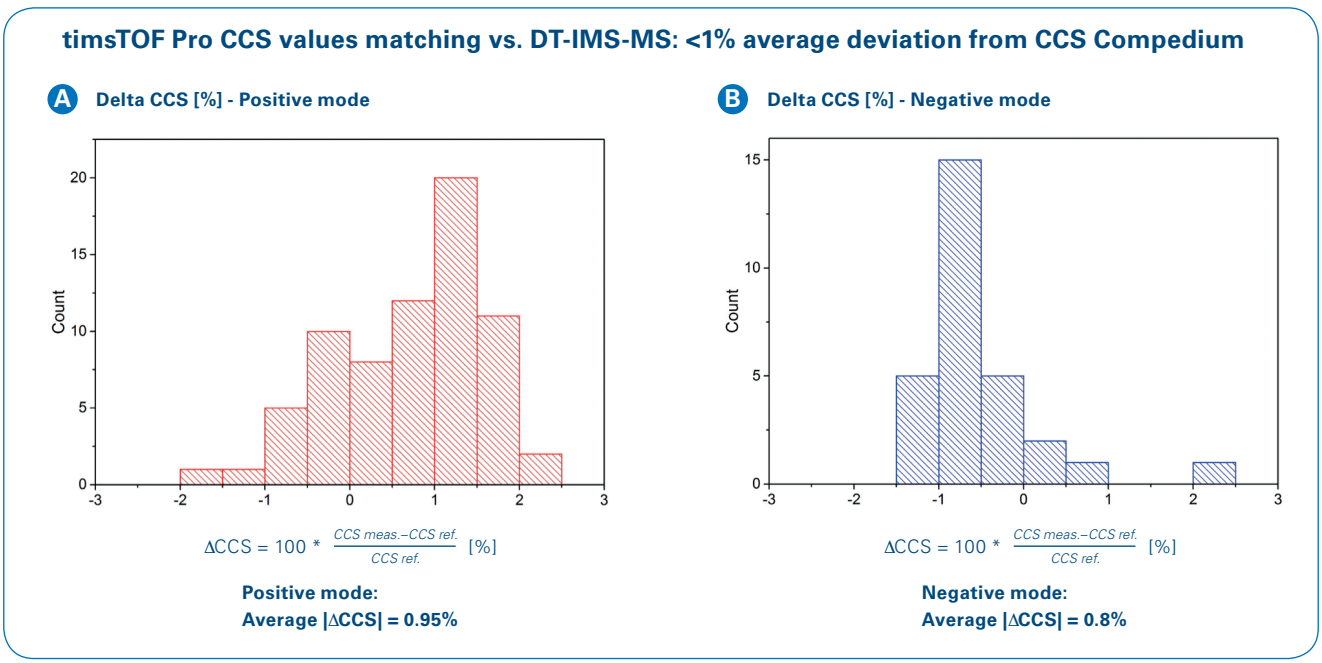


Figure 7: Deviation of CCS values measured in timsTOF Pro versus CCS values from public CCS Compendium repository [14]. (A) CCS values of 70 measured lipids in C. elegans *vs. CCS compendium. (B) CCS values of 30 measured lipids in* C. elegans *vs. CCS compendium.*

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.
- Confident identification is crucial and for biological interpretation. The MetaboScape 4D-Lipidomics processing workflow addresses this need by providing unique benefits such as:
    - Feature extraction by T-ReX 4D providing accurate mass, isotopic pattern, retention time, clean PASEF MS/MS spectra and CCS values as basis for annotation with 5 complementary confidence criteria
    - Rule based lipid annotation routines for identification of lipid species taking into consideration the Lipidomics Standards Initiative guidelines
    - Verification of annotations by CCSPredict, providing additional confidence in lipid identifications
    - 4D Kendrick Mass Defect plots, turning complex mass spectral information into a compositional map with informative clustering of points based on lipid specific homologous repeating units for validation of lipid assignments
    - Complementary to rule based annotation, MetaboScape supports LipidBlast [14], the largest Lipidomics open source *in silico* MS/MS & CCS library for >550,000 lipid structures
- Characteristic lipids can be detected readily by complementary statistical tools, such as PCA, in the MetaboScape. 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.

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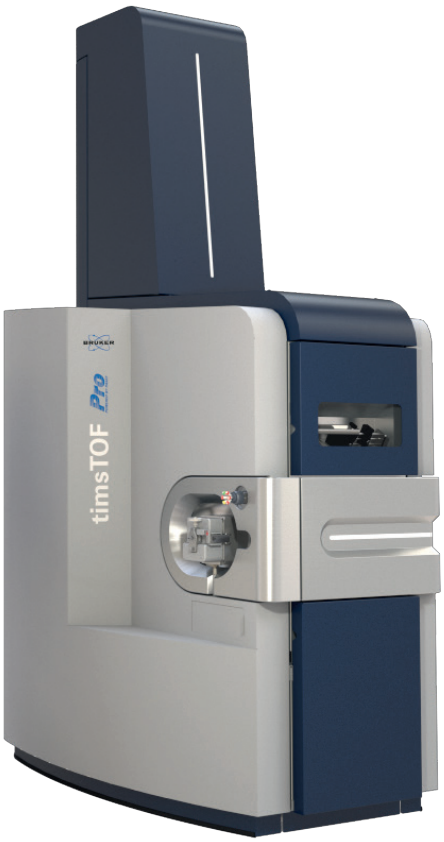

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