**UHPLC-IMS-Q-ToF-MS analysis of Maradolipids, found exclusively in *Caenorhabditis elegans* dauer larvae**

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**Abstract**

The biomedical model organism *Caenorhabditis elegans* reacts to harsh environmental conditions by interrupting its normal life cycle and entering an alternative developmental stage called dauer stage. Dauer larvae show distinct changes in metabolism and morphology to survive unfavorable environmental conditions and are able to survive for a long time without feeding. Only at this developmental stage, dauer larvae produce a specific class of glycolipids called maradolipids. Herein, we present the analysis of maradolipids using Ultrahigh Performance Liquid Chromatography-Ion Mobility Spectrometry-Quadrupole-Time of Flight-Mass Spectrometry (UHPLC-IMS-Q-ToF-MS). The results show that combination of liquid chromatography and ion mobility spectrometry separation together with data independent AllIons fragmentation represent a valuable tool for lipid identification. Using this analytical tool, marado- and lysomaradolipids have been identified from *C. elegans* dauer larvae extracts.

**Keywords**

* Ion Mobility Spectrometry
* Lipidomics
* Lipid identification
* *Caenorhabditis elegans*

**Introduction**

The small nematode *Caenorhabditis elegans* (*C. elegans*) is one of the premier model organisms in biomedical research. *C. elegans* normally develops from the fertilized egg through four larval stages into reproductive adults. In order to react to changing environments organisms *C. elegans* can interrupt its normal life cycle and enter an alternative developmental stage called dauer stage (German for enduring). As compared to normal larvae, dauer larvae show distinct changes in metabolism and morphology to survive unfavorable environmental conditions. Dauer larvae are able to survive for a long time without feeding. Once conditions ameliorate, they develop into normal adults without compromises in lifespan or fertility. *C. elegans* harbours a complex metabolome and lipidome with several different lipid classes, including lipids specific to *C. elegans* (1).

Changes in metabolism enable improved usage of energy resources and includes the rerouting of several metabolic pathways (2). One interesting aspect of dauer larvae is the production of specific glycolipids distinct from glucosylceramides. They have been named maradolipids and are found exclusively in the dauer stage of *C. elegans*. Chemically they are defined as 6,6’-diacyltrehaloses and have been identified for the first time by Penkov et al. They performed an extraction and purification of glycolipids followed by shotgun analysis of the obtained lipids. Maradolipids contain a high amount of branched chain fatty acids, mostly C15:0iso (> 20 mol%) (3). An additional study identified lysomaradolipids, containing only a single acyl group (4).

So far, maradolipids have been only analyzed by shotgun lipidomics. However, LC-MS based workflows are often used for lipid profiling and allow the separation and detection of new lipids and lipid classes (5). Reversed-phase based separation enables the separation of lipids based on their hydrophobicity, hence their different acyl chains. This allows a more detailed description of lipid species and their composition. Another emerging tool for lipid analysis is the ion mobility separation (IMS). Separation in IMS is based on the differential traveling of ions in a drift gas along an electric field. The velocity of ions is based on their molecular shape, which is expressed as rotational averaged collision cross section (CCS). CCS values help to add further confidence in lipid identification (6).

This investigation presents the analysis of maradolipids using chromatographic and ion mobility separation. Maradolipids standards have been analyzed to determine their chromatographic and ion mobility behavior as well as fragmentation in positive and negative ionization mode. Results served as basis for analysis of maradolipids from *C. elegans* dauer larvae lipid extract. Based on RT, CCS and data independent AllIons fragmentation different maradolipids could be identified. Additionally, a collection of lysomaradolipids has been identified. The obtained results show, how RT, CCS and AllIons fragmentation can help in the identification of lipids.

**Material and Methods**

*Chemicals*

Maradolipid standards have been synthesized by the Knölker group according to Pässler et al. (7). A mix standard consisting of 6-*O*-myristoyl-6’-*O*-myristoyltrehalose (Mar(14:0/14:0)), 6-*O*-(13-methylmyristoyl)-6’-O-(13-methylmyristoyl)trehalose (Mar(15:0/15:0)), 6-O-myristoyl-6’-O-oleoyltrehalose (Mar(14:0/18:1)), 6-O-palmitoyl-6’-O-palmitoyltrehalose (Mar(16:0/16:0)), 6-O-(13-methylmyristoyl)-6’-O-(15-methylpalmitoyl)trehalose (Mar(15:0/17:0)), 6-O-(13-methylmyristoyl)-6’-O-oleoyltrehalose (Mar(15:0/18:1)), 6-O-palmitoyl-6’-O-oleoyltrehalose (Mar(16:0/18:1)), 6-O-(15-methylpalmitoyl)-6’-O-oleoylterhalose (Mar(17:0/18:1)), 6-O-oleoyl-6’-O-oleyoltrehalose (Mar(18:1/18:1)) and 6-O-oleoyl-6’-O-(2-octyl-cyclopropaneoctanoyl)trehalose (Mar(18:1/19:1)) was dissolved in methanol. All solvents and additives were obtained from Merck / Sigma-Aldrich and were of highest available purity, typically LC-MS grade.

*C. elegans cultivation and extraction*

*daf-2*(e1370) mutants were obtained from the Caenorhabditis Genetics Center (CGC) and grown under standard conditions according to Brenner et al on Nematode Growth Medium (NGM) (8). To obtain dauer larvae synchronized L1 larvae were obtained by bleaching and seeded onto NGM plates and grown at 25°C. Once sufficient amounts of dauer larvae were obtained, worms were washed off the plates using an M9 buffer and washed three times. Lipids were extracted according to Bligh and Dyer (9). The chloroform phase was evaporated to dryness and redissolved in 60% iPrOH/35% ACN/5% H2O (v/v/v).

*Multifield analysis of Maradolipid standards*

Collisional Cross Sections (CCS) of maradolipid standards were collected using the multifield method similar to Stow et al. using an Agilent 6560 DT-IMS-Q-ToF-MS (10). The instrument was operated with N2 at 3.95 Torr. The maradolipid standard mix was diluted in a 50/50 mixture of eluent A and eluent B (see below) and infused using a syringe pump with a flow rate of 500 µL / min. Data were analyzed using the Agilent MassHunter Workstation IM-MS Browser 10.0. Ion mobility data have been referenced using either m/z 922 or 1033 from the reference mass solution in positive and negative ionization mode respectively.

*UHPLC-IMS-Q-ToF-MS analysis*

Chromatographic separation was performed as described by Witting et al. (11). Lipids were separated using an Agilent 1290 Infinity II UHPLC (Agilent Technologies, Waldbronn, Germany) equipped with Waters CORTECS UPLC C18 column (150 mm x 2.1 mm ID, 1.7 µm particle size) (Waters, Eschborn, Germany). Separation was achieved by a linear gradient from 68% eluent A (40% H2O / 60% ACN, 10 mM ammonium formate and 0.1% formic acid) to 97% eluent B (10% ACN / 90% iPrOH, 10 mM ammonium formate and 0.1% formic acid). Mass spectrometry detection was performed using an Agilent 6560 IM-Q-TOF-MS (Agilent Technologies, Waldbronn, Germany). Ion mobility separation was performed under uniform field conditions with AllIons fragmentation using an alternating scheme, switching between low and high collision energy using either 10, 20 or 40 eV. In order to obtain CCS values, calibration of the IMS dimension was performed using the Agilent Low Concentration Tune Mix infused prior to the worklist. Data were preprocessed using the PNNL preprocessing tool with a smoothing in RT direction using 3 data points and in drift direction using 5 data points. Additional saturation repair has been performed.

Fragmentation data from AllIons fragmentation was examined using the Agilent MassHunter Workstation IM-MS Browser 10.0 and non-targeted 4-dimensional peak picking has been performed using the Agilent MassHunter Workstation Mass Profiler 10.0 software. Minimum peak intensity was set at 100 counts and common organic formula without halogens were used as isotope model. Alignment parameters: RT tolerance +/- 10% + 0.5 min, DT tolerance +/- 1.5 % and mass tolerance +/- 15 ppm + 2.0 mDa. Calculation of Kendrick mass defects and all further data handling were performed in Microsoft Excel.

<Figure 1>

Figure 1: Structure of maradolipid standards synthesized and used in this study.

**Results and Discussion**

*Determination of reference CCS values and RTs*

In order to characterize the ion mobility separation of maradolipids CCS values of authentic reference standards were determined. Maradolipid standards were infused in a 50/50 mixture of eluent A and B of the later employed chromatographic separation to the same adducts. In positive ion mode maradolipids are ionizing as [M+NH4]+ adducts during direct infusion as well as [M+FA-H]- adducts in negative mode. This is in agreement with Penkov et al., who detected acetate adducts of maradolipids in negative ion mode. Although [M+Na]+ adducts were detected during chromatographic analysis they were not detected in the direct infusion experiments.

CCS values of the maradolipid standards were determined using the multifield method according to Stow et al. (10). The mixture contained one pair of isobaric maradolipids (Mar (16:0/16:0) and Mar(15:0/17:0)). Using IMS they could not be resolved and therefore only a single CCS value for both was obtained. Table 1 summarizes the obtained values. Three independent replicates were performed and results from the individual measurements are in good agreement. Consistent with other lipid classes, increasing chain length led to increased CCS. In the next step LC-IMS-MS was performed using a single field drift tube experiment. This allowed us to collect CCS and RT in parallel. CCS values from the single field-experiment were in good agreement with values derived from the multifield method (Table 1). In positive mode relative deviation ranged from -0.40 to -0.65%, while in negative mode they ranged from –0.29 to 0.43%. In order to identify potential trends for investigations in natural samples we plotted the Kendrick mass defect for CH2 against the *m/z*. As expected, homologous series form horizontal lines. Furthermore, the CCS was used as the size of data points and RT a color (Figure 1A and 1B).

In contrast to glycerophospholipids, the maradolipids have no distinct sn1 or sn2 position since the 6 and 6’ position on the trehalose are equal. Therefore, only single peaks will be measured throughout the measurements, while for glycerophospholipids two peaks might be found in the chromatography and ion mobility. Similar to PCs or PEs, maradolipids show a linear increase in CCS with growing chain length. Slopes of trendlines for CCS vs *m/z* plots are slightly smaller for maradolipids compared to PCs and PEs (data not shown). In contrast to IMS alone, UHPLC-IMS-Q-ToF-MS was able to separate the isobaric structures Mar(16:0/16:0) and Mar(15:0/17:0).

Putative isomeric overlap within a 5mDa window in negative ion mode with theoretical PE-Cers and SMs with a high number of hydroxyl groups was found using the LipidMaps search against CompDB (12). Since such lipids are currently not known in *C. elegans* and not expected, the collective information on the MS1 level (*m/z*, KMD and CCS) allow to identify putative maradolipids in lipid extracts.

Table 1: Summary of CCS and RT values obtained maradolipid standards. CCS values were derived from direct infusion multifield measurements and UHPLC-IMS-QToF-MS. Means and standard deviations were calculated from triplicate measurements. Deviations of single field CCS from multifield CCS are indicated in brackets.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Ion mode** | **Name** | **adduct** | **m/z** | **CCS +/- SD (multi field)** | **CCS +/- SD (single field)** | **RT +/- SD** |
| pos | Mar(14:0/14:0) | [M+NH4]+ | 780.5467 | 282.87 +/- 0.25 | 284.00 +/- 0.26 (-0.40 %) | 12.98 +/- 0.03 |
| Mar(15:0/15:0) | 808.578 | 289.13 +/- 0.21 | 290.60 +/- 0.20 (-0.51 %) | 13.90 +/- 0.02 |
| Mar(14:0/18:1) | 834.5937 | 293.00 +/- 0.20 | 294.20 +/- 0.26 (-0.41 %) | 14.38 +/- 0.02 |
| Mar(16:0/16:0) | 836.6093 | 294.93 +/- 0.25 | 296.57 +/- 0.38 (-0.55 %) | 15.10 +/- 0.02 |
| Mar(15:0/17:0) | 836.6093 | 294.93 +/- 0.25 | 296.63 +/- 0.15 (-0.58 %) | 15.47 +/- 0.02 |
| Mar(15:0/18:1) | 848.6093 | 296.03 +/- 0.23 | 297.37 +/- 0.35 (-0.45 %) | 14.78 +/- 0.02 |
| Mar(16:0/18:1) | 862.625 | 298.77 +/- 0.25 | 300.70 +/- 0.26 (-0.65 %) | 15.52 +/- 0.02 |
| Mar(17:0/18:1) | 876.6406 | 301.67 +/- 0.15 | 303.17 +/- 0.23 (-0.50 %) | 15.85 +/- 0.02 |
| Mar(18:1/18:1) | 888.6406 | 302.87 +/- 0.32 | 304.20 +/- 0.00 (-0.44 %) | 15.56 +/- 0.02 |
| Mar(18:1/19:1) | 902.6563 | 306.80 +/- 0.17 | 307.67 +/- 0.21 (-0.28 %) | 16.23 +/- 0.02 |
| Mar(14:0/14:0) | [M+Na]+ | 785.5021 | --- | 282.70 +/- 0.26 | 12.98 +/- 0.02 |
| Mar(15:0/15:0) | 813.5334 | --- | 289.43 +/- 0.32 | 13.90 +/- 0.02 |
| Mar(14:0/18:1) | 839.5491 | --- | 291.47 +/- 0.32 | 14.38 +/- 0.02 |
| Mar(16:0/16:0) | 841.5647 | --- | 295.30 +/- 0.36 | 15.09 +/- 0.02 |
| Mar(15:0/17:0) | 841.5647 | --- | 295.37 +/- 0.21 | 15.47 +/- 0.02 |
| Mar(15:0/18:1) | 853.5647 | --- | 294.93 +/- 0.31 | 14.78 +/- 0.02 |
| Mar(16:0/18:1) | 867.5804 | --- | 298.83 +/- 0.31 | 15.52 +/- 0.02 |
| Mar(17:0/18:1) | 881.596 | --- | 301.73 +/- 0.21 | 15.85 +/- 0.02 |
| Mar(18:1/18:1) | 893.596 | --- | 303.33 +/- 0.15 | 15.56 +/- 0.02 |
| Mar(18:1/19:1) | 907.6117 | --- | 306.70 +/- 0.20 | 16.23 +/- 0.02 |
| neg | Mar(14:0/14:0) | [M+FA-H]- | 807.5111 | 284.65 +/- 0.35 | 284.40 +/- 0.10 (-0.09 %) | 12.94 +/- 0.04 |
| Mar(15:0/15:0) | 835.5424 | 290.55 +/- 0.64 | 290.77 +/- 0.06 (0.07 %) | 13.87 +/- 0.04 |
| Mar(14:0/18:1) | 861.5581 | 293.95 +/- 0.64 | 294.37 +/- 0.40 (0.14 %) | 14.35 +/- 0.03 |
| Mar(16:0/16:0) | 863.5737 | 296.35 +/- 0.07 | 297.03 +/- 0.51 (0.23 %) | 15.06 +/- 0.03 |
| Mar(15:0/17:0) | 863.5737 | 296.35 +/- 0.07 | 297.07 +/- 0.38 (0.24 %) | 15.44 +/- 0.04 |
| Mar(15:0/18:1) | 875.5737 | 297.20 +/- 0.57 | 297.10 +/- 0.53 (-0.03 %) | 14.74 +/- 0.04 |
| Mar(16:0/18:1) | 889.5894 | 299.90 +/- 0.28 | 300.07 +/- 0.59 (0.06 %) | 15.48 +/- 0.04 |
| Mar(17:0/18:1) | 903.605 | 301.85 +/- 0.35 | 303.17 +/- 0.46 (0.44 % ) | 15.82 +/- 0.03 |
| Mar(18:1/18:1) | 915.605 | 304.00 +/- 0.42 | 303.13 +/- 0.42 (-0.29 %) | 15.53 +/- 0.03 |
| Mar(18:1/19:1) | 929.6207 | 306.70 +/- 0.57 | 307.17 +/- 0.40 (0.15 %) | 16.20 +/- 0..03 |

*Fragmentation pattern of maradolipids*

Next fragmentation spectra of maradolipids were investigated. UHPLC-IMS-Q-ToF-MS data were collected applying the data independent AllIons fragmentation mode using alternating frames switching between low and high collision energy. Three different runs with either 10, 20 or 40 eV collision energy were produced. First, fragmentation in negative mode was investigated. Fragmentation pathways of acetate adducts of maradolipids has been described by Papan et al. (4). Upon fragmentation, first the [M-H]- ion is formed from which the fatty acids are lost and can be detected as free acyl or as neutral fragments. Subsequently, fragments with *m/z* 323.0984 and 305.0878 derived from trehalose are formed. Investigating the fragmentation of [M+FA-H]- adducts similar fragmentation was observed. Fragmentation data of Mar(14:0/14:0) and Mar(14:0/18:1) was closer examined, both representing a symmetrical and an unsymmetrical maradolipid that had a clean (no overlapping interference) spectrum in the AllIons fragmentation data.

Similar to the fragmentation observed by Papan et al. first the fragmentation of the [M+FA-H]- to the [M-H]- ion was observed. This fragment further fragments by losing one of the two possible fatty acids attached at the 6- or 6’-position which leads to [M-R1COOH]- or [M-R2COOH]- fragments. In case of Mar(14:0/14:0) only one single fragment and in case of Mar(14:0/18:1) two fragments have been observed. The corresponding [R1COO]- and [R2COO]- fragments were also observed. The fragments [M-R1COOH]- and [M-R2COOH]- were only observed upon fragmentation with 20 eV. 40 eV yielded higher intensities of [R1COO]- and [R2COO]-. An interesting feature for the screening and identification for maradolipids are the fragments *m/z* 323.0984 and 305.0878 which correspond to [trehalose-H2O-H]- and [trehalose-2H2O-H]-. In order to confirm results from the AllIons fragmentation, targeted isolation of maradolipid standards was performed. Comparison showed that the same fragments are obtained. However, due to targeted isolation in the quadrupole the observed spectra were cleaner (data not shown).

Investigation of positive ion mode fragmentation data from the AllIons fragmentation data showed that major fragments derived from [M+NH4]+ adducts are [M-H2O+H]+ as well as [R1CO]+ and [R2CO]+ of the two respective acyl groups. Since no additional information can be derived from combined positive and negative mode analysis, only negative mode data was further investigated. Based on the obtained results, 20 eV seem to be the most informative collision energy, when performing non-targeted analysis and search for maradolipids since it yielded the most explainable fragments. 40 eV yielded the highest intensity for FA and trehalose fragments. Since AllIons fragmentation can be combined with UHPLC and IMS it allows to obtain sufficient information for maradolipid identification. Co-elution and similarity in drift times allow to filter the data independent MS2 data and exclude false positive fragments.

<Figure 2>

Figure 2: (A) Plots of the KMD against the m/z shows horizontal trendlines that can be used for identification of maradolipids. The CCS value is shown as size of the point, while RT is represented as color. Increasing chain length leads to larger molecular structures hence higher CCS values and an increased RT on the used RP separation. Different degrees of unsaturation are seen as parallel lines. (B) Different fragmentation energies yield different amount of fragments with different intensities. Plots of fragment against the collision energy shows that 20 eV yields the highest number of fragments and represents the best collision energy for characterization of novel maradolipids (C) Reconstructed fragmentation spectra derived from UHPLC-IMS-QToF-MS/MS for fragments shown in panel B.

*UHPLC-IMS-Q-ToF-MS analysis of C. elegans dauer larvae*

In order to proof that the combination of LC, IMS and AllIons fragmentation is able to identify maradolipids, *C. elegans* dauer larvae were generated from *daf-2(e1370)* mutants by growing them at 25 °C. Worms were harvested and extracted using a Bligh and Dyer extraction. Analysis of dauer larvae was performed by UHPLC-IMS-Q-ToF-MS using AllIons fragmentation with either 10, 20 and 40 eV. Since the positive mode did not offer additional information on the identification of maradolipids only the negative mode data were used. To see first if maradolipids are found in the lipid extract negative mode data were used and extracted ion chromatograms for *m/z* 323.0972 and 305.0877 in the high collision energy frames were generated. Coelution of these two *m/z* indicates presence of potential maradolipids. Since 20 eV spectra contained the highest information content, they were investigated first. Indeed, coelution of the two m/z was observed in the range of 12 to 17 minutes, being in the same range where the standards have been eluted. Interestingly, additional peaks for the *m/z* 323.0972 were observed in the range from 2.5 to 6.5 minutes, but not *m/z* 305.0867.

Indicating the presence of maradolipids in the dauer extract, non-targeted peak picking of lipid features was performed. In total 1349 features were detected in all three replicates of dauer larvae lipid extract in negative ion mode. From the measured masses the Kendrick mass and KMD were calculated. To identify species of maradolipids the referenced KMD was used with a value of 0.6094 (13). This narrowed down the list of lipid features to 123 candidates. The list was further condensed by filtering on the RT region of eluting maradolipid standards and compared against a computer-generated list of potential maradolipids using potential fatty acids present in maradolipids based on results from Penkov et al. (3) (SI Table 3). Using MS1 annotation to filter potential maradolipids 48 candidates remained. Of these, 10 could be matched with the used standards based on *m/z*, RT and CCS values.

Investigating peaks that are putatively annotated as additional maradolipids several interesting candidates were found. For example, for *m/z* 835.5424 a small side peak, which might represent an isobaric species with a different fatty acid composition. Investigating the AllIons fragmentation data, it can be putatively identified as Mar(14:0/16:0). To further confirm this identification, we checked trends along RT and CCS values. Data were checked for maradolipids that contained 14:0 and 16:0 fatty acyl side chains. Mar(14:0/14:0) and Mar(16:0/16:0) have been measured as standard. The putative Mar(14:0/16:0) falls between these standards in regard to RT and CCS. Although deviation of the Mar(16:0/16:0) standard from the RT trendline was higher, trends along CCS trend lines were fitting. Generally, a higher deviation of RT from standards was observed for maradolipids in *C. elegans* samples, but errors were generally below 2%, while the highest error for CCS was 0.4%. Furthermore, CCS trend lines showed good linear trends, while for RT this was only the case for very limited examples and typically showed quadratic behavior. Combining all available information, the peak was confirmed to be Mar(14:0/16:0) based on AllIons fragmentation, RT and CCS. A list with all putatively identified maradolipids, their RT, *m/z* and CCS values can be found in SI table 1.

<Figure 3>

Figure 3 (A) Extracted ion chromatograms for m/z 305.0877 and 323.0972 in high collision energy frames of the UHPLC-IMS-Q-ToF-MS AllIons experiments. Coelution of both masses indicates presence of of maradolipids, while m/z 323.0972 alone indicates potential lysomaradolipids (B) RT and CCS trendlines used for identification of Mar(14:0/16:0).

*Lysomaradolipids*

While searching for potential maradolipids using AllIons fragmentation, an additional region between 2.5 and 6.5 minutes showing the fragment *m/z* 323.0972 was identified. However, no corresponding fragment *m/z* 305.0877 was found. Therefore, it was hypothesized that the peaks in this area represent lysomaradolipids. Papan et al. have identified lysomaradolipids using shotgun based lipidomics analysis of lipid extracts from *C. elegans* dauer larvae. The fragmentation pattern they have obtained show strong similarities compared to the ones found in the present publication (4). Their proposed fragmentation is matching the observation of the peaks eluting in this RT range. Using the obtained AllIons fragmentation data it was observed that a collision energy of 10 eV is more useful for the non-targeted search because the [M+FA-H]- and [M-H]- ions, as well as the [trehalose-H2O-H]- fragments are present in the high collision energy data. For further structural elucidation 20 eV collision energy was used, since both the [trehalose-H2O-H]- fragment as well as fatty acyl fragments were visible, while 40 eV mostly produced the fatty acyl fragment.

Using a similar filtering approach and putative annotation on the MS1 level with masses of theoretical lysomaradolipids we identified a list of 12 potential candidates. Coelution of MS1 *m/z*, the [trehalose-H2O-H]- fragment as well as specific fatty acyl fragments were used for identification. Fragment *m/z* EICs were isolated for the specific drift time regions of the intact molecule.

Interestingly, for the *m/z* of LysoMar(17:0) two chromatographic peaks were found. While for the first and higher peak fragmentation data identified a fragment at *m/z* 269, no fragmentation data confirming the putative ID was available due to low intensity of the precursor. However, while checking for co-elution with *m/z* 323.0972, perfect coelution could be observed for both peaks. *C. elegans* is able to produce mono-methyl-branchend chain fatty acids on its own and most maradolipids contain a branched chain fatty acid. It might be possible that one peak represents a mmBCFA C17 and the other one a straight chain version. Both fatty acids have been detected in the analysis of total fatty acids, but heptadecanoic acid only in low amounts (14). Investigating trendlines for both RT and CCS using odd numbered LysoMar showed that both peaks are matching the trends between LysoMar(15:0) and LysoMar(19:0). However, if only higher peak eluting earlier is used trends increased. The CCS value of the second peak is slightly higher (247.72 compared to 247.44), which indicates a slightly larger structure. Since the two peaks showed good chromatographic separation the logP value for both possibilities was calculated as a measure of hydrophobicity. The logP of the straight chain LysoMar(17:0) is 2.66 and the logP of the iso-branched chain version is 2.50. This would fit with the trends seen based on CCS, indicating that the branched chain version is eluting before the straight chain version. Table 2 summarizes all identified lysomaradolipids. Since no reference standards are currently available for lysomaradolipids these identifications cannot be further validated.

<Figure 4>

Figure 4 (A) RT and CCS trendlines constructed for lysomaradolipids. For LysoMar(17:0) two peaks are visible. (B) Extracted ion chromatograms from low and high collision energy frames from AllIons fragmentation data. The two LyosMar(17:0) isomers were clearly separated in the RT dimension. Co-elution for both peaks the fragment m/z 323.0972 is observed. However, only for the first isomer the respective FA(17:0) fragment was detected.

Table 2: Summary of CCS and RT values of detected lysomaradolipids. CCS values were derived UHPLC-IMS-QToF-MS. Means and standard deviations were calculated from triplicate measurements.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Ion mode** | **Name** | **adduct** | **m/z** | **CCS +/- SD (multi field)** | **CCS +/- SD (single field)** | **RT +/- SD** |
| neg | LysoMar(19:1) | [M+FA-H]- | 665.3748 | --- | 251.45 +/- 0.35 | 5.839 +/- 0.06 |
| LysoMar(16:1) | 623.3281 | --- | 240.94 +/- 0.14 | 2.9 +/- 0.01 |
| LysoMar(18:1) | 651.3596 | --- | 247.47 +/- 0.36 | 4.506 +/- 0.07 |
| LysoMar(17:1) | 637.3444 | --- | 245.22 +/- 0.1 | 3.819 +/- 0.01 |
| LysoMar(17:0) | 639.3581 | --- | 247.72 +/- 0.41 | 5.26 +/- 0.01 |
| LysoMar(20:0) | 681.4054 | --- | 256.82 +/- 0.37 | 8.63 +/- 0.01 |
| LysoMar(18:0) | 653.3747 | --- | 250.72 +/- 0.43 | 6.407 +/- 0.05 |
| LysoMar(16:0) | 625.3436 | --- | 244.3 +/- 0.40 | 4.254 +/- 0.03 |
| LysoMar(17:0) | 639.3593 | --- | 247.44 +/- 0.27 | 4.995 +/- 0.03 |
| LysoMar(19:0) | 667.3906 | --- | 253.90 +/- 0.13 | 7.518 +/- 0.00 |
| LysoMar(14:0) | 597.3125 | --- | 237.07 +/- 0.45 | 2.697 +/- 0.02 |
| LysoMar(15:0) | 611.3282 | --- | 240.83 +/- 034 | 3.182 +/- 0.02 |

**Conclusion**

Lipid analysis and identification represents a delicate, but important task. Besides MS and MS/MS orthogonal information such as RT and CCS can be helpful in identifying members of homologous series or to clean up fragmentation patterns. We described the analysis of maradolipids, a class of lipids found exclusively in the dauer stage of *C. elegans*, using UHPLC-IMS-Q-ToF-MS. Previous analysis of maradolipids used high resolution shotgun lipidomics. In this work lipid extracts from *C. elegans* dauer larvae were directly analyzed without prior prefractionation and enrichment of glycolipids. Based on authentic reference standards CCS values using the multifield method could be determined. Furthermore, RT and CCS trendlines have been established. Combination of KMD, RT and CCS analysis as well as AllIons fragmentation data allowed the identification of several members of the maradolipid family. Furthermore, several lysomaradolipids for which no reference standards are currently available could be identified, including two putative isomers of LysoMar(17:0). The obtained results show how RT, CCS and AllIons fragmentation can be combined for the identification of lipid species.

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**Conflicts of interest**

No conflict of interests.

**Availability of data and material**

CCS and RT values of marado- and lysomaradolipids detected in *C. elegans* are summarized in SI Table 1 and 2. Masses, formulae, m/z and fragment m/z of theoretical marado- and lysomaradolipids are summarized in SI Table 3.

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**Supplemental Information**

SI Table 1: RT, CCS and m/z values of all detected maradolipids in *C. elegans* dauer larvae

SI Table 2: RT, CCS and m/z values of all detected lysomaradolipids in *C. elegans* dauer larvae

SI Table 3: m/z and fragment m/z of theoretical marado- and lysomaradolipids