Chapter 13

Quantification of Acyl-Acyl Carrier Proteins for Fatty Acid Synthesis Using LC-MS/MS

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Abstract

The fatty acid biosynthetic cycle is predicated on an acyl carrier protein (ACP) scaffold where two carbon acetyl groups are added in a chain elongation process through a series of repeated enzymatic steps. The chain extension is terminated by hydrolysis with a thioesterase or direct transfer of the acyl group to a glycerophospholipid by an acyltransferase. Methods for analysis of the concentrations of acyl chains attached to ACPs are lacking but would be informative for studies in lipid metabolism. We describe a method to profile and quantify the levels of acyl-ACPs in plants, bacteria and mitochondria of animals and fungi that represent Type II fatty acid biosynthetic systems. ACPs of Type II systems have a highly conserved Asp-Ser-Leu-Asp (DSLD) amino acid sequence at the attachment site for 4-′-phosphopantetheinyl arm carrying the acyl chain. Three amino acids of the conserved sequence can be cleaved away from the remainder of the protein using an aspartyl protease. Thus, partially purified protein can be enzymatically hydrolyzed to produce an acyl chain linked to a tripeptide via the 4-′-phosphopantetheinyl group. After ionization and fragmentation, the corresponding fragment ion is detected by a triple quadrupole mass spectrometer using a multiple reaction monitoring method. ^{15}N isotopically labeled acyl-ACPs generated in high amounts are used with an isotope dilution strategy to quantify the absolute levels of each acyl group attached to the acyl carrier protein scaffold.

Key words Acyl-carrier protein, Aspartyl protease, Fatty acid synthesis, Isotope dilution, Lipid metabolism, Liquid chromatography, Mass spectrometry, Multiple reaction monitoring

1 Introduction

Fatty acids are energy dense molecules important for many biological processes. As an energy reserve, fatty acids comprise most of the carbon in the storage lipid, triacylglycerol, and as components of phospho- and galactolipids, the fatty acids establish the molecular species of membrane lipids and affect fluidity [1]. Acyl lipids can also act as signaling molecules and regulate gene expression and protein activity [2, 3]. In plants, fatty acids are synthesized predominantly in the chloroplast and to a lesser extent in the mitochondria [4–6]. Acyl chains are elongated by two carbons at a time through a series of reactions starting with condensation of a

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two-carbon acetyl group to produce the ketoacyl derivative that is subsequently reduced, dehydrated, and reduced again to produce a fully saturated acyl chain (Fig. 1) [7]. In plants, these reactions are repeated until the fatty acid is 16–18 carbons in length [8]. The elongating acyl chains are shuttled through the fatty acid synthesis reactions by a scaffold protein called acyl-carrier protein (ACP). A certain proportion of the 18:0-ACP is desaturated by stearoyl-ACP desaturase to produce oleoyl-ACP (18:1-ACP). Chain elongation is terminated by a thioesterase or acyltransferase. A majority of the fatty acids are cleaved by a thioesterase and exported from the chloroplast and reactivated to acyl-CoAs to be used in lipid production at the ER [1, 9, 10]. Alternatively, an acyltransferase can directly

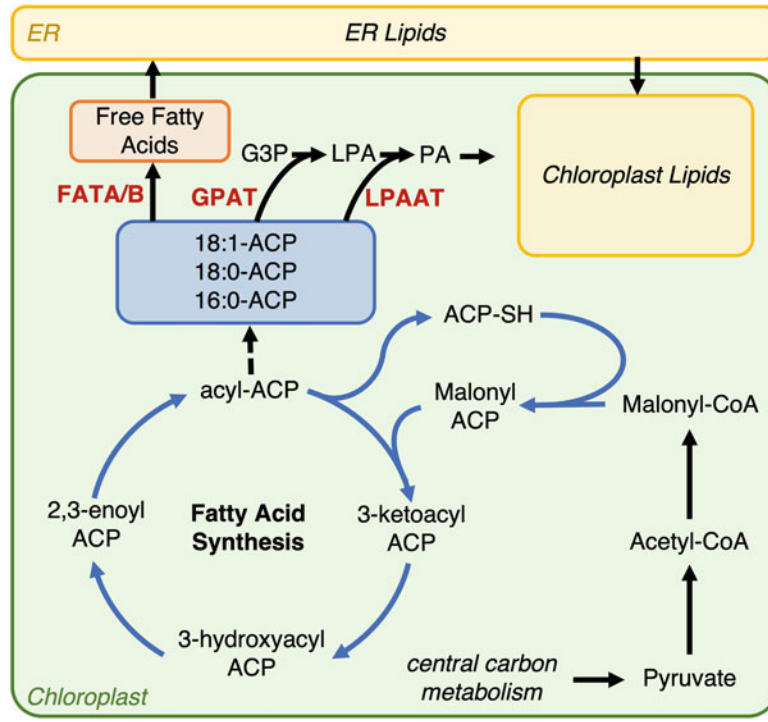


Fig. 1 Fatty acid metabolism in a plant cell. Fatty acid synthesis connects central carbon metabolism with the production of lipids. Acyl-chains are produced by elongating an acyl-ACP backbone with two carbons at a time, involving a cycle of four reactions per acetyl addition. The primary products of fatty acid synthesis are fully saturated 16 and 18 carbon acyl-ACPs (16:0- and 18:0-ACP) and monounsaturated 18 carbon acyl-ACP (18:1-ACP). Most of these acyl chains are cleaved from the ACP protein via acyl-ACP thioesterases (FATA/FATB) and the free fatty acids (i.e., nonesterified fatty acids. NEFA) produced are exported from the chloroplast and used to synthesize lipids in the endoplasmic reticulum (ER). Alternatively, the acyl chains can be directly transferred from ACP to glycerol-3-phosphate (G3P) and lysophosphatidic acid (LPA) by G3P and LPA acyltransferases (GPAT and LPAAT) to form phosphatidic acid (PA)

transfer fatty acyl chains from acyl-ACPs to glycerol-3-phosphate (G3P) and lysophosphatidic acid (LPA) in the chloroplast as part of the prokaryotic Kennedy pathway [1, 11–18].

Fatty acids as major components of lipid molecules are a significant sink for carbon and therefore represent the crucial node connecting primary and lipid metabolism. The current methods of analyzing acyl-ACPs are confounded by low abundance, multiple isoforms of ACPs, and other sources of acyl-chains (i.e., acyl-CoA and nonesterified fatty acids) [6, 19, 20]. We provide a protocol for analyzing fatty acid synthesis intermediates (acyl-ACPs) by using synthesized isotopically labeled standards, an aspartyl protease, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) based on a recent publication [21]. Plants and bacteria carry out fatty acid synthesis using a discrete acyl-carrier protein (9–15 kDa) (Type II FAS), in contrast to yeast and animals that use a large multidomain enzyme (Type I FAS) [22–25]. The ACP involved in Type II FAS contains highly conserved amino acid residues (Asp, Ser, Leu, Asp) at the acyl-chain attachment site. By using an aspartyl protease (Asp-N), the acyl-ACP molecule is effectively reduced in size to a three amino acid peptide with the acyl-chain attached at the serine through a 4′-phosphopantatheine prosthetic group (Fig. 2a). This molecule is sensitively detected and each acyl chain length is well resolved by LC-MS/MS (Fig. 2b) [21]. Acyl-ACPs can be accurately quantified through isotope dilution techniques with enzymatically synthesized acyl-ACPs [21].

The strategy described in this chapter allows for a deeper insight into the steps of fatty acid synthesis. The method is capable of quantifying all acyl-ACPs commonly found in plant tissues and was developed with saturated standards for carbon lengths of 2–18 and monounsaturated 16 and 18 carbon acyl chains [21]. Presumably, other acyl chains could be quantified in a similar way if standards were made and if the basic chemical structure remained the same thus resulting in an equivalent mass loss and fragmentation pattern in the mass spectrometer. Additionally, the 3-hydroxyacyl and 2,3-enoyl fatty acid synthesis intermediates (Fig. 1) can be measured, allowing for precise studies on regulation of the fatty acid synthesis cycle. The method can also be used as a discovery tool to probe for novel acyl-ACP species, such as polyunsaturated hexadecatrienoyl-ACP (16:3-ACP) which was observed in seeds and leaves of *Camelina sativa* (false flax) and could potentially be involved in novel lipid remodeling events occurring within the chloroplast [21].

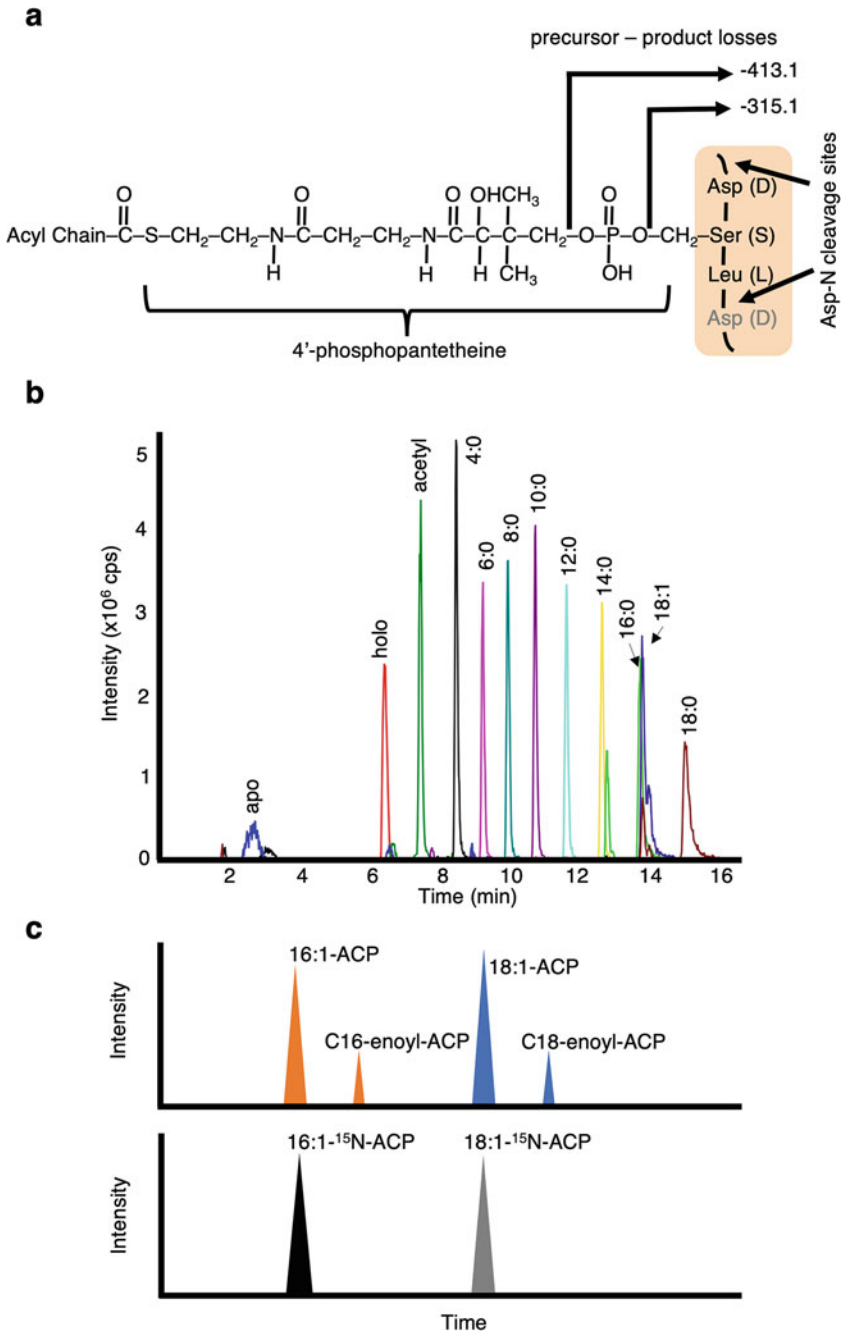


Fig. 2 Acyl-ACP detection via LC-MS/MS. (**a**) Acyl-ACP structure with Asp-N protease cleavage sites and the precursor–product fragmentation indicated. The numbers 315.1 and 413.1 are equivalent to the neutral losses after fragmentation on the protein or pantetheine sides, respectively, of the phosphate group from the acyl-ACP. (**b**) Representative chromatograph of acyl-ACP standards following LC-MS/MS analysis. For each ACP analyte, the MRM signal as shown in Table 1 is plotted. Reproduced from [21] with permission from The Plant Cell (www.plantcell.org; copyright American Society of Plant Biologists). (**c**) Verification of peak identities. ^{15}N labeled standards (lower panel) are used to validate sample peaks by retention time. 16:1-Δ9cis-ACP and the

2 Materials

For all solutions, use ultrapure water (18 MΩ H_2O). Use analytical grade chemicals. Clean and autoclave all glassware used for growth media and buffer storage.

2.1 General Material and Equipment

1. UV and visible spectrum compatible cuvettes.
2. Sterile filters for buffer sterilization.
3. 2 mL lined screw cap tubes.
4. Shaking incubator(s) (17 and 37 °C).
5. Adjustable wavelength spectrophotometer (UV and visible).
6. Ultrasonic microtip probe (6–12 mm).
7. Heating block and/or water bath for 1.5 and 2 mL tubes.
8. Vacuum centrifugal concentrator.
9. Bead mill.
10. Laminar flow hood.

2.2 Overexpression of apo-ACP and Sfp Transferase in E. coli and Purification

1. 1 × 2 L baffled shake flask per culture medium, autoclaved.
2. LB Agar growth medium for selection plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g agar, H_2O to adjust volume, autoclaved.
3. Sterile petri dishes containing a selective medium for single colony isolation.
4. Sterile inoculation loop(s).
5. 50 mg/mL filter-sterilized kanamycin, store at −20 °C.
6. LB Growth Medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, H_2O to volume, autoclave, store at 4 °C.
7. 500 mL flask for starter culture medium preparation.
8. Sterile culture tubes for starter cultures.
9. 1 × 2 L bottle per buffer (cleaned and autoclaved) for buffer storage.
10. Ni^{2+} Immobilized Metal Affinity Chromatography (IMAC) Binding Buffer: 10 mM imidazole, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, filter-sterilize, store at 4 °C.

Fig. 2 (continued) 16:1-Δ2trans-enoyl-ACP elongation intermediate (both in orange) are isomers which can be separated by chromatography. Labeling and LC-MS/MS analysis of ^{15}N 16:1-Δ9cis-ACP internal standard identifies 16:1-Δ9cis-ACP as eluting earlier than C16-Δ2trans-enoyl-ACP. The same result is observed for 18:1-Δ9cis-ACP and 18:1-Δ2trans-enoyl-ACP (both in blue). Not drawn to scale

11. Ni^{2+} IMAC elution buffer: 250 mM imidazole, 20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% (v/v) glycerol, filter-sterilize, store at 4 °C.
12. Desalting/Storage Buffer: 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 10% (v/v) glycerol, filter-sterilize, store at 4 °C.
13. 10× 3-Morpholinopropane-1-sulfonic acid (MOPS) base: In the order listed, 0.4 mM MOPS, 0.04 mM Tricine–KOH, pH 7.4, 0.5 mM NaCl, 2.76 mM K_2SO_4, 0.005 mM $CaCl_2$, 5.25 mM $MgCl_2$, 10× Hunter's Trace Element Solution (Chlamydomonas Resource Center), 10× Gibco® Minimum Essential Medium (MEM), Vitamin solution (10 mg/L choline chloride, 10 mg/L calcium D-pantothenate, 10 mg/L folic acid, 10 mg/L nicotinamide, 10 mg/L pyridoxal hydrochloride, 1 mg/L riboflavin, 10 mg/L thiamine hydrochloride, 20 mg/L myoinositol, 850 mg/L sodium chloride) in water, filter-sterilize, store at −20 °C (*see* Subheading 3.1, **step 12**).
14. Modified MOPS minimal medium (for isotopic labeling): 10× MOPS base, 0.528 g/L $^{15}NH_4Cl$, 1.32 mM monopotassium phosphate (KH_2PO_4), and 0.2% glucose (sterile) in water, filter-sterilize.
15. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG), filter-sterilize, store at −20 °C.
16. 1 × 250 mL centrifuge bottle per culture, preweigh.
17. 1 × 50 mL round bottom centrifuge tube per culture, preweigh.
18. Lysozyme, store at −20 °C.
19. cOmplete™ Mini EDTA-free Protease Inhibitor Tablets (1 tablet per 10 mL), store at 4 °C.
20. ÄKTA FPLC system (GE Healthcare), or equivalent, with 2 and 20 mL sample loops.
21. 5 mL HisTrap™ High Performance column.
22. 2 × 5 mL HiTrap™ Desalting column.
23. 3 and 20 mL Luer lock syringes.
24. Amicon® Ultra Centrifugal Filters (3000 and 10,000 molecular weight cutoff, MWCO).

2.3 Acyl-ACP Standard Synthesis

1. Dimethyl sulfoxide (DMSO).
2. 500 mM MOPS–NH_4OH stock solution (pH 6.5).
3. 50 mM Tris(2-caroxyethyl)phosphine hydrochloride (TCEP) solution in water.
4. 1 mM apo-ACP protein (isolated from *E. coli* BL21 [New England Biolabs] overproducing apo-ACP; *see* Subheading 3.1) in storage buffer, store at −80 °C.

5. 20% (w/v) Polysorbate 20 (synonymous: Tween 20).
6. 5 mM acyl-CoA (*see* **Note 1**).
7. 100 mM Magnesium chloride ($MgCl_2$).
8. 100 mM Manganese chloride ($MnCl_2$).
9. 0.2 mM Sfp transferase (isolated from *E. coli* BL21 [DE3] [Novagen] overproducing Sfp transferase; *see* Subheading 3.1) in storage buffer, store at −80 °C.

2.4 Analysis of Acyl-ACPs by SDS-PAGE

1. 16.5% Mini-PROTEAN® Tris-Tricine Gel (Bio-Rad) or equivalent.
2. 10× Tris/Tricine/SDS Running Buffer: 100 mM Tris–NaOH, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.3 after dilution to 1× in water.
3. Tricine Sample Buffer for Protein Gels: 200 mM Tris–HCl, pH 6.8, 40% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) Coomassie Brilliant Blue G-250.
4. 0.5 M TCEP.
5. Prestained Protein Standard marker proteins.
6. Colloidal Coomassie Brilliant Blue G-250 Stain: 0.05% (w/v) Coomassie Brilliant Blue G-250, 5% (w/v) aluminum sulfate-(14–18)-hydrate [$Al_2(SO_4)_3 \times (14–18)\ H_2O$] (or 10% [w/v] ammonium sulfate), 10% (v/v) ethanol (96%), and 2% (v/v) orthophosphoric acid (85%) (*see* **Note 2**).
7. Destaining Solution: 10% (v/v) ethanol (96%) and 2% (v/v) orthophosphoric acid (85%).

2.5 Extraction of Acyl-ACP Standards

1. 20% (w/v) trichloroacetic acid (TCA).
2. 1% (w/v) TCA.
3. 50 mM MOPS–NH_4OH (pH 7.5) with 5% (v/v) DMSO.

2.6 Extraction of Acyl-ACP from Plant Tissues

1. Liquid nitrogen for sample collection.
2. 2 mL lined screw cap tubes (2 per sample).
3. Metal grinding beads.
4. 100% (w/v) TCA solution (minimum 0.3 μL per sample) (for later use to make 5% and 1% solutions).
5. 5% (w/v) TCA (minimum 1.5 mL per sample).
6. 1% (w/v) TCA (minimum 2.5 mL per sample).
7. 50 mM MOPS–NH_4OH (pH 7.5) with 5% (v/v) DMSO (minimum 1.6 mL per sample).
8. pH strips.
9. Gel loading pipette tips.
10. 0.8 μm polyethersulfone (PES) centrifuge filters.

2.7 Endoproteinase Asp-N Digestion

1. Endoproteinase Aspartate-N (Asp-N) (Sigma-Aldrich, from *Pseudomonas fragi*, P3303-1VL; lyophilized powder) in chilled ddH_2O, prepare immediately before use, keep on ice.
2. Methanol.
3. 0.5 M TCEP solution.

2.8 LC-MS/MS

1. HPLC vials (with inserts).
2. Shimadzu HPLC, or equivalent.
3. AB SCIEX QTRAP® 6500 mass spectrometer, or equivalent.
4. Column oven.
5. Discovery® BIO Wide Pore C18 reversed phase column (10 cm × 2.1 mm; 3 μm) (Millipore Sigma), or equivalent.
6. Buffer A: acetonitrile/10 mM ammonium formate and formic acid, pH 3.5 (10:90, v/v).
7. Buffer B: acetonitrile/10 mM ammonium formate and formic acid, pH 3.5 (90:10, v/v).

2.9 Software

1. Fast Protein Liquid Chromatography (FPLC) control and evaluation software: Unicorn™ (GE Healthcare), or equivalent.
2. LC-MS/MS Integration software: Analyst (SCIEX), or equivalent.
3. Image processing software: ImageJ, or equivalent.
4. Microsoft® Office Excel, or equivalent.

3 Methods

3.1 Overexpression of apo-ACP and Sfp Transferase in E. coli and Harvesting of Cells

An isotope dilution-based quantitation relies on individual standards for each analyte being measured (*see* Subheading 3.9, **steps 6–10**). However, no acyl-ACP standards are commercially available. This approach uses recombinant proteins: Sfp transferase (Sfp, WP_003234549) from *Bacillus subtilis and* apo-ACP (AcpP, NP_415612) from *Escherichia coli* expressed in *E. coli* expression strains to enzymatically synthesize acyl-ACP standards (Fig. 3). The Sfp transferase and apo-ACP described in this method are expressed as C-terminal hexahistidine (His_6) fusion proteins. The plasmids contain ColE1 origins, kanamycin resistance cassettes, and inducible lac promoters.

To produce the unlabeled apo-ACP substrate and the Sfp transferase required for acyl-ACP standard synthesis, LB growth medium is used. However, the ^{15}N labeled apo-ACP substrate is obtained using a minimal medium with $^{15}NH_4Cl$ as nitrogen source. Growth of *E. coli* within this medium results in ^{15}N labeled proteins. For the following steps, perform all inoculations under sterile conditions in a laminar flow hood and use flasks that are at least two times the volume of the media being prepared.

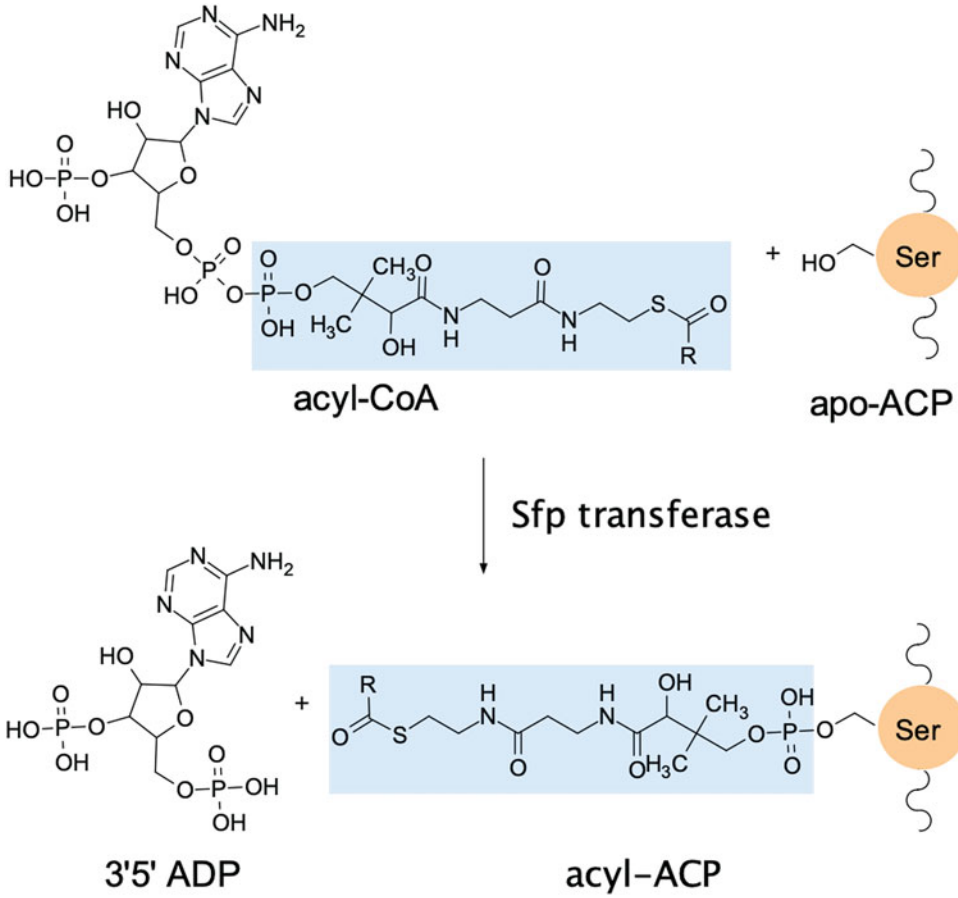


Fig. 3 Acyl-ACP standard synthesis using Sfp transferase. Sfp transferase covalently transfers the 4-′-phosphopantetheine group with an acyl chain attached (highlighted in blue) from acyl-CoA to apo-ACP at a conserved serine residue. Reproduced from [21] with permission from The Plant Cell (www.plantcell.org; copyright American Society of Plant Biologists)

1. Generate LB agar medium for single colony isolation and autoclave.
2. Cool the medium to approximately 60 °C and then add kanamycin to 50 μg/mL final concentration.
3. Pour medium into plates (~30 mL per plate) in a laminar flow hood and allow to cool for approximately 30 min with the lids slightly off to avoid condensation.
4. Use plates immediately or seal with Parafilm and store at 4 °C.
5. Plate *E. coli* expression strains (for expression of apo-ACP or Sfp transferase) for single colony isolation using a streak plate method.
6. Incubate plates, inverted, at 37 °C for 24 h.

7. Generate 1 L of LB growth medium in a 2 L flask to express the unlabeled proteins and 250 mL in a 500 mL flask to aliquot to starter cultures (*see* **Note 3**).
8. Autoclave the LB medium, baffled flasks, and buffer storage bottles.
9. Store the 1 L of LB medium at 4 °C until needed. Allow the 250 mL LB medium to reach room temperature before using.
10. For ^{15}N labeled apo-ACP: Make 500 mL 10× MOPS medium by adding components in the order listed (*see* Subheading 2.2, **item 13** for concentrations): add MOPS, Tricine, NaCl, and water to 150 mL, adjust pH to 7.4 with 10 M KOH as necessary. Add K_2SO_4, $CaCl_2$, $MgCl_2$, Hunters Trace Element Solution, MEM vitamin solution and water to a final volume of 500 mL.
11. Filter-sterilize 10× MOPS base medium, aliquot 100 mL aliquots, and store at −20 °C.
12. Start the production process by aliquoting 5 mL of the LB medium for starter cultures to sterile culture tubes and add 5 μL of 50 mg/mL kanamycin and inoculate with a single colony from a selection plate to serve as starter culture (starter cultures for ^{15}N labeling of apo-ACP are also made with LB medium). Make a separate starter culture for each overexpression condition (i.e., one each for unlabeled apo-ACP and Sfp transferase, and for ^{15}N culturing of apo-ACP expressing cells).
13. Incubate starter cultures overnight at 37 °C with shaking at 100–250 rpm.
14. The following day, warm 1 L LB growth medium to room temperature before inoculation.
15. Make 1 L of 1× Modified MOPS Minimal medium (for ^{15}N isotopic labeling of apo-ACP expressing cells), filter-sterilize, and transfer to an autoclaved baffled flask (*see* **Note 4**).
16. Add 1 mL of 50 mg/mL kanamycin and 5 mL starter cultures to each 1 L growth medium (LB medium or 1× Modified MOPS Minimal medium).
17. Incubate the cultures at 37 °C with shaking at 100–250 rpm (*see* **Note 5**).
18. Monitor the O.D. at 600 nm until it reaches approximately 0.6 (between 0.4 and 0.8 is acceptable) (*see* **Note 6**).
19. Cool the cultures by placing them on ice or at 4 °C.
20. Add 1 mL of 1 M IPTG to 1 L cultures to induce expression.
21. Reduce the incubation temperature to 16–18 °C and continue shaking at 100–250 rpm for expression overnight.

22. The next morning, place the cultures on ice (or at 4 °C) and keep on ice from this point onward.
23. Preweigh 50 mL round bottom and/or 250 mL centrifuge tubes (one per culture).
24. For each culture, spin down the cells at 4 °C in 250 mL centrifuge tubes for 5 min at 15,344 × *g*, dump out supernatant (preserve cell pellet), add more culture to bottle and repeat until the cells of the entire culture are concentrated in one pellet.
25. Consider transferring the cell pellets to preweighed 50 mL round bottom centrifuge tubes or maintain in the 250 mL centrifuge tubes (*see* **Note 7**).

3.2 Purification of apo-ACP and Sfp Transferase by Ni^{2+} IMAC Chromatography

Apo-ACP and Sfp transferase that have been expressed with C-terminal His_6 fusions are isolated by Ni^{2+} IMAC affinity purification. The proteins are retained on 5 mL HisTrap™ High Performance Columns (GE Healthcare) and eluted by increasing the concentration of imidazole over time.

1. Make Ni^{2+} IMAC Binding Buffer, Ni^{2+} IMAC Elution Buffer, and Desalting/Storage Buffer.
2. Filter-sterilize buffers and store in autoclaved bottles at 4 °C.
3. Reweigh the tubes with the *E. coli* cells expressing apo-ACP or Sfp transferase to obtain a pellet mass.
4. Add 3 mL of Ni^{2+} IMAC Binding Buffer per 1 g of cells, then add 10 mg/1 mL of lysozyme and 1 cOmplete™ Mini EDTA-free Protease Inhibitor Tablet per 10 mL.
5. Resuspend cells by pipetting up and down, vortexing, or shaking for 30 min (*see* **Notes 8** and **9**).
6. Return the tubes to ice.
7. Sonicate for 1 min at 50% duty cycle at the microtip limit followed by 1 min of cooling on ice, repeat six times.
8. Spin down cell debris by centrifuging at 4 °C at 48,384 × *g* for 30–45 min and collect the soluble fraction for protein purification.
9. For Ni^{2+} IMAC purification on the ÄKTA FPLC system, equilibrate the pumps and the 5 mL HisTrap™ High Performance Column with Ni^{2+} IMAC Binding Buffer.
10. Attach 20 mL sample loop.
11. Elution buffer gradient: 0–200 mL, 0% Ni^{2+} IMAC Elution buffer; 200–210 mL, 0–100% Ni^{2+} IMAC Elution buffer; 210–220 mL, 100% Ni^{2+} IMAC Elution buffer; 220–255 mL, 0% Ni^{2+} IMAC Elution buffer; 255 mL, stop.

12. Flow rate profile: 0–10 mL, 5 mL/min; 10–30 mL, 1 mL/min; 20–200 mL, 5 mL/min; 200–220 mL, 1 mL/min; 220–255, 5 mL/min; 255 mL, stop.
13. Set valve position to inject at 10 mL, set valve position to load at 200 mL.
14. Collect 1 mL fractions from 200 to 220 mL.
15. Measure the absorbance at 280 nm for each fraction.
16. Combine the protein containing fractions, indicated by an increase in absorbance at 280 nm.

3.3 Concentration and Desalting of Recombinant apo-ACP and Sfp Transferase

1. Concentrate combined protein fractions of Ni^{2+} IMAC column purification (*see* Subheading 3.2) to less than 2 mL using Amicon® Ultra Centrifugal Filters (3000 and 10,000 MWCO for apo-ACP and Sfp transferase, respectively).
2. For desalting, use 2 × 5 mL HiTrap™ Desalting Columns in tandem connected to the ÄKTA FPLC system:
3. Equilibrate the pumps and column to Desalting/Storage Buffer.
4. Connect 2 mL sample loop.
5. Elute proteins in Desalting/Storage Buffer.
6. Flow rate profile: 0–25 mL, 5 mL/min; 25–37 mL, 1 mL/min; 37–62 mL, 5 mL/min; 62 mL, stop.
7. Set valve position to inject at 25 mL.
8. Collect 1 mL fractions from 25 to 37 mL.
9. Measure the absorbance at 280 nm for each fraction.
10. Combine the protein containing fractions and concentrate to less than 2 mL using Amicon® Ultra Centrifugal Filters (3000 and 10,000 MWCO for apo-ACP and Sfp transferase, respectively).
11. Measure the absorbance at 280 nm (A_{280}) using UV-transparent cuvettes with the storage buffer as blank.
12. Calculate the molar concentration (c) of the protein by dividing A_{280} by the molar extinction coefficient (ε) and the pathlength of the cuvette (b) (*see* **Note 10**).

$$c = \frac{A_{280}}{\varepsilon b}.$$

13. Run a small amount of protein with several dilutions on an SDS-PAGE gel (*see* Subheading 3.6) to validate the purification of the protein and determine purity.
14. Dilute the apo-ACP to 1 mM and the Sfp transferase to 200 μM with storage buffer.
15. Aliquot (e.g., 500 μL) and store at −80 °C.

3.4 Acyl-ACP Standard Synthesis

1. Per 100 µL of reaction add 14 µL (for standards up to ten carbons in chain length) or 10 µL (for standards 12 or more carbons in chain length) of water, 10 µL of DMSO (10%, v/v final concentration), 10 µL of 500 mM MOPS–NH_4OH (pH 6.5) (50 mM final) 8 µL of 50 mM TCEP (4 mM final), 20 µL of 1 mM recombinant apo-ACP (200 µM final), 4 µL of 20% (w/v) Polysorbate 20 (1%, v/v, final) to reactions for standards of 12 carbon chain lengths or longer only (omit Polysorbate 20 from reactions up to ten carbons in chain length),and 10 µL of the respective 5 mM acyl-CoA (500 µM final). Mix thoroughly (*see* **Note 11**). Add 8 µL of 100 mM $MgCl_2$ (10 mM final) and 8 µL of 100 mM $MnCl_2$ (10 mM final). Mix thoroughly. Add 12 µL of recombinant Sfp transferase (24 mM final). Mix well and quickly spin the reaction tube to collect liquid from the sides of the tube.
2. React at 37 °C for 3 h with vortexing every hour.
3. Quench by precipitating the acyl-ACPs from the reaction by adding TCA and preforming *Clean Up* of *Acyl-ACP Standards* (*see* Subheading 3.7).
4. Determine reaction efficiency by SDS-PAGE analysis (*see* Subheading 3.6).
5. Calculate the concentration of acyl-ACP (C_{acyl}) in each reaction by multiplying the fraction of acyl-ACP (determined from densitometry of the corresponding lane in the SDS gel) by the concentration of the ACP in the reactions.

$$C_{acyl} = \frac{A_{acyl}}{A_{holo} + A_{acyl}} \times C_{f(ACP)}.$$

$$\text{Example}: C_{acyl} = 0.80 \times 200\ \mu M = 160\ \mu M.$$

3.5 Making the Calibration Curve for Quantification of Acyl-ACPs

Before making the calibration curve and performing isotope dilution-based quantification, perform a single point quantification analysis on your samples to estimate the amount of internal standard necessary. Though a multipoint isotope dilution-based quantification will measure across a range of peak area ratios of analyte to internal standard, it is good practice to operate at a ratio as close to 1 as possible.

3.5.1 Single Point Quantification for Estimation

1. Isolate acyl-ACPs from plant tissues (*see* Subheading 3.8). At **step 2**, add a range of concentrations of ^{15}N labeled acyl-ACP standards for each acyl-ACP to be quantified that spans 1–2 orders of magnitude (e.g., 0.01–1 µM final concentration) to 30–40 mg fresh weight (FW) of replicate biomass samples.
2. Carry out *LC-MS/MS Analysis* (*see* Subheading 3.10).
3. Estimate the amount of ^{15}N standard needed for each acyl-ACP species for isotope dilution (*see* Subheading 3.11, **steps 1–5**).

3.5.2 ^{15}N Labeled Standard Mix/^{15}N Standard Diluent

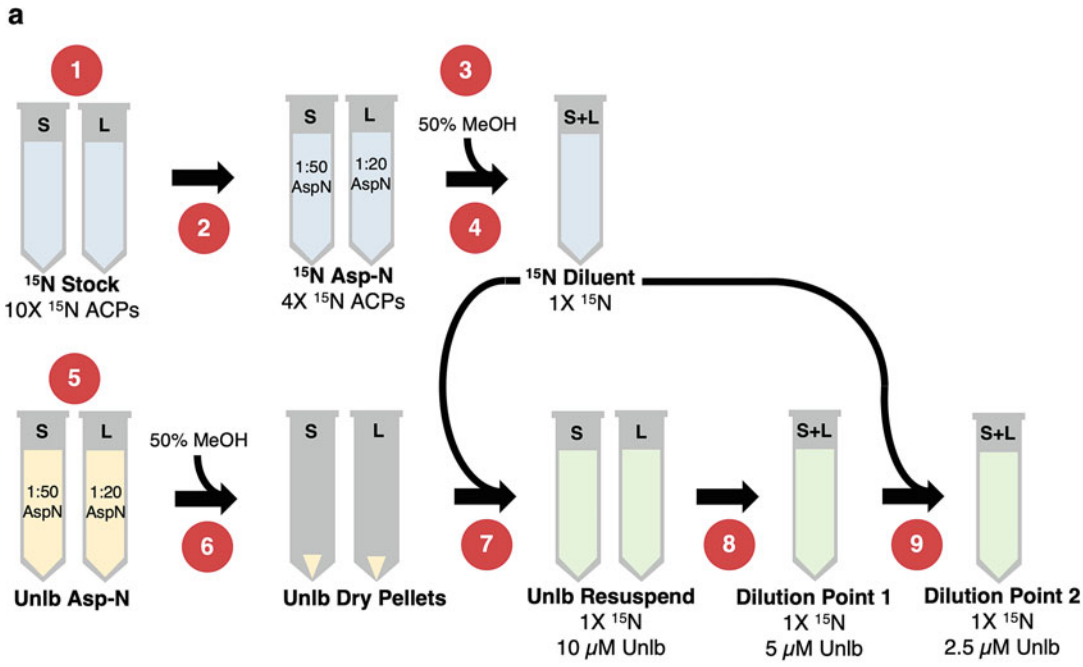
1. Make two separate stock mixes of ^{15}N labeled acyl-ACP standards at 10 times the concentration determined for quantification (the calculated estimate from the single point quantification in Subheading 3.5.1). One stock will contain each ^{15}N labeled standard with acyl chains up to ten carbons in length (10× short ^{15}N standard mix, usually contains: apo form and C2, C4, C6, C8, C10 saturated acyl chains), and a second stock will contain each ^{15}N labeled standard with acyl chains of 12 carbons or longer (10× long ^{15}N standard mix, usually contains: C12, C14, C16, C18 saturated and C16:1 and C18:1 acyl chains). These two acyl-ACP standard mixes will serve as the ^{15}N labeled stock mixes used for the calibration curve as well as for internal standards in plant tissue samples. (Fig. 4a, step 1).
2. Calculate the total volume of 1× ^{15}N standard needed for serial dilution of the calibration curve (*see* Subheading 3.5.4) and carry out **steps 3–5** (this section, below) to achieve this volume in **step 5**.
3. Digest the 10× short and long ^{15}N labeled acyl-ACP mixes separately as outlined in Subheading 3.9, **steps 1–3**. For **step 2** of Subheading 3.9, digest an aliquot of the long and short ^{15}N acyl-ACP standard mixes at 4× concentrations with ratios of 1:50 (short mix) or 1:20 (long mix) of Asp-N enzyme and acyl-ACPs (w/w) in 50 mM MOPS–NH_4OH (pH 7.5) with 5% (v/v) DMSO to volume (fourth of the volume calculated in **step 2** of this section) (Fig. 4a, step 2).
4. For each mix, add methanol to a final concentration of 50% (v/v). This will bring the two standard mix concentrations to 2× each (Fig. 4a, step 3).
5. Mix equal volumes of the 2× short ^{15}N standard mix and 2× long ^{15}N standard mix together to produce the 1× ^{15}N standard diluent (Fig. 4a, step 4). This is called "1× ^{15}N standard diluent" because it is later used to dilute the unlabeled acyl-ACP standard mix to generate the standard curve (Fig. 4b, c).

3.5.3 Unlabeled Standard Mix

1. Calculate the volume of each individual acyl-ACP standard ($V_{\text{Ind Std}}$) necessary for your most concentrated point of the standard curve (dilution point 1) by multiplying the final concentration (C_f) by the final volume (V_f) and dividing by the concentration of acyl-ACP (C_{acyl}; calculated in Subheading 3.4, **step 5**). Include an unlabeled standard for each acyl-ACP to be quantified in the biological sample.

$$V_{\text{Indv Std}} = \frac{C_f \times V_f}{C_{acyl}}.$$

For example, for a dilution point 1 with 5 μM of each standard in a final volume of 400 μL:



b

Dilution Point	Fold Dilution	Previous Dilution (μL)	1X ^{15}N Diluent (μL)
1	NA	NA	400
2	2	200	200
3	2	200	200
4	2	200	200
5	5	75	300
6	5	75	300
7	5	75	300
8	5	75	300
9	5	75	300
10	5	75	300

c

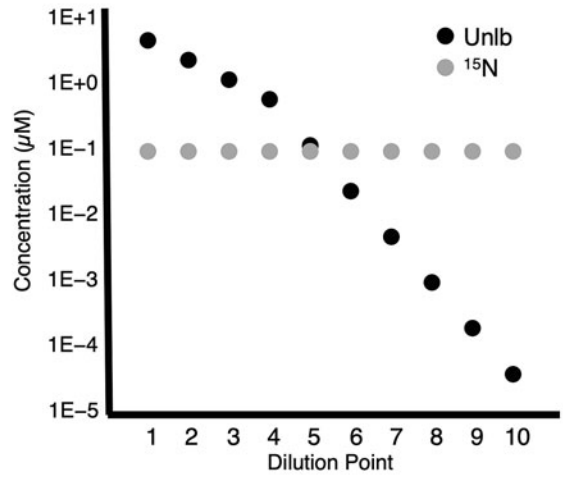


Fig. 4 Generating calibration curves for quantification. (**a**) Steps 1–4 (in red), making the ^{15}N labeled standard mix/diluent (Subheading 3.5.2). Steps 5 and 6, preparing the Unlabeled Standard Mix (Subheading 3.5.3). Steps 7 and 9, serial dilution of the unlabeled standards into the ^{15}N standard diluent (Subheading 3.5.4). Repeat **step 9** until the full standard curve is made. MeOH, methanol; L, long chain acyl-ACPs (acyl chains of 12 carbons or longer); S, short chain acyl-ACPs (acyl chains up to 10 carbons); NA, not applicable; Unlb, unlabeled. (**b**) Table showing fold dilution of each dilution point. (**c**) Graphical representation of standard concentrations

$$V_{\text{Indv Std}} = \frac{5\ \mu\text{M} \times 400\ \mu\text{L}}{160\,\mu\text{M}} = 12.5\ \mu\text{L each standard.}$$

2. Add the calculated amount of each unlabeled acyl-ACP standard into two separate mixes, one for standards with acyl chains up to ten carbons in length (short unlabeled standard mix), and a second for standards with acyl chains of 12 carbons or longer (long unlabeled standard mix) (Fig. 4a, step 5).
3. Digest each mix as outlined in Subheading 3.9, **steps 1–3** (Fig. 4a, step 5).
4. Validate Asp-N protease digest via SDS-PAGE. Include a negative control (undigested acyl-ACP). A successful digestion results in the absence of the acyl-ACP band (Fig. 5).

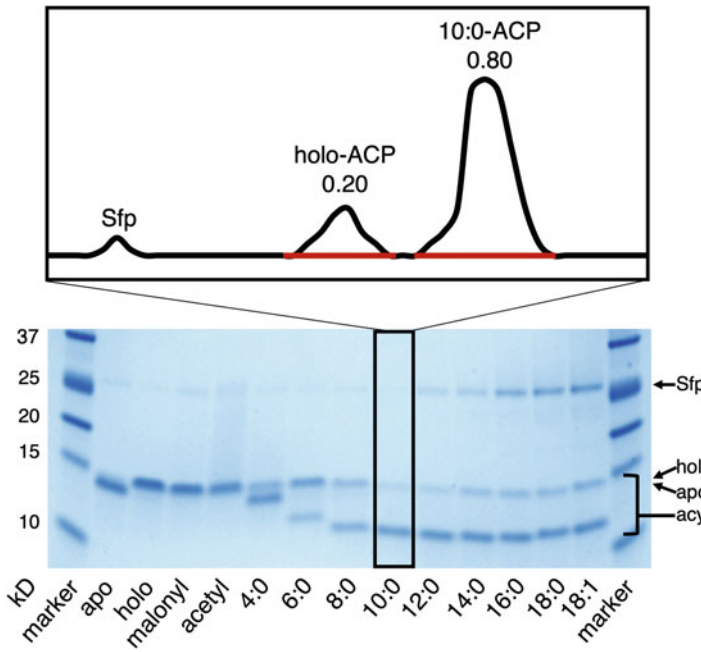


Fig. 5 SDS-PAGE analysis of acyl-ACP standards. Acyl-ACP separation on a 16.5% Tris-Tricine SDS-PAGE gel with lane by lane densitometry analysis using ImageJ software. The ACP's acidity and acyl-chain lengths both affect migration patterns. Acylated forms of ACP with chains of four carbons or more migrate faster on the gel than apo-ACP, though holo-, acetyl-, and malonyl-ACP migrate slower and cannot be distinguished from each other by migration pattern. Acyl chains of 10 to 18 carbons in length are also indistinguishable from each other based on migration patterns. After scanning the gel and densitometric image analysis, integrate the signal in the lane to obtain the peak areas for each band (e.g., of 10:0-ACP reaction analysis in blowout pane). Use the peak areas to determine the fraction of contaminating holo-ACP (0.20, i.e., 20%) and validate reaction efficiency (0.80, i.e., 80% 10:0-ACP). Reaction efficiency is observed by the complete disappearance of the apo-ACP substrate that has been turned into acyl-ACP

5. For each mix, add methanol to a final concentration of 50% (v/v) and dry down using a vacuum centrifugal concentrator (Fig. 4a. step 6).

3.5.4 Serial Dilution

1. Resuspend both the short unlabeled standard mix and the long unlabeled standard mix that were dried down (from Subheading 3.5.3, **step 5**) to a 2× final concentration (e.g., 10 μM) in the 1× ^{15}N standard diluent produced in Subheading 3.5.2, **step 5** (Fig. 4a, step 7).
2. Mix equal volumes of the 2× short and long unlabeled standard mixes together to obtain a 1× total unlabeled standard mix in 1× ^{15}N standard diluent (this is dilution point 1) (Fig. 4a, step 8).
3. Serially dilute this 1× total unlabeled standard mix from **step 2** into the 1× ^{15}N standard diluent (Fig. 4a, step 9) to obtain 10–12 dilutions that span six orders of magnitude (Fig. 4b, c) (*see* **Notes 12** and **13**).

3.5.5 Example Standard Curve Generation

1. Individual unlabeled and ^{15}N labeled acyl-ACP standards are synthesized from 200 μM purified *E. coli* ACP and commercial 500 μM acyl-CoA substrates (Subheading 3.4).
2. SDS-PAGE analysis (Fig. 5) determines that all apo-ACP (80% of total; remaining 20% is holo-form) is converted to acyl-ACP form (160 μM) (i.e., each acyl-ACP as an individual stock at 160 μM).
3. A single point quantification with 0.05, 0.5 and 5 μM ^{15}N acyl-ACP standards per 30 mg biomass in a final volume of 100 μL determines each sample analyte to be close to 0.1 μM (i.e., biomass contains ~10 pmol per analyte per 30 mg FW).
4. Make the 10× short and long ^{15}N labeled acyl-ACP standard stock mixes, combining the individual ^{15}N acyl-ACP standards: Two separate ^{15}N acyl-ACP standard mixes are made at 10× the concentration needed for the plant sample, one containing 25 μL of each individual ^{15}N acyl-ACP up to ten carbons (10× short ^{15}N standard mix) in length and one containing 25 μL of each individual ^{15}N acyl-ACP 12 carbons or longer (10× long ^{15}N standard mix) and bringing both mixes to 4 mL with 50 mM MOPS–NH_4OH (pH 7.5) and with 5% (v/v) DMSO (Fig. 4a, step 1).

$$C_f = 10 \times 0.1\,\mu M = 1\,\mu M \text{ each.}$$

$$Vol_{\text{indv std}} = \frac{C_f \times V_f}{C_{\text{acyl}}} = \frac{1\ \mu M \times 4\,mL}{160\ \mu M} = 0.025\,mL = 25\,\mu L \text{ each.}$$

5. An aliquot of each ^{15}N acyl-ACP standard mix is used to make the 1× ^{15}N standard diluent for the standard curve (*see* **step 7**

below), the rest is put aside for use as internal standards for quantification in biological samples.

6. 2.8 mL of 1× (here, 0.1 μM as determined in **step 3**) ^{15}N standard diluent is needed for the standard curve in Fig. 4b, c.
7. Approximately 3 mL of 1× (0.1 μM) ^{15}N standard diluent is made by first separately diluting 300 μL of each 10× (1 μM) short and long ^{15}N labeled acyl-ACP mixes to 750 μL by adding Asp-N at 1:50 and 1:20 (w/w) to short and long mixes, respectively and bring to volume with 50 mM MOPS–NH_4OH (pH 7.5) containing 5% (v/v) DMSO (Fig.4a, step 2). The mixes are then at 4× (0.4 μM) concentration and are reacted. Then 750 μL of methanol is added to quench each reaction (Fig. 4a, step 3)—the two standard mixes are then at 2× (0.2 μM) concentration. 1.48 mL of both the short and the long ^{15}N labeled acyl-ACP mixes are combined to make 1× (0.1 μM) ^{15}N standard diluent (2.96 mL, i.e., ~3 mL) (Fig. 4a, step 4). This is used for the serial dilution in the standard curve.
8. Now, make the unlabeled standard for the standard curve: The amount of each individual unlabeled standard (160 μM) necessary for a final concentration of 5 μM in a final volume of 400 μL for the highest dilution point on the standard curve (dilution point 1) is calculated to be 12.5 μL.

$$V_{\text{Indv Std}} = \frac{C_f \times V_f}{C_{\text{acyl}}} = \frac{5\ \mu\text{M} \times 400\ \mu\text{L}}{160\,\mu\text{M}} = 12.5\ \mu\text{L}.$$

9. Make short unlabeled standard mix by adding 12.5 μL of each individual unlabeled acyl-ACP with up to ten carbons in length. Make long unlabeled standard mix by adding 12.5 μL of each individual unlabeled acyl-ACP with 12 carbons or longer are added to another tube. 1:50 and 1:20 (w/w) Asp-N is added to the short and long mixes, respectively, and 50 mM MOPS–NH_4OH (pH 7.5) with 5% (v/v) DMSO is added to a final volume of 500 μL and both tubes reacted (Fig. 4a, step 5). Then 500 μL of methanol is added to quench each reaction and the samples are dried using a vacuum centrifugal concentrator (Fig. 4a, step 6).
10. Dilution point 1 (5 μM) is made by resuspending each of the two digested, dried, unlabeled standard mixes in 200 μL of 1× (0.1 μM) ^{15}N standard diluent (Fig. 4a, step 7)—the unlabeled standards are then at 2× (10 μM) concentration. 190 μL of each are combined to a final concentration of 5 μM unlabeled standards in 1× (0.1 μM) ^{15}N standard diluent (Fig. 4a, step 8). This is dilution point 1.
11. The standard curve is generated by serial diluting dilution point 1) using 1× (0.1 μM) ^{15}N standard diluent (Fig. 4a, step 9, b, c).

3.6 SDS-PAGE Analysis of Synthesized Acyl-ACP Standards

1. Dilute 9 parts of acyl-ACP sample (synthesized standard), 1 part 0.5 M TCEP, and 10 parts Tricine Sample Buffer (*see* **Notes 14** and **15**).
2. Heat at 60 °C for 10 min.
3. Set up the gel electrophoresis chamber with16.5% Mini-PROTEAN® Tris-Tricine Gel (Bio-Rad) or equivalent and 1× Tris/Tricine/SDS Running Buffer.
4. Load 10 μL (1–3 μg) of acyl-ACP in each lane of the gel and 7 μL Precision Plus Protein™ Dual Xtra Prestained Protein Standard, or equivalent.
5. Run the gel at 90–100 V until the dye front has completely cleared (*see* **Note 16**).
6. Clean the gel by placing it in ~200 mL deionized water and microwave for 1 min or alternatively shake for 15 min at room temperature then replace the water and repeat three more times.
7. Add Colloidal Coomassie Brilliant Blue G-250 Stain and microwave for 15 s then shake overnight.
8. De-stain by rinsing several times with deionized water, then add destaining solution and shake gently until the protein bands are clearly visible.
9. Rinse the gel with deionized water.
10. Image the gel using a gel imager with white light.
11. Analyze the image and perform densitometry using image analysis software (Fig. 5 for an example).
12. Obtain the peak areas of densitometric scans for each band and use to assess the fractions of holo-ACP apo-ACP or acyl-ACP and reaction efficiency (Fig. 5).

3.7 Clean-Up of Acyl-ACP Standards by TCA Extraction

Trichloroacetic acid (TCA) precipitation is used for protein concentration and partial purification prior to proteomic or other analysis [26] and effectively cleans up protein by removing contaminating metabolites and other components of biomass.

1. Add 20% (w/v) TCA to the individual acyl-ACP standard reactions (from Subheading 3.4, **steps 1** and **2**) to a final concentration of 5% (w/v) and vortex.

$$V_{20\%\mathrm{TCA}} = V_{\mathrm{sample}} \times 0.333.$$

2. Centrifuge samples at 14,000 rpm (~15,000–20,000 × *g* depending on rotor) for 10 min at 4 °C.
3. Discard the supernatant and add 1 mL of 1% (w/v) TCA to wash the pellet.
4. Centrifuge samples at 14,000 rpm for 10 min at 4 °C.

5. Discard the supernatant and resuspend the pellet in 50 mM MOPS–NH_4OH (pH 7.5) with 5% (v/v) DMSO to the original reaction volume from Subheading 3.4, **steps 1** and **2**, to achieve a final concentration of ($C_{f\ (ACP)}$) of 200 μM ACP.

3.8 Acyl-ACP Extraction from Plant Tissues

Throughout the extraction process, keep samples on ice and perform all steps (centrifugation, bead milling, and mixing) at 4 °C. Minimize disruption of the pellet while handling at all times to reduce losses; it is recommended that gel loading pipette tips are used to remove/collect the supernatant close to the pellet to minimize disruption of the pellet and remove/collect as much of the supernatant as possible. At least three biological replicates are recommended for this analysis.

1. Harvest and homogenize fresh tissue to a fine powder in the presence of liquid nitrogen (*see* **Notes 17** and **18**).
2. Add isotopically labeled acyl-ACP standards (*see* Subheading 3.5.1 for details on amounts), 1.5 mL of 5% (w/v) TCA, and two steel beads to 30–40 mg of frozen, homogenized tissue and then bead mill the samples for 5 min (*see* **Note 19**).
3. Remove the beads and centrifuge at 14,000 rpm for 10 min at 4 °C.
4. Discard the supernatant and preserve the biomass pellet (*see* **Notes 20** and **21**). Then add 1.5 mL 1% (w/v) TCA and two steel beads and vortex (or bead mill if necessary) to break up the pellet.
5. Remove the beads and centrifuge at 14,000 rpm for 10 min at 4 °C.
6. Discard the supernatant (*see* **Notes 20** and **22**) and suspend the pellet in 1.5 mL 50 mM MOPS–NH_4OH (pH 7.6) with 5% (v/v) DMSO, add two steel beads, and vortex well (or bead mill if necessary).
7. Check that the pH is above 6.5 with 1 μL for a few samples and incubate the samples on a mixer for 1 h in a cold room. Use diluted NH_4OH to raise the pH as required.
8. Remove the beads and centrifuge at 14,000 rpm for 10 min at 4 °C.
9. Collect the supernatant in a new tube (*see* **Note 23**) and add 100% (w/v) TCA to achieve a final concentration of 10% (w/v) and gently invert the samples 2–3 times after this addition (do not vortex).

$$V_{100\%\mathrm{TCA}} = \frac{V_{\mathrm{sample}}}{9}.$$

$$V_{100\%\mathrm{TCA}} = \frac{1.5\,\mathrm{mL}}{9} = 0.167\,\mathrm{mL}.$$

10. Chill samples to −80 °C overnight (or for a minimum of 1 h if continuing extraction the same day).
11. Thaw the samples on ice without mixing and centrifuge at 14,000 rpm for 10 min at 4 °C.
12. Discard the supernatant and wash the pellet carefully with 1 mL 1% TCA. Do not vortex. Centrifuge again and return to ice.
13. Discard the supernatant and dissolve the pellet in a minimal volume of 50 mM MOPS–NH_4OH (pH 7.6) with 5% (v/v) DMSO. Record the volume added (*see* **Note 24**).
14. Check that the final pH is at or above 7.5 but below 9 to allow for optimal protease activity in the following steps.
15. Proceed to endoproteinase Asp-N digestion of acyl-ACPs and sample cleanup (*see* Subheading 3.9).

3.9 Endoproteinase Asp-N Digestion of Acyl-ACPs and Sample Clean-Up

1. Resuspend endoproteinase Asp-N (lyophilized powder) in chilled water to 0.04 μg/μL just before use (2–5 μL per sample needed). Keep on ice.
2. Add endoproteinase Asp-N to samples at a 1:50–1:20 enzyme–protein (w/w) ratio (*see* **Note 25**).
3. Vortex, quickly spin the reaction contents to the bottom of the tube, and digest the samples in a water bath at 37 °C overnight (~16 h) with periodic mixing.
4. Quench the reactions by adding 0.5 M TCEP to a final concentration of 1–5 mM and methanol to 50% (v/v). Record the final volume of the sample.
5. Store at −80 °C until LC-MS/MS analysis.
6. The day of LC-MS/MS analysis, thaw samples and remove any insoluble materials using 0.8 μm PES centrifuge filters.
7. Transfer the filtrate to LC autosampler vials for analysis.

3.10 LC-MS/MS Analysis

After digestion and sample clean-up, acyl-ACPs are separated on a C18 column with a Shimadzu HPLC or equivalent and detected by LC-MS/MS using an AB Sciex QTRAP 6500 instrument with Analyst software or equivalent.

1. Set up LC-MS/MS with C18 column and appropriate buffers (Subheading 2.8).
2. Set flow rate to 0.2 mL/min, 100% buffer A.
3. LC buffer gradient: 0–4 min, 0–10% B; 4–12 min, 10–100% B, 12–17 min, hold at 100% B; 17–18 min, 100–0% B; 18–27 hold at 0% B, stop at 27 min.
4. MS parameters: positive ion and multiple reaction monitoring (MRM) mode; curtain gas, 30 psi; ion spray voltage, 4.5 kV;

source temperature, 400 °C; nebulizing gas (GS1), 30 psi; focusing gas (GS2), 30 psi. Routine declustering potential (DP), collision energy, (CE), cell exit potential (CXP), and the m/z parameters for the mass filters (Q1 and Q3) are presented in Table 1.

5. Inject 10 μL of sample.
6. Run three technical replicates (replicate injections of the same sample) for each standard concentration of the calibration curve.
7. During the calibration curve, make a twofold dilution of the lowest detectable concentration (above background) and run to determine true limits of detection (LOD) and quantification (LOQ) values.
8. For subsequent analyses, only a single dilution point that lies within the linear range is necessary to validate the standard curve.

Table 1
LC-MS/MS Parameters for acyl-ACPs and elongation intermediates on the AB Sciex QTRAP 6500 mass spectrometer

Acyl-ACP	Acyl Chain	Q1 (*m/z*)	Q3 (*m/z*)	DP, CE, CXP (V, eV, V)	RRT (min)
apo	NA	334.2	203.1	61, 15, 24	2.2
Holo	NA	674.2	261.1	80, 40, 10	5.9
Malonyl	$C_3H_3O_3$	760.2	303.2	90, 50, 10	[a]
Acetyl	C_2H_3O	716.3	303.2	80, 40, 10	7.0
3-Ketobutyryl	$C_4H_5O_2$	758.3	345.2	90, 50, 10	[a]
3-Hydroxybutyryl	$C_4H_7O_2$	760.3	347.2	90, 50, 10	7.1
2,3-*trans*-Butenoyl	C_4H_5O	742.3	329.2	90, 50, 10	8.0
Butyryl (4:0)	C_4H_7O	744.3	331.2	90, 40, 10	8.2
3-Ketohexanoyl	$C_6H_9O_2$	786.3	373.2	100, 50, 10	[a]
3-Hydroxyhexanoyl	$C_6H_{11}O_2$	788.3	375.2	100, 50, 10	8.1
2,3-*trans*-Hexenoyl	C_6H_9O	770.3	357.2	100, 50, 10	8.9
Hexanoyl (6:0)	$C_6H_{11}O$	772.3	359.2	100, 40, 10	9.1
3-Ketooctanoyl	$C_8H_{13}O_2$	814.3	401.2	100, 50, 10	[a]
3-Hydroxyoctanoyl	$C_8H_{15}O_2$	816.3	403.2	100, 45, 10	8.9
2,3-*trans*-Octenoyl	$C_8H_{13}O$	798.3	385.2	100, 40, 10	9.8
Octanoyl (8:0)	$C_8H_{15}O$	800.4	387.3	100, 45, 10	10.0
3-Ketodecanoyl	$C_{10}H_{17}O_2$	842.4	429.3	100, 40, 10	[a]

(continued)

Table 1
(continued)

Acyl-ACP	Acyl Chain	Q1 (*m/z*)	Q3 (*m/z*)	DP, CE, CXP (V, eV, V)	RRT (min)
3-Hydroxydecanoyl	$C_{10}H_{19}O_2$	844.4	431.3	100, 40, 10	9.8
2,3-*trans*-Decenoyl	$C_{10}H_{17}O$	826.4	413.3	100, 40, 10	10.7
Decanoyl (10:0)	$C_{10}H_{19}O$	828.4	415.3	100, 45, 10	11
3-Ketododecanoyl	$C_{12}H_{21}O_2$	870.4	457.3	100, 40, 10	[a]
3-Hydroxydodecanoyl	$C_{12}H_{23}O_2$	872.4	459.3	100, 40, 10	10.7
2,3-*trans*-Dodecenoyl	$C_{12}H_{21}O$	854.4	441.3	100, 40, 10	11.7
Dodecanoyl (12:0)	$C_{12}H_{23}O$	856.4	443.3	100, 45, 10	12.0
3-Ketotetradecanoyl	$C_{14}H_{25}O_2$	898.4	485.3	100, 45, 10	[a]
3-Hydroxytetradecanoyl	$C_{14}H_{27}O_2$	900.4	487.3	100, 45, 10	11.7
2,3-*trans*-Tetradecenoyl	$C_{14}H_{25}O$	882.4	469.3	100, 45, 10	12.8
Tetradecanoyl (14:0)	$C_{14}H_{27}O$	884.4	471.3	100, 45, 10	13.1
3-Ketohexadecanoyl	$C_{16}H_{29}O_2$	926.5	513.4	100, 45, 10	[a]
3-Hydroxyhexadecanoyl	$C_{16}H_{31}O_2$	928.5	515.4	100, 45, 10	12.8
2,3-*trans*-Hexadecenoyl	$C_{16}H_{29}O$	910.5	497.4	100, 45, 10	14.0
Hexadecanoyl (16:0)	$C_{16}H_{31}O$	912.5	499.4	100, 50, 10	14.3
cis-9-Hexadecanoyl (16:1)	$C_{16}H_{29}O$	910.5	497.4	100, 50, 10	13.3
cis, *cis*-7, 10-Hexadecanoyl (16:2)	$C_{16}H_{27}O$	908.4	495.3	100, 50, 10	[a]
cis, *cis*, *cis*-7,10,13-Hexadecanoyl (16:3)	$C_{16}H_{25}O$	906.4	493.3	85, 46, 14	12.0
3-Ketooctadecanoyl	$C_{18}H_{33}O_2$	954.5	541.4	100, 45, 10	[a]
3-Hydroxyoctadecanoyl	$C_{18}H_{35}O_2$	956.5	543.4	100, 45, 10	14.0
2,3-*trans*-Octadecenoyl	$C_{18}H_{33}O$	938.5	525.4	100, 45, 10	15.1
Octadecanoyl (18:0)	$C_{18}H_{35}O$	940.5	527.4	100, 50, 10	15.5
cis-9-Octodecanoyl (18:1)	$C_{18}H_{33}O$	938.5	525.4	100, 50, 10	14.4
cis, *cis*-9, 12-Octadecanoyl (18:2)	$C_{18}H_{31}O$	936.5	523.4	100, 50, 10	[a]
cis, *cis*, *cis*-9,12,15-Octodecanoyl (18:3)	$C_{18}H_{29}O$	934.5	521.3	100, 50, 10	[a]

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CE collision energy, *CXP* collision cell exit potential, *DP* declustering potential, *NA* not applicable, *Q1* quadrupole 1 mass filter, *Q3* quadrupole 3 mass filter, *RRT* relative retention time

[a]Unvalidated

3.11 LC-MS/MS Data Analysis and Quantification

Peaks are integrated using the compatible integration software. Analytes can be estimated by single point quantification or quantified by isotope dilution based quantification, both methods using ^{15}N-labeled acyl-ACP internal standards.

1. Verify peak identities by internal standards and retention times (Fig. 2b, c).
2. Integrate the peak areas for each analyte and standard using compatible integration software (Fig. 6a, b).
3. For single point quantification (for estimation), obtain the peak area ratios of sample analyte to internal standard.
4. For each analyte use the sample with a ratio closest to 1 to calculate the amount of sample analyte:

$$C_{\text{analyte}} = \frac{A_{\text{analyte}}}{A_{\text{standard}}} \times C_{\text{standard}}.$$

5. Normalize the calculated concentration to the sample weight:

$$\text{Example}: \frac{\text{pmol}}{\text{mg}} = \frac{C_{\text{analyte}}\left(\frac{\text{pmol}}{\mu\text{L}}\right) \times \text{sample vol } (\mu\text{L})}{\text{sample weight (mg)}}.$$

6. For isotope dilution-based quantification, plot the ratios of the peak areas of the unlabeled to labeled standards versus the concentrations of the unlabeled standards for each analyte (Fig. 6c).
7. Perform a linear regression analysis on each analyte to generate standard curves, determine linear ranges, and calculate limits of detection (LOD) and quantification (LOQ) (Fig. 6d) (*see* **Notes 26** and **27**):
8. LOD and LOQ: Calculate the standard deviation (SD) of the ratio of peak areas for the technical replicates of the lowest concentration of standard with peak areas above background. Multiply the standard deviation by 3.3 (for LOD) and 10 (for LOQ) and divide by the slope (m) of the linear regression line.

$$\text{LOD} = \frac{3.3 \times \text{SD}}{m}.$$

$$\text{LOQ} = \frac{10 \times \text{SD}}{m}.$$

9. Calculate the ratio of peak areas of the sample analyte (unknown) to internal standard (^{15}N labeled) (Fig. 6e).
10. Calculate the sample analyte (unknown) concentration using the linear regression line obtained from the standard curve (Fig. 6f).
11. Normalize the calculated concentration to the sample weight:

$$\text{Example}: \frac{\text{pmol}}{\text{mg}} = \frac{C_{\text{analyte}}\left(\frac{\text{pmol}}{\mu\text{L}}\right) \times \text{sample vol } (\mu\text{L})}{\text{sample weight (mg)}}.$$

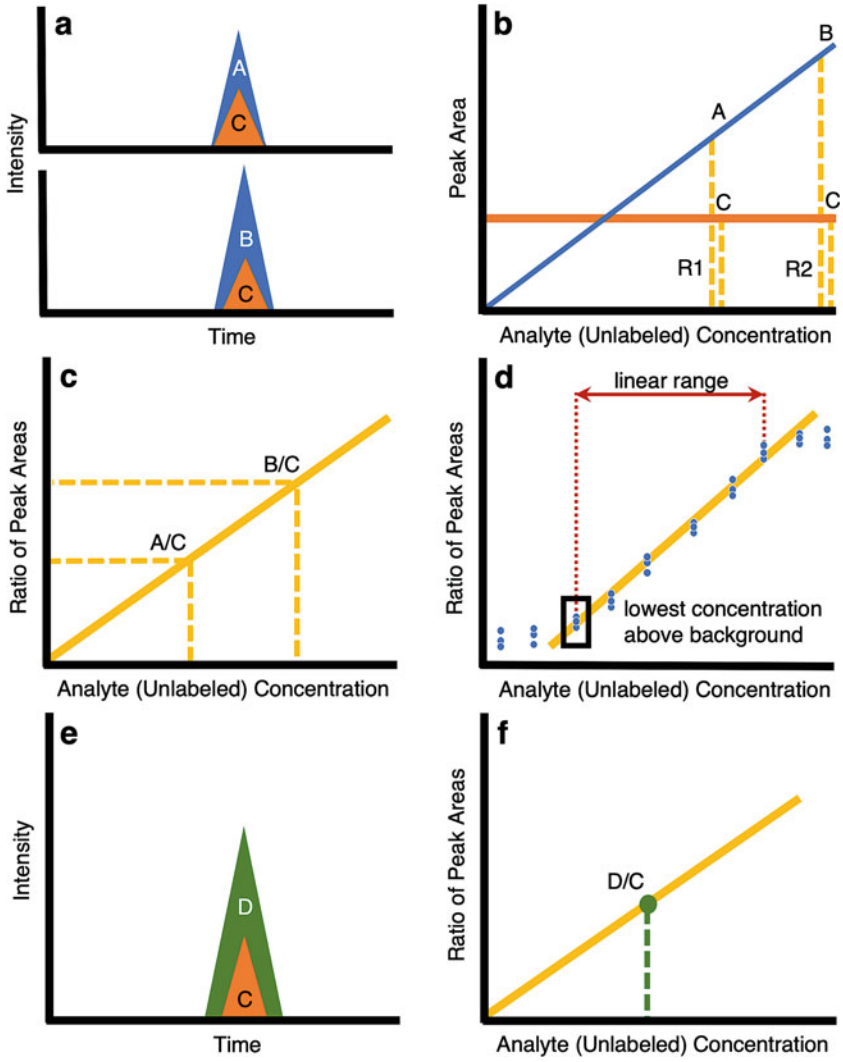

Fig. 6 Isotope dilution-based quantification. (**a**) Peak area integration of standards from the standard curve. A and B, one unlabeled standard at different concentrations; C, ^{15}N standard. (**b**) Plots of unlabeled (blue) and ^{15}N (orange) standard peak areas across the standard curve. A, B and C, peak areas from panel (a); R1 and R2, link between peak areas to calculate ratios of peak areas for unlabeled to ^{15}N standard. (**c**) Plots of the ratios of peak areas (unlabeled to ^{15}N standard) over the concentration of the unlabeled standard across the standard curve. (**d**) Plots of the ratios of peak areas (unlabeled to ^{15}N standard) over the concentration of the unlabeled standard across the standard curve with replicate injections. Peak areas of the lowest concentration above background (black box) are used to calculate LOD and LOQ. The linear range is the range in which the peak area increases linearly in response to changes in concentration. (**e**) Peak area integration of biological sample analyte with internal standard. C, ^{15}N standard; D, sample analyte (concentration unknown). (**f**) The ratio of sample analyte D (unknown) to internal standard C is used to calculate the concentration of the sample analyte (unknown concentration) using the standard curve

4 Notes

1. Acyl-CoA may be dissolved in chilled ddH_2O or 50% (v/v) DMSO solution which may increase solubility of longer acyl-chains. Aliquot and store at −20 °C.
2. The order in which each component is added must be maintained to retain stain sensitivity. First dissolve aluminum sulfate in water, then add ethanol and mix, then mix in Coomassie Brilliant Blue G-250. Once the Coomassie Brilliant Blue G-250 is dissolved, add phosphoric acid and add water to reach the final volume. The staining solution is a colloidal solution so particulates should not be filtered out.
3. LB Medium can be made the day before inoculation and stored at 4 °C.
4. 1× Modified MOPS Minimal medium should be made the day of inoculation.
5. The temperature can be lowered as far as 30 °C to slow the growth process if necessary. Lower than 30 °C will result in too little growth.
6. *E. coli* cells double every 30–60 min at 37 °C (depending on conditions). LB medium will result in faster growth than modified MOPS minimal medium. The O.D. can be checked infrequently until the culture looks turbid. Once the culture is near the target O.D., frequent O.D. monitoring is necessary. Beyond an O.D. of 0.8, the cells enter the stationary phase which results in reduced protein expression. An O.D. below 0.4 will not be sufficient to produce high yields of recombinant protein.
7. Transferring the cells breaks up the pellet, speeding up the resuspension process; however, this method results in minute losses.
8. The shaker method works well for stable proteins and allows time for the lysozyme to take effect.
9. If there is not enough time to carry out the protein purification, resuspend the cells in Ni^{2+} IMAC Binding Buffer and store them at −80 °C. Add lysozyme and protease inhibitor upon thawing to help with cell lysis.
10. Do not attempt to quantify ACP using Bradford assay-based methods due to differences in dye binding capacities between ACP and bovine serum albumin (BSA).
11. Polysorbate 20 (Tween 20) is crucial for solubilization of the medium- and long-chain acyl-CoA (12 and longer) in the presence of $MgCl_2$ and $MnCl_2$ [27] and omitted from reactions where solubilization is not an issue (10 and shorter) to

decrease loading of polysorbate 20 onto the reverse phase column during LC-MS/MS analysis.

12. It is recommended to increase the fold dilution for the lower concentrations.
13. During LC-MS/MS analysis, make a twofold dilution of the lowest detectable concentration (above background) to determine true LOD and LOQ values.
14. Prepare samples at approximately 1–3 μg/10 μL final concentration for best detection and resolution with Coomassie stain.
15. TCEP is included to prevent disulfide bonding between the holo-forms of ACP (ACP-4′-phosphopantetheine-SH).
16. Run the gel in a cold-room to avoid overheating and prevent curvatures in the band pattern. This will enhance the resolution when performing densitometry.
17. An increasing amount of tissue during isolation does not always result in higher yields of acyl-ACPs. This case is observed in leaf tissue where 30 mg FW yields higher amounts of acyl-ACPs than >60 mg FW. It is generally better to extract less tissue and concentrate the sample either by resuspending in less volume at **step 13** (Subheading 3.8) or by vacuum centrifugal concentration. Always note the final volumes for later calculations.
18. A fine powder must be obtained after homogenization of the tissue. Anything not well homogenized will result in losses and poor sample handling.
19. It is recommended that you estimate the amount of each acyl-ACP standard necessary for your samples by adding differing amounts to replicate samples and performing a single point quantification before performing isotope dilution-based quantification for best results.
20. Use a 1 mL pipet tip first, then follow using a gel loading tip when close to the bottom to avoid sucking up any of the pellet.
21. It is more important to maintain the integrity of the pellet than to remove every last drop of TCA solution at this step.
22. It is important to remove as much of the TCA solution as possible to achieve a pH above 6.5 in the next step.
23. If the supernatant is cloudy or discolored, filtering may be helpful for downstream steps.
24. Resuspension in 30–50 μL is ideal for achieving adequate mass spectrometry detection (~100 μL final volume in autosampler vial for MS after Asp-N endoproteinase digestion and quenching).
25. Short and medium chain acyl-ACPs (up to ten carbons) can be digested efficiently using 1:50 (w/w) and medium to long chain acyl-ACPs (12 carbons or longer) require 1:20 (w/w) for efficient digestion.

26. Generally, the evaluation of six data points within the linear range is desirable for quantification.
27. A weighted linear regression can be performed to account for variance in data points that range across multiple orders of magnitude. An integration software (like Analyst) or other available online worksheets [28] can do this.

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