Chapter 8

Fatty Acid Composition by Total Acyl Lipid Collision-Induced Dissociation Time-of-Flight (TAL-CID-TOF) Mass Spectrometry

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Abstract

Total acyl lipid collision-induced dissociation time-of-flight (TAL-CID-TOF) mass spectrometry uses a quadrupole time-of-flight (QTOF) mass spectrometer to rapidly provide a comprehensive fatty acid composition of a biological lipid extract. Samples are infused into a QTOF instrument, operated in negative mode, and the quadrupole is used to transfer all, or a wide mass range of, precursor ions to the collision cell for fragmentation. Time-of-flight-acquired mass spectra provide mass accuracy and resolution sufficient for chemical formula determination of fatty acids in the complex mixture. Considering the limited number of reasonable CHO variants in fatty acids, one can discern acyl anions with the same nominal mass but different chemical formulas. An online application, LipidomeDB Data Calculation Environment, is employed to process the mass spectral output data and match identified fragments to target fragments at a resolution specified by the user. TAL-CID-TOF methodology is a useful discovery or screening tool to identify and compare fatty acid profiles of biological samples.

Key words TAL-CID-TOF, LipidomeDB Data Calculation Environment (DCE), QTOF, Collision-induced dissociation, Fatty acid composition, Fatty acid analysis, Fatty acyl, Lipidomics, Mass spectrometry

1 Introduction

Fatty acids are integral building blocks of phospholipids, galactolipids, triacylglycerols, sphingolipids, cholesteryl esters, and other lipids. They also occur as free fatty acids. Normal-chain fatty acids and their oxidized counterparts (oxylipins) play diverse roles in cellular structures, cell biology, development, signaling, stress responses, and defense mechanisms in plants and animals, both as free fatty acids and as components of complex lipids [1–12]. Characteristic alterations in fatty acid composition occur in many human pathophysiologies, such as inflammation, diabetes, coronary heart disease, and colorectal cancer [13–19].

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The fatty acid composition of a lipid extract is commonly determined by gas chromatography (GC), with either flame ionization detection (GC-FID) or mass spectrometry (GC-MS) detection, after derivatization to fatty acid methyl esters. Liquid chromatography and various other separation techniques have also been used, with special handling sometimes required for easily degradable oxylipin species [20–25]. Alternatively, we have developed a simple method utilizing direct infusion of a crude lipid extract into a quadrupole time-of-flight (QTOF) mass spectrometer to provide rapid identification of component fatty acyl species [26]. Because it is performed on a high-mass-resolution QTOF instrument, this technique, "total acyl lipid collision-induced dissociation time-of-flight mass spectrometry" (TAL-CID-TOF MS), can identify fatty acyl anion fragments to the level of the chemical formula. Anions with the same nominal mass (*m/z*), but different chemical formulas, can be differentiated by small *m/z* differences. Optionally, internal standards can be added to the samples for approximate quantification.

In TAL-CID-TOF MS analysis, a lipid extract is directly infused into a QTOF instrument, operated in negative mode with an electrospray ionization source, and either the quadrupole is turned off or it is adjusted to allow ions of a wide *m/z* range to enter the collision cell. If there is no selection by the quadrupole, fragments from collision-induced dissociation result from both complex lipids and free fatty acids in the extract. If the QTOF method is designed such that primarily ions of $m/z > 400$ enter the collision cell, the fatty acyl fragments result mainly from complex lipids; free fatty acids are attenuated or eliminated from the analysis.

TAL-CID-TOF MS is a precise and reproducible tool for screening biological samples for fatty acid composition. Differences among samples due to experimental treatments, genetic mutations, pathological conditions, and so on can be easily determined with minimal sample processing. Figure 1 shows results from an experiment investigating the effects of mechanical wounding on *Arabidopsis thaliana* leaves [8]. Forty-five minutes after wounding, the levels of ten fatty acid species have significantly changed from the levels in unwounded leaves, with most oxylipins increasing and most unoxidized fatty acids decreasing.

Due to the mass accuracy of this technique, clear distinctions among anion fragments with the same nominal mass can be made and formulas determined. Figure 2 displays centroided mass spectra from another experiment studying the effect of wounding on the lipid profile of *A. thaliana* leaves [2]. Left insets in both Panels a and b of Fig. 2 show that a glycerol-galactose anion at *m/z* 253.0929 ($C_9H_{17}O_8$), a fragment from monogalactosyldiacylglycerol (MGDG) molecular species, is distinguishable from the 16:1 fatty acyl anion at *m/z* 253.2173 ($C_{16}H_{29}O_2$). The right inset in Panel b documents the appearance in the wounded sample of an

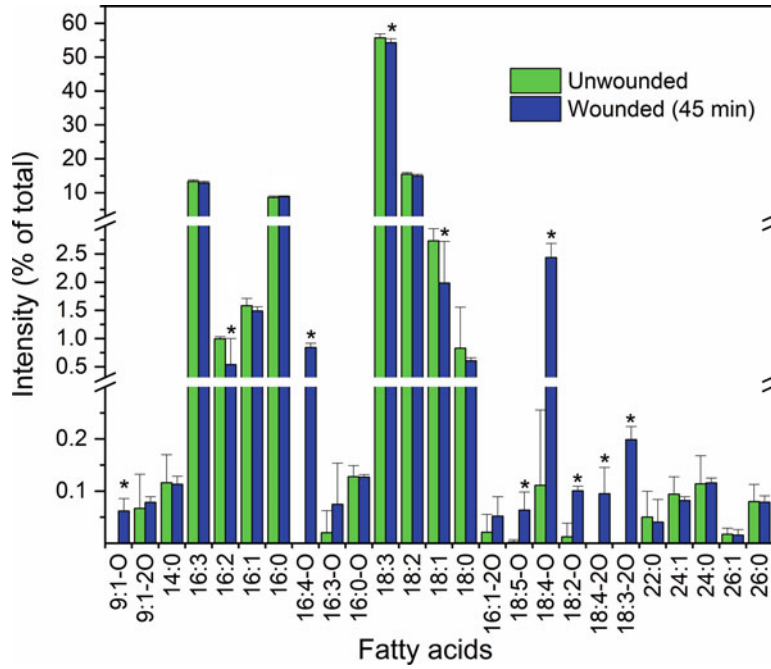

Fig. 1 Levels of fatty acids in Arabidopsis Col-0 leaves, unwounded and 45 min after mechanical wounding, as detected by TAL-CID-TOF methodology. Fatty acid nomenclature is indicated by the number of acyl carbons: number of double bonds or double bond equivalents beyond the acid carbonyl carbon—number of "extra" oxygens in addition to the two in the carboxylic acid group. Levels of lipids in wounded leaves were compared to those in unwounded leaves using a Student's *T*-test with correction for false discovery rate (FDR), and levels which were significantly different ($p < 0.05$) are labeled with an asterisk (*). Error bars indicate standard deviation, $n = 10$. This figure is modified from Fig. S4 in Vu et al. [8]

oxylipin fragment at *m/z* 281.1758 (C16H25O4), which correlates to a 16-carbon ketol fatty acyl anion, 16:3-2O. (The 16:3-2O nomenclature indicates 16 carbons, 3 double bonds or double bond equivalents beyond the acid carbonyl carbon, and 2 "extra" oxygens in addition to the two in the carboxylic acid group.) The peak for 16:3-2O is distinct from the unoxidized 18:1 fatty acyl anion at *m/z* 281.2486 (C18H33O2). These spectra also clearly show increases in three additional oxylipin species in wounded plants: dinor-oxophytodienoic acid (dnOPDA, 16:4-O, *m/z* 263.1653, C16H23O3), oxophytodienoic acid (OPDA, 18:4-O, *m/z* 291.1966, C18H27O3), and an 18-carbon ketol compound (18:3-2O, *m/z* 309.2071, C18H29O4).

Once the TAL-CID-TOF MS data are obtained, they are exported from the mass spectrometer's acquisition software as peak lists of anion fragments (*m/z* and intensity), formatted appropriately, and uploaded into an online application, LipidomeDB

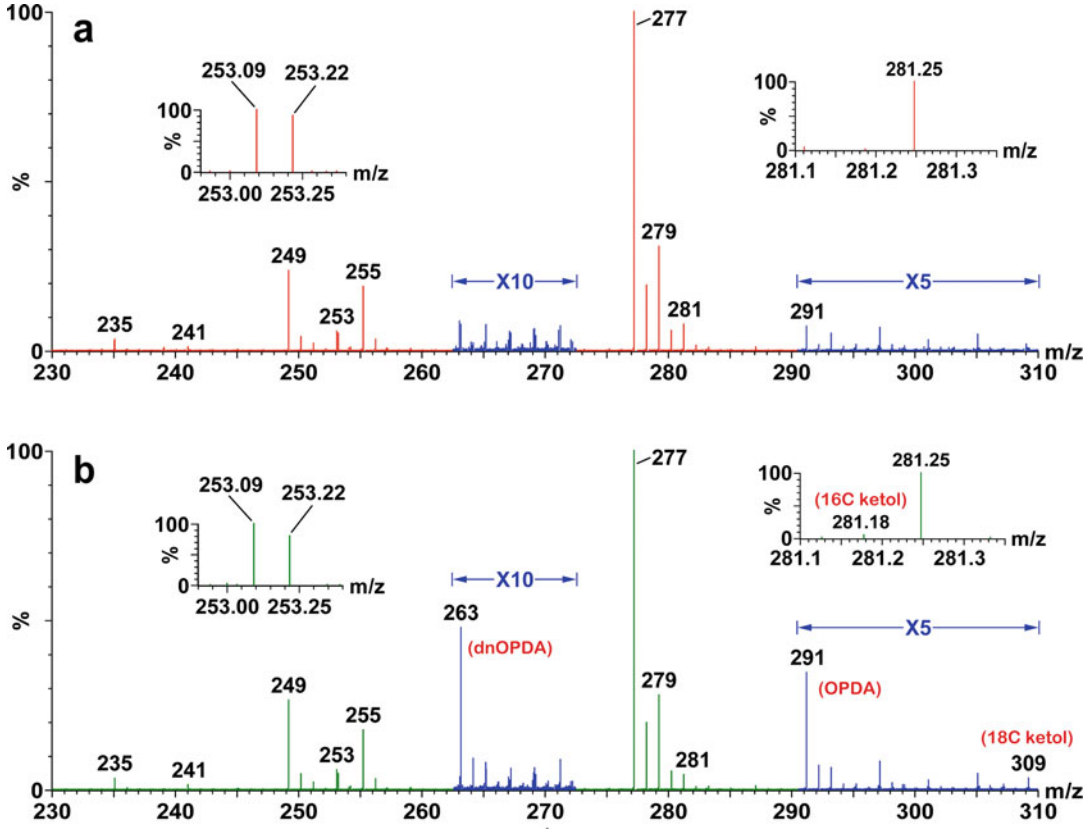

Fig. 2 Centroided TAL-CID-TOF anion mass spectra from fragmentation of lipids in Arabidopsis Col-0 leaves, unwounded (**a**) and 5 h after wounding (**b**). Spectra are expanded 10× from approximately *m/z* 262–273 and 5× from *m/z* 290–310. Insets show *m/z* ranges where there are two peaks at the same nominal mass. Peak intensities are plotted relative to the base (largest) peak in the spectrum. This figure is modified from Fig. 2 in Buseman et al. [2]

Data Calculation Environment (DCE). LipidomeDB DCE is a web-based tool, which was designed to process mass spectral data obtained from direct infusion of lipid-containing biological extracts into a triple quadrupole mass spectrometer [27]. The initial capabilities provided interpretation and quantification of data from multiple precursor and neutral loss scans on multiple samples, and a later update added multiple reaction monitoring (MRM) functionalities [28]. LipidomeDB DCE has now been updated to process CID-TOF data. A list of target fragment ions, e.g., a list of fatty acyl anions, is defined within the application in terms of fragment names and chemical formulas, which indicate *m/z*. LipidomeDB DCE matches fragments in the uploaded TAL-CID-TOF mass spectral sample data with those in the target list at a mass resolution specified by the user. The output is the intensity of each target fragment for each sample. Users may design their own target lists and further interpret the data based on experimental goals.

If internal standards are added to samples before analysis, TAL-CID-TOF MS provides semiquantitative elucidation of fatty acid levels, the limitations of which are detailed in Esch et al. [26]. Confirming earlier studies [29, 30], it was shown that the *sn2* position acyl moiety on the glycerol backbone in phospholipids tends to fragment more readily than the *sn1* acyl moiety [26]. Fragmentation of MGDG molecular species conversely produces higher intensity peaks from the *sn1* fatty acyl group. Additionally, different classes of complex lipids (i.e., those with different head groups) vary in their ability to ionize and/or form fragments in negative mode; changes in collision energy alter the response. Although the fragmentation disparities among different complex lipids render this technique only approximately quantitative, it is useful for making sample-to-sample acyl composition comparisons within an experiment, as demonstrated in Fig. 1 [8].

TAL-CID-TOF MS can be used as a "discovery" tool, providing rapid screening of new tissues, genetic variants, or physiological states. Accurate identification of the fatty acyl moieties enables subsequent quantitative probes of the lipidome through triple quadrupole scanning for their intact lipid precursor ions [2, 8]. Utilization of this methodology avoids lengthy purification, derivatization, and potential lipid degradation and effectively provides comprehensive fatty acid composition analysis of lipid extracts from diverse biological samples.

2 Materials

1. Lipid extract from a biological sample (*see* **Note 1**).
2. 2 mL glass vials with Teflon-lined lids (Thermo Scientific, B7800-1).
3. Nitrogen gas stream evaporator, in hood.
4. Methanol–chloroform–300 mM ammonium acetate in water (665:300:35, v/v/v).
5. Chloroform–methanol (1:1, v/v), for washing between samples.
6. Methanol–acetic acid (9:1, v/v), for occasional washing between samples.
7. Phosphatidylethanolamine (PE)(46:0) [di23:0], available by transphosphatidylation of phosphatidylcholine (PC)(46:0) [di23:0] from Avanti [31], for use as a mass spectrometer calibrant or internal standard.
8. External syringe pump for infusing sample into the mass spectrometer, capable of delivering 25 μL/min, unless the mass spectrometer has an integrated syringe pump.

9. Glass syringe for use in the syringe pump, such as a 1 mL Hamilton gastight syringe (Fisher Scientific, 13-684-94).
10. Quadrupole time-of-flight mass spectrometer (QTOF MS), such as Q-Tof 2, Q-Tof Premier, Xevo QTof, or Synapt (Waters/Micromass, Ltd., Milford, MA), equipped with an electrospray ionization source (*see* Subheading 3.1). A method for MDS/Sciex QStar Elite (Applied Biosystems, Foster City, CA) is provided in Subheading 3.2. The instrument must be capable of allowing all ions, or ions of a wide mass range, into the collision cell before TOF analysis.
11. Access to the online web-based application LipidomeDB Data Calculation Environment (DCE) at http://lipidome.bcf.ku.edu:8080/Lipidomics/.

3 Methods

3.1 Sample Preparation and Mass Spectrometry (Waters Mass Spectrometers) (See Note 2)

1. To prepare the sample for analysis, transfer a volume equal to approximately 5–50 nmol of lipids from a complex biological lipid extract to a 2 mL glass vial (*see* **Note 3**). Add internal standard(s) if desired (*see* **Note 4**). Evaporate the solvent with a nitrogen gas stream. Add 1.2 mL of methanol–chloroform–300 mM ammonium acetate in water (665:300:35, v/v/v) (*see* **Note 5**). Prepare an additional "background solvent" vial containing 1.2 mL of the solvent mixture.
2. Calibrate the QTOF MS (*see* **Note 6**).
3. Design an acquisition method for the QTOF MS that will either turn off mass selection in the Q1 quadrupole entirely or will allow ions of a specified wide *m/z* range to enter the collision cell before TOF analysis. Set up the method, with electrospray ionization in negative mode, to continuously infuse sample at 25 μL/min. The TOF analyzer should be tuned for at least 8000 full width at half height (FWHH) resolving power. Masses of fragments should be determined to four decimal places and be accurate to within 2 millimass units (±0.002 u). Create methods in continuum (individual scans saved) and multiple channel analyzer (MCA) modes. In MCA mode, delay data collection until adequate time has passed for sample to move into the mass spectrometer and for signal to stabilize (*see* **Note 7**).
4. Infuse the "background solvent" into the QTOF MS, acquiring TAL-CID-TOF data with the MCA method developed above. Potential background noise and contamination in the solvent and/or instrument can be visualized in the resulting spectra.

5. Infuse the lipid extract sample into the QTOF MS. Perform a preliminary test acquisition examining data in individual scans (not MCA mode), and make sure that the sample concentration is appropriate (*see* **Note 8**).
6. Continue to infuse the sample, and acquire the TAL-CID-TOF data in MCA mode.
7. In between samples, infuse chloroform–methanol (1:1, v/v) to rinse the tubing and source and remove residue from the ion path. If many samples are being analyzed, periodically infuse methanol–acetic acid (9:1, v/v) to remove residual, "sticky" fatty acyl groups and contaminants.
8. Select background subtraction, smoothing, and centroiding parameters such that each resolved ion is represented as one *m/z* and intensity pair. Obtain a peak list from the spectrum. Copy the peak mass (*m/z*) and intensity columns, and paste them into an Excel file (*see* **Note 9**).
9. Format the Excel file appropriately for uploading into LipidomeDB DCE. The peak mass and intensity columns should be pasted into Columns A and B, respectively. Two additional rows should be inserted above the data. Cell A1 should be the word "Mass," and Cell B1 should be "Signal." Cells A2 and B2 should be the number "0." Fragment data from all samples in an experiment should be pasted into one Excel workbook, with individual samples on separate sheets. Each sample should have a unique name or number indicated on the sheet tab. There is no limit to the number of sheets (samples) or rows of fragment data for TAL-CID-TOF analysis.

3.2 Mass Spectrometry (MDS/Sciex QStar Elite)

Here we provide alternate instructions for the QStar Elite QTOF MS (MDS/Sciex).

1. Using Analyst QS 2.0 software, design an acquisition method, with electrospray ionization, which allows ions of a specified wide *m/z* range to enter the collision cell before TOF analysis. Our laboratory uses an installed script developed by Applied Biosystems, "Q1 Resolving Overrides." When operated in product ion mode with the script activated, the Q1 quadrupole allows only ions larger than the mass filled in at the "Products of ___" prompt, plus slightly smaller ions, to pass through to the collision cell. Experiment to clarify this quadrupole ion selection range for your potential sample components. We have found that "Products of 540" allows only ions of $m/z > 430$ to enter the collision cell; smaller ions are filtered out. Fragmentation of lipid extracts in negative mode should thus produce fatty acyl anions from complex lipid precursors; free fatty acids should be eliminated from analysis.

2. Develop the acquisition method in negative product ion mode ("Products of 540"), continuously infusing sample at 30 μL/min with the integrated Harvard syringe pump. The ion spray voltage should be set at −4.5 kV, the source temperature at 150 °C, the curtain gas at 25 psi, and the ion source gases at 20 and 30 psi. The declustering potential should be −80 V, the declustering potential 2, −15 V, and the focusing potential, −300 V. The collision gas, nitrogen, should be set at 3 psi, and the collision energy at −45 V. Multiple channel acquisition (MCA) mode (cumulative scans) is best if any of the lipid extract components are in low abundance; continuum mode (individual scans) is adequate if the sample is concentrated. Data should be collected over *m/z* 100–1200, 60–300 scans over 1–5 min, and accurate masses of fragments should be determined to four decimal places.
3. Calibrate the QTOF MS externally with one or more lipid standards, which when fragmented in TAL-CID-TOF analysis, will produce both low and high mass anions that bracket the fatty acyl range of interest. A good choice for a calibrant is PE (di23:0). Upon TAL-CID-TOF fragmentation, PE(di23:0) produces a glycerol phosphate-related peak at *m/z* 152.9958 and a 23:0 fatty acyl anion peak at *m/z* 353.3425. If the calibrant concentration is unknown, determine the concentration of the calibrant by phosphate assay [32], and add approximately 1 nmol to 1.2 mL of methanol–chloroform–300 mM ammonium acetate in water (665:300:35, v/v/v). Infuse the calibrant solution, acquire the data with the TAL-CID-TOF method developed above, and calibrate the QTOF MS on the produced anion fragments. Perform calibration daily or as needed.
4. In between calibrants/samples, infuse chloroform–methanol (1:1, v/v) to rinse the tubing, source, and ion path. If many samples are being analyzed, periodically infuse methanol–acetic acid (9:1, v/v) for more thorough rinsing.
5. Prepare samples as indicated in Subheading 3.1, **step 1**.
6. Infuse the lipid extract sample into the QStar Elite. Perform a preliminary test acquisition, examining data in individual scans (not MCA mode), to make sure that the concentration is appropriate. The intensity of each peak in a single scan should be ≤5000 counts to ensure that the detector is not saturated. If the sample is too concentrated, dilute by adding methanol–chloroform–300 mM ammonium acetate in water (665:300:35, v/v/v) until the peaks are on scale. Alternatively, if the peak intensities are weak, prepare a more concentrated sample or adjust the method to accumulate more scans than usual.

7. Continue to infuse the sample, and acquire the TAL-CID-TOF data. Analyze the "background solvent" sample with the same method, acquisition time, and mode (continuum or MCA) as the lipid extract samples to determine background noise and contamination in the solvent and/or instrument.
8. Utilize Analyst QS 2.0 software to open the data file. If the acquisition was performed in MCA mode, the spectrum will open immediately. If the acquisition was performed in continuum mode, left-click and drag across the total ion current chromatogram to average all scans, right-click, and choose "Show Spectrum." Smooth with a 0.4–1–0.4 setting (previous point weight-current point weight-next point weight), choose "List Data," then "Peak List," then "Save as Text," and save the table as a ".txt" file. Open the ".txt" file with Excel, and copy the columns "Centroid Mass (Da)" and "Centroid Intensity" to a new Excel file.
9. *See* Subheading 3.1, **step 9**, to continue with data set-up and subsequent processing.

3.3 LipidomeDB Data Calculation Environment (DCE)

1. Utilize LipidomeDB DCE (http://lipidome.bcf.ku.edu:8080/Lipidomics/) to process the mass spectral data and match identified fragments in the sample extract to designated target fragments (*see* **Note 10**). After logging in, choose "Add CID-TOF Experiment." The database will assign an experiment number. Enter an experiment name and description. Enter a mass unit (u) value in the "Mass Tolerance" box, which will define the *m/z* window for peak searching. A mass tolerance of 0.004 u is recommended; this setting will locate sample fragment ion peaks within ±0.004 u of the theoretical *m/z* value of each designated target fragment ion. Select a "Mass to find" option from the three choices in the drop-down menu: "Find the Nearest Mass" (nearest to the theoretical *m/z* value of the designated target fragment ion), "Find the Largest Signal" (largest within the *m/z* window designated by the mass tolerance setting), and "Find the Sum of all Signals" (sum within the *m/z* window designated by the mass tolerance setting). For TAL-CID-TOF data sets, "Find the Nearest Mass" is recommended. Press "Continue."
2. The next step involves entering a CID-TOF Target Fragment list, which will be used to mine the sample spectral data. Choose an existing, preformulated list (*see* **Note 11**), modify and save such a list with a new name (*see* **Note 12**), or build a new list (*see* **Note 13**). It is possible and sometimes desirable, especially with many modifications, to directly add fragments to the database or to add or edit existing CID-TOF Target Fragment lists independent of an actual experimental workflow (*see* **Notes 14** and **15**, respectively).

3. After the CID-TOF Target Fragment list has been entered and "Continue" selected, upload the spectral data. These data must be in an Excel file, formatted as detailed above in Subheading 3.1, **step 9**. Press "Continue" to display the results on screen, which will appear as a summary table of the target fragment intensities for all samples. Press "Export to Excel" at the bottom of the table to download the results.
4. If an internal standard was added to the samples, approximate quantification of fatty acyl fragments is possible. Calculate the amount of each target fragment, in relation to the internal standard (IS) (*see* **Note 16**). Alternatively, determine the level of each target fragment, as a percentage of the total fatty acyl fragment intensities (*see* **Note 17**).

4 Notes

1. Many extraction methods can be used to isolate lipids from biological tissues. A modified Bligh–Dyer method [33, 34] and a rapid one-step extraction from plant tissue [35] are both excellent for phospho- and galactolipids and have been commonly used in our laboratory for Arabidopsis, sorghum, maize, grasses, and other plants. Methods can be modified for different tissues [36]. Once the extract is obtained, it is best to evaporate to dryness and redissolve in 1 mL chloroform. *See* also Chapters 1 and 2 in this book.
2. Mass spectrometry directions in Subheadings 3 and 4 are written primarily for Waters QTOF instruments (Subheading 3.1). An alternative method for MDS/Sciex QStar Elite is provided in Subheading 3.2.
3. For *A. thaliana* leaves, a lipid extract prepared from 0.2 mg dry leaf tissue is used. An equivalent amount is necessary for other Arabidopsis tissues, such as roots and pollen. For more fibrous tissues (stems, grasses, thick leaves, etc.), a larger dry weight equivalent is necessary, perhaps double or more. In terms of protein, an extract from tissue containing 35 μg is suggested. The sample aliquot required for analysis must often be determined by trial and error.
4. If approximate quantification of the fatty acids in the sample is desired, an internal standard can be added. Internal standards are synthetic or purified compounds that are added to the sample before analysis. Their concentration should be accurately determined by traditional analytical methods, such as phosphate assay [32] or gas chromatography of fatty acid methyl esters. Internal standards for TAL-CID-TOF analysis should be lipids which ionize well in negative mode and

produce non-naturally occurring fatty acyl anion fragments upon collision-induced dissociation. Good choices are complex lipids with a single, uncommon fatty acid, such as PC(24:0) [di12:0], from Avanti, or PE(46:0) [di23:0]. After phosphate assay [32] and preparation of a 0.5 mM solution in chloroform, add 2 μL (1 nmol) to the extract aliquot.

5. Alternatively, instead of evaporating, add chloroform to the aliquot up to a total volume of 360 μL. Add 840 μL methanol–300 mM ammonium acetate in water (95:5, v/v). The resultant volume and solvent composition (1.2 mL of methanol–chloroform–300 mM ammonium acetate in water, 665:300:35, v/v/v) will be the same with either method.
6. On Waters instruments, you may calibrate in V mode using sodium formate and its reference file, over *m/z* 50–1000 (a range in which negatively charged sodium formate cluster ions are formed [37]), making sure to obtain an ion below *m/z* 100. Keep ion counts of all utilized calibrant ions below 0.1 ions per push (ipp). Sodium formate (0.5 mM) can be prepared by adding 100 μL of a 10% formic acid solution (in HPLC-grade water) to 25 mL of 0.5 mM sodium hydroxide (in isopropanol–water, 9:1, v/v) (ToF G2-S Standard Kit-2, Waters, 700008892). Sonicate for 5 min.
7. Parameters should be optimized for your instrument and sample concentration. On the Waters QTOF instruments, with MassLynx as the operating and data processing software, needle voltage should be set at 2.6 kV, cone voltage at 35 V, and collision cell offset at 28 V. The quad profile should be set up to start at *m/z* 400; larger anions will enter the collision cell, but smaller ions will be subject to Q1 transmission attenuation. To set up the quad profile, select the RF Settings or Quad Profile tab (depending on the instrument/software version) on the tune page, choose "Manual Fixed" as the quadrupole option, and fill in "400" as the quadrupole MS profile mass. Fragmentation of lipid extracts in negative mode should produce fatty acyl anions from primarily complex lipid precursor ions; detection of free fatty acids should be attenuated. Data can be acquired with TOF MS function for 5 min in continuum mode over *m/z* 50–1000, 10 s per cycle, or in MCA mode (additive scans). In either continuum or MCA mode, analyze only scans where the total ion current is high and stable; the time when this occurs depends on the length and diameter of tubing from syringe to the mass spectrometer.
8. If the peak intensities are too high (>0.1 ions per push (ipp) on Waters instruments) and the detector is saturated, the exact mass (*m/z*) determination and the intensity will not be accurate. Dilute the sample by adding methanol–chloroform–

300 mM ammonium acetate in water (665:300:35, v/v/v), and test again. Repeat the dilution/test run as necessary until the peak intensities in a single scan are on scale. This detector saturation level is specific to each mass spectrometer. If the peak intensities in the test run are weak, prepare a more concentrated sample or adjust the method to accumulate more scans than usual.

9. Verify that the centroid parameters identify only one "stick" per resolved ion in the acyl range (*m/z* 100–400). Lock on a mass of a known acyl anion in the spectrum (e.g., 18:3 at *m/z* 277.2173). Display the spectrum from *m/z* 100–400. Consider setting a threshold at 0.01–1% of the base (largest) peak to reduce noise. Switch from spectrum view to peak list. Copy two columns, mass and intensity, and paste into an Excel sheet.
10. A tutorial, which details the function and capabilities of the application and provides example data files to illustrate its use, is available at the LipidomeDB DCE website.
11. Select an existing list from the drop-down menu. The list "fatty1 (95fa)" is a list of 95 unoxidized and oxidized fatty acyl anions suitable for analysis of lipids derived from many plants (Table 1). The second option, "fatty2 (39fa)", includes only the unoxidized fatty acyl anions from the "fatty1" list. The third list, "fatty3 (34 animal fa)", is optimized for mammalian lipids. After selection, the boxes will populate; the mode is negative. Press "Continue" at the bottom of the page.
12. To modify an existing list, select it from the drop-down menu. New fragments can be entered by typing in a fragment name already stored in the database, which will retrieve the proper fragment formula. The maximum number of fragments in a target list is 100 (designated as #0–99), and they do not need to be listed in any particular order. If a fragment is not in the database, entering both the fragment name and chemical formula is required. Elements that can be interpreted in the formula are C, H, O, N, P, and S. Chemical formulas should be entered as CmHnOqNrPsSt, where the large letters represent the elements, and the small letters indicate the number of atoms of each element. Leaving an element out of the formula indicates 0 atoms of that element, and no number following an element indicates 1 atom of that element. If a fragment contains atoms due to formation of an adduct ion, these atoms must be included in the fragment formula. Fragments derived from any internal standards in the sample must be included in the target fragment list. The "Mass of Detected Ion" box will calculate automatically when the chemical formula is retrieved or entered. After the modified target fragment list is complete, give it a new name in the "Save current interface" box at the

Table 1
CID-TOF target fragment list of fatty acyl anions, "fatty1 (95 fa)", from LipidomeDB DCE

Fragment name	Fragment formula	Detected ion, *m/z*	Fragment name	Fragment formula	Detected ion, *m/z*
6:0	C6H11O2	115.0765	18:1-O	C18H33O3	297.2435
7:1-O	C7H11O3	143.0714	16:2-3O	C16H27O5	299.1864
8:0	C8H15O2	143.1078	18:0-O	C18H35O3	299.2592
7:1-2O	C7H11O4	159.0663	16:1-3O	C16H29O5	301.2020
9:1-O	C9H15O3	171.1027	16:0-3O	C16H31O5	303.2177
10:0	C10H19O2	171.1391	18:5-2O	C18H25O4	305.1758
9:1-2O	C9H15O4	187.0976	20:3	C20H33O2	305.2486
12:3-O	C12H17O3	209.1183	18:4-2O	C18H27O4	307.1915
13:0	C13H25O2	213.1860	20:2	C20H35O2	307.2643
12:2-2O	C12H19O4	227.1289	16:5-4O	C16H21O6	309.1344
14:0	C14H27O2	227.2017	18:3-2O	C18H29O4	309.2071
15:2	C15H25O2	237.1860	20:1	C20H37O2	309.2799
15:1	C15H27O2	239.2017	16:4-4O	C16H23O6	311.1500
15:0	C15H29O2	241.2173	18:2-2O	C18H31O4	311.2228
16:3	C16H25O2	249.1860	16:3-4O	C16H25O6	313.1657
16:2	C16H27O2	251.2017	18:1-2O	C18H33O4	313.2384
16:1	C16H29O2	253.2173	16:2-4O	C16H27O6	315.1813
16:0	C16H31O2	255.2330	18:0-2O	C18H35O4	315.2541
16:5-O	C16H21O3	261.1496	16:1-4O	C16H29O6	317.1970
16:4-O	C16H23O3	263.1653	16:0-4O	C16H31O6	319.2126
17:3	C17H27O2	263.2017	18:5-3O	C18H25O5	321.1707
16:3-O	C16H25O3	265.1809	18:4-3O	C18H27O5	323.1864
16:2-O	C16H27O3	267.1966	19:3-2O	C19H31O4	323.2228
17:1	C17H31O2	267.2330	21:1	C21H39O2	323.2956
16:1-O	C16H29O3	269.2122	18:3-3O	C18H29O5	325.2020
17:0	C17H33O2	269.2486	21:0	C21H41O2	325.3112
16:0-O	C16H31O3	271.2279	18:2-3O	C18H31O5	327.2177
16:5-2O	C16H21O4	277.1445	18:1-3O	C18H33O5	329.2333
18:3	C18H29O2	277.2173	18:0-3O	C18H35O5	331.2490
16:4-2O	C16H23O4	279.1602	22:3	C22H37O2	333.2799
18:2	C18H31O2	279.2330	22:2	C22H39O2	335.2956

(continued)

Table 1
(continued)

Fragment name	Fragment formula	Detected ion, *m/z*	Fragment name	Fragment formula	Detected ion, *m/z*
16:3-2O	C16H25O4	281.1758	18:5-4O	C18H25O6	337.1657
18:1	C18H33O2	281.2486	22:1	C22H41O2	337.3112
16:2-2O	C16H27O4	283.1915	18:4-4O	C18H27O6	339.1813
18:0	C18H35O2	283.2643	22:0	C22H43O2	339.3269
16:1-2O	C16H29O4	285.2071	18:3-4O	C18H29O6	341.1970
16:0-2O	C16H31O4	287.2228	18:2-4O	C18H31O6	343.2126
18:5-O	C18H25O3	289.1809	18:1-4O	C18H33O6	345.2283
18:4-O	C18H27O3	291.1966	18:0-4O	C18H35O6	347.2439
19:3	C19H31O2	291.2330	23:1	C23H43O2	351.3269
16:5-3O	C16H21O5	293.1394	23:0	C23H45O2	353.3425
18:3-O	C18H29O3	293.2122	24:1	C24H45O2	365.3425
19:2	C19H33O2	293.2486	24:0	C24H47O2	367.3582
16:4-3O	C16H23O5	295.1551	25:1	C25H47O2	379.3582
17:3-2O	C17H27O4	295.1915	25:0	C25H49O2	381.3738
18:2-O	C18H31O3	295.2279	26:1	C26H49O2	393.3738
19:1	C19H35O2	295.2643	26:0	C26H51O2	395.3895
16:3-3O	C16H25O5	297.1707			

LipidomeDB DCE is an online application which processes mass spectral data from direct infusion of lipid extracts into a tandem mass spectrometer (http://lipidome.bcf.ku.edu:8080/Lipidomics/). The displayed target list is preloaded within the application and is suitable for TAL-CID-TOF analysis of lipids derived from many plants. LipidomeDB DCE searches uploaded sample data for these target fragments and returns lists of identified targets and their intensities for all samples. Fatty acyl anion fragment nomenclature is indicated by the number of acyl carbons: number of double bonds or double bond equivalents beyond the acid carbonyl carbon—number of "extra" oxygens in addition to the two in the carboxylic acid group. "Detected Ion *m/z*" is the theoretical exact mass of the fatty acyl anion fragment, calculated from the listed fragment formula

top of the page. It will then be available for immediate and future use and is visible only to the user who created it. Press "Continue" at the bottom of the page.

13. A new CID-TOF Target Fragment List can be designed by following the instructions in **Note 12**, without selecting an existing list to start. Press "Continue" at the bottom of the page.
14. Fragments may be added to the database by selecting "Add or Edit Fragments" on the page following log-in. Already-entered fragment names and formulas are listed in the drop-down menus. To edit an existing fragment, the "Copy Current

Fragment" button can be used. To enter a new fragment, type the name and formula into the boxes. Alternatively, two columns of up to 100 rows at a time (fragment names and formulas) can be copied and pasted from Excel into the box at the bottom of the page; the "Fill Boxes" button will then generate the numbered list. When ready to commit the new fragments to the database, click "Add Fragments." Further instructions can be found on the website and in the tutorial.

15. CID-TOF Target Fragment lists can be added or edited by selecting "Add or Edit CID-TOF Interfaces" on the page following log-in. The instructions in **Notes 12** and **13** above can generally be followed to make these changes. Additionally, a box at the bottom of the page allows for copying and pasting two columns of up to 100 rows (fragment names and formulas) from Excel; "Fill Boxes" will then generate the numbered list. After the new or edited CID-TOF Interface is ready to save to the database, give it a name in the "Interface name" box at the top of the page, and press "Save" at the bottom. The new interface (target fragment list) will be available for use only by the user who created it.
16. To determine the amount of a fatty acyl fragment in comparison to the IS, calculate as follows:

$$\begin{aligned}\text{nmol of target fragment} = &(\text{intensity of target fragment})\\ &\times (\text{nmol IS target fragment})\\ &/(\text{intensity of IS target fragment}).\end{aligned}$$

If the IS has two identical target fragments (e.g., two identical fatty acyl chains), *nmol IS target fragment* in the above formula should be double the IS concentration added to the sample.

17. To determine the level of a fatty acyl fragment intensity as a percentage of the total intensity of all fragments, calculate as follows:

$$\begin{aligned}\%\text{ of target fragment} = &(\text{intensity of target fragment})\\ &\times 100/(\text{sum of target fragment intensities}).\end{aligned}$$

Note that *sum of target fragment intensities* should not include any intensities of internal standards.

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