Chapter 19

Analysis of Lipid Profiles in Skeletal Muscles

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Abstract

The lipidome of skeletal muscles is a worthwhile target of research, as it affects a multitude of biological functions, and is, in turn, affected by factors such as diet, physical activity, and development. We present two methods for the analysis of the main lipid classes in skeletal muscles of humans and other animals, that is, triacylglycerols and phospholipids. The methods differ in that the former concerns total phospholipids, while the latter concerns individual phospholipids. In both methods, lipids are extracted from muscle after the addition of internal standards, and they are separated by one-dimensional (1D) thin-layer chromatography (TLC). This is sufficient for the separation of triacylglycerols and total phospholipids. In the first method, the two classes are subsequently subjected to methanolation to produce methyl esters of fatty acids (and, to a lesser extent, dimethyl acetals of fatty aldehydes derived from plasmalogens), which are analyzed by gas chromatography (GC). Quantitation is achieved on the basis of the internal standards. In the second method, 1D TLC is used for the analysis of triacylglycerols only, whereas individual phospholipids are separated by two-dimensional TLC. This results in the isolation of phosphatidyl choline, lysophosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, cardiolipin, and sphingomyelin. Methanolysis and subsequent analysis by GC results in the determination of the fatty acid and aldehyde profiles of the individual muscle phospholipids.

Key words: Dimethyl acetals, Fatty acids, Gas chromatography, Lipids, Methyl esters, Phospholipids, Plasmalogens, Skeletal muscle, Thin-layer chromatography, Triacylglycerols

1. Introduction

Lipids are integral components of all cells. The plasma membrane forming the boundary of a cell with its surroundings is basically lipid (in particular, phospholipid) in nature. The same holds true for all membranes delimiting the subcellular organelles of eukaryotic cells (the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes, etc.) and specialized structures like the sarcoplasmic reticulum of muscle fibers and the disks of the
rod outer segment. Lipids, mainly in the form of triacylglycerols, are stored in cells of several types, including muscle fibers, hepatocytes, and adipocytes, the latter consisting of triacylglycerols by about 80%. In addition, lipids are present in biological fluids. For example, the blood plasma hosts fatty acids, triacylglycerols, phospholipids, and sterols, transporting them to tissues.

Lipid analysis presents a challenge emanating from the great heterogeneity of a class of biological compounds united only by their poor solubility in water. Indeed, there is little chemical similarity between, say, a triacylglycerol and cholesterol. (By contrast, a protein will always consist of amino acid residues that are variations on a theme, and nucleic acids will always consist of nucleotide residues that are variations on another theme). Hence, one needs a variety of methods to study the full lipid complement in a biological sample.

The aim of this chapter is to provide detailed methods for the analysis of the main lipid classes in the skeletal muscles of humans and other animals, that is, triacylglycerols and phospholipids, both of which are affected by factors such as diet (1), physical exercise (2), and development (3, 4), and both of which, in turn, affect a multitude of biological functions, including ion homeostasis, gene expression, and signal transduction (5, 6) (see Note 1). Two alternative methods are described below (see Fig. 1). In both, lipids are extracted from muscle with a powerful organic solvent (chloroform-methanol, 2:1), followed by the addition of water that results in the formation of two phases, one containing the lipids

![Fig. 1. Major steps in the two methods of analysis of lipid profiles in skeletal muscles described in this chapter. 1D one-dimensional; 2D two-dimensional; GC gas chromatography; TLC thin-layer chromatography.](image-url)
and another containing nonlipid constituents (7). Then, in the first method, triacylglycerols (represented schematically in Fig. 2a) and phospholipids (see Fig. 2b–j) as a whole are separated from each other and from minor lipid constituents of skeletal muscles by one-dimensional (1D) thin-layer chromatography (TLC). The two

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Fig. 2. Schematic representation of skeletal muscle lipids determined by the methods described in this chapter. (a) Triacylglycerol, (b) phosphatidyl choline, (c) lysophosphatidyl choline, (d) phosphatidal choline (a plasmalogen), (e) phosphatidyl ethanolamine, (f) phosphatidal ethanolamine (a plasmalogen), (g) phosphatidyl serine, (h) phosphatidyl inositol, (i) cardiolipin, (j) sphingomyelin.
fractions are subsequently subjected to methanolysis to produce methyl esters (MEs) of fatty acids (and, to a lesser extent, dimethyl acetals, DMAs, of fatty aldehydes derived from plasmalogens), which are analyzed by gas chromatography (GC).

Internal standards added at the beginning of the method compensate for losses during the entire procedure. As internal standards, we use a triacylglycerol and a phospholipid with acyl groups (heptadecanoyl, abbreviated 17:0, see Note 2) that are absent from the natural muscle triacylglycerols and phospholipids. The internal standards comigrate with the natural lipids of the same class during TLC and, upon methanolysis, yield methyl heptadecanoate (17:0 ME), which serves as reference for calculating the amounts of the endogenous MEs and DMAs after they are separated by GC.

The method outlined above is useful if one is content with determining the fatty acid composition of total phospholipids. However, an added complexity of the lipidome is diversity within the class of phospholipids. This diversity stems from:

1. The presence of either of two possible alcohols, namely, glycerol (see Fig. 2b–i) and sphingosine (see Fig. 2j), as the backbones to which the acyl groups and phosphate are linked.
2. The presence of either of several alcohols, such as choline (see Fig. 2b–d, j), ethanolamine (see Fig. 2c, f), serine (see Fig. 2g), and inositol (see Fig. 2h), at the end of the polar head group.
3. The unusual structure of cardiolipin (CL) (see Fig. 2i), being almost a diphosphatidyl glycerol.
4. The presence of lysophospholipids (see Fig. 2c), that is, phospholipids lacking one acyl group.
5. The presence of ether, rather than ester, linkages in certain phospholipids (see Fig. 2d, f).

If one is then interested in separating all of the individual phospholipids that are present in skeletal muscles in appreciable amounts, one has to resort to two-dimensional (2D) TLC, followed by methanolysis of each phospholipid and by GC of the resulting MEs and DMAs. These procedures constitute the second method.

Description of the two methods is preceded by instructions on how to establish the method for the gas chromatographic analysis of MEs and DMAs, which is a prerequisite for both.

2. Materials

Use analytical grade reagents. To prevent contamination of reagents and samples by lipids present on the skin and to protect hands from hazardous organic solvents, wear gloves throughout all steps.
Lipid Profiles in Muscle

1. Gas chromatograph equipped with flame ionization detector. Carrier gas may be helium or hydrogen.
2. Column. Many commercially available capillary GC columns that are suitable for analysis of fatty acid MEs will do. We recommend a length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 μm.
3. Analytical balance displaying four decimal points of the gram.
4. Glass screw top vials, 2 and 4 mL, along with perforated (open top) screw caps and PTFE/silicone septa.
5. Hexane.
6. ME standards. We have found 22 MEs to be present at appreciable amounts (that is, at least 0.1% of total) in skeletal muscle triacylglycerols and phospholipids. These and 17:0 ME (derived from methanolysis of the internal standards) are listed in Table 1, along with their relative molecular masses (Mr) to aid you in the calculations (see Subheadings 3.2.3 and 3.3.4). Obtain all MEs at the lowest available quantities. Make a stock solution of each ME standard at an approximate concentration of 4 mg/mL (no need to be accurate) by weighing 4–8 mg inside a 2-mL screw top vial and dissolving in 1–2 mL of hexane. Cover the vial with a septum (glossy side facing the rim of the vial) and cap tightly. Use the standard to establish the retention time of the ME in your gas chromatographic system as described in Subheading 3.1. Store at −20°C (see Note 3).
7. Hexadecanal dimethyl acetal standard. Hexadecanal dimethyl acetal (16:0 DMA, Mr 286.5) is produced by methanolysis of plasmalogens (see Fig. 1d, f) carrying a 1-hexadecenyl group (see Note 4). Make a 4 mg/mL stock solution in hexane and store as described above (see Note 5).
8. Syringe, 10 μL, with pointed needle.

1. Liquid nitrogen, mortar, and pestle for tissue pulverization.
2. Glass test tubes, small (3–5 mL). Use new tubes in each case and discard the used ones, as washing them with organic solvents to remove their lipids may be more expensive than buying them (let alone the effort).
3. Glass Pasteur pipettes and rubber bulb.
4. Glass screw top test tubes, small (5–7 mL), with PTFE-lined screw caps.
5. Glass funnel, small (fitting the opening of the screw top test tubes).
Table 1
Methyl esters usually detected by gas chromatographic analysis of skeletal muscle lipids

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation of acyl group</th>
<th>$M_r$ of methyl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl laurate</td>
<td>12:0</td>
<td>214.4</td>
</tr>
<tr>
<td>Methyl myristate</td>
<td>14:0</td>
<td>242.4</td>
</tr>
<tr>
<td>Methyl myristoleate</td>
<td>14:1ω9</td>
<td>240.4</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>16:0</td>
<td>270.5</td>
</tr>
<tr>
<td>Methyl palmitoleate</td>
<td>16:1ω7</td>
<td>268.4</td>
</tr>
<tr>
<td>Methyl heptadecanoate$^a$</td>
<td>17:0</td>
<td>284.5</td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>18:0</td>
<td>298.5</td>
</tr>
<tr>
<td>Methyl oleate</td>
<td>18:1ω9</td>
<td>296.5</td>
</tr>
<tr>
<td>Methyl <em>cis</em>-vaccenate</td>
<td>18:1ω7</td>
<td>296.5</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>18:2ω6</td>
<td>294.5</td>
</tr>
<tr>
<td>Methyl γ-linolenate (all- <em>cis</em>-6,9,12)</td>
<td>18:3ω6</td>
<td>292.5</td>
</tr>
<tr>
<td>Methyl α-linolenate (all- <em>cis</em>-9,12,15)</td>
<td>18:3ω3</td>
<td>292.5</td>
</tr>
<tr>
<td>Methyl stearidonate</td>
<td>18:4ω3</td>
<td>290.5</td>
</tr>
<tr>
<td>Methyl arachidate</td>
<td>20:0</td>
<td>326.5</td>
</tr>
<tr>
<td>Methyl eicosenoate</td>
<td>20:1ω9</td>
<td>324.5</td>
</tr>
<tr>
<td>Methyl dihomo-γ-linolenate</td>
<td>20:3ω6</td>
<td>320.5</td>
</tr>
<tr>
<td>Methyl arachidonate</td>
<td>20:4ω6</td>
<td>318.5</td>
</tr>
<tr>
<td>Methyl 5,8,11,14,17-eicosapentaenoate</td>
<td>20:5ω3</td>
<td>316.5</td>
</tr>
<tr>
<td>Methyl behenate</td>
<td>22:0</td>
<td>354.5</td>
</tr>
<tr>
<td>Methyl all- <em>cis</em>-7,10,13,16-docosatetraenoate</td>
<td>22:4ω6</td>
<td>346.5</td>
</tr>
<tr>
<td>Methyl all- <em>cis</em>-4,7,10,13,16-docosapentaenoate</td>
<td>22:5ω6</td>
<td>344.5</td>
</tr>
<tr>
<td>Methyl all- <em>cis</em>-7,10,13,16,19-docosapentaenoate</td>
<td>22:5ω3</td>
<td>344.5</td>
</tr>
<tr>
<td>Methyl all- <em>cis</em>-4,7,10,13,16,19-docosahexaenoate</td>
<td>22:6ω3</td>
<td>342.5</td>
</tr>
</tbody>
</table>

The methyl esters detected in a muscle depend on species and diet. Certain methyl esters may be only available by the name [fatty acid] methyl ester, for example, *cis*-vaccenic acid methyl ester, rather than methyl *cis*-vaccenate.

$^a$Derived from methanolysis of internal standards.
6. Syringe, 10 μL, with blunt needle.
7. Scalpel with round blade.
8. Organic solvents: chloroform, methanol, ethanol (absolute), petroleum ether (boiling point range, about 40–60°C), diethyl ether, acetic acid.
10. Lipid extraction solvent: chloroform-methanol 2:1 (v/v) with 0.005% (w/v) 2,6-di-tert-butyl-4-methylphenol (aka butylated hydroxytoluene, or BHT) to prevent lipid peroxidation.
11. Triheptadecanoyl glycerol (triheptadecanoin) as triacylglycerol internal standard (abbreviated as 17:0 TG).
12. Diheptadecanoyl phosphatidyl choline (17:0 PC) as phospholipid internal standard (see Note 6).
13. Triacylglycerol and phospholipid internal standard solutions: Weigh out about 20 mg of each 17:0 TG and 17:0 PC into separate 4-mL screw top vials. Note weight to the fourth decimal point of the gram and place the vials on ice. Prepare two screw caps with septa in place. Then add 3 mL of cold chloroform to each vial and cap immediately and tightly (see Note 7). This will give two standard solutions of about 7 mg/mL, or 7 μg/μL, concentration. For greater accuracy, take into account the purity of the substances used (usually 99%). For the sake of subsequent calculations, let \( a \) and \( b \) be the concentrations of the two standard solutions, respectively. Store at −20°C. Stable for 1 year.
14. High-performance TLC plates of silica gel. Plates usually come in boxes of 25. Plate dimensions can be 10×10, 10×20, or 20×20 cm depending on the number of samples to be analyzed in each run. We recommend plates that are 20 cm on at least one dimension if you intend to run more than ten samples simultaneously. Another parameter to consider is the silica gel’s support: it may be glass, plastic, or aluminum. The main advantage of plastic and aluminum is that they can be cut to the desired dimensions with scissors. If you use a 20×20 cm plate with glass support, at least half of it will be wasted, since the developer needs only migrate by 10 cm (see Note 8). Handle TLC plates by their sides and supports. Do not touch the delicate silica gel surface.
15. Spotting guide: On a sheet of paper, draw a straight line parallel to one side at a distance of 1.5 cm. On that line, make 12 black dots with a marker pen that are 1.5 cm apart, starting 1.5 cm from one end. Number the dots (see Fig. 3).
16. TLC tank. Choose tank dimensions to accommodate the plates you have chosen.
17. Multiplate rack, optional.
19. Dichlorofluorescein spray reagent: 0.2% (w/v) dichlorofluorescein in ethanol. Make 100 mL at a time and store indefinitely at room temperature.
20. Spray bottle.
21. Spray box to prevent dichlorofluorescein from staining the lab’s surfaces.
22. Hair dryer, optional.
23. UV lamp.
24. Eye goggles against UV radiation.
25. Methanolic sodium methoxide, 0.5M.
26. Methanolic boron trifluoride, 10–15% (w/w) (1.3–2M).
27. Heating block reaching 100°C. A water bath reaching 50°C and a boiling water bath will do instead.
28. Microvolume inserts (if the gas chromatograph is equipped with autosampler).

You will need all materials described under Subheading 2.2. You will need a second TLC tank (see Subheading 2.2, item 16) for 2D TLC. Because 2D TLC requires 10 × 10 cm, we do not recommend TLC plates of other dimensions (10 × 20 or 20 × 20 cm) with glass support. Additionally, obtain the following:

1. Acetone.
2. Phospholipid standards: We have found seven phospholipids to be present at appreciable amounts (that is, at least 1% of total) in skeletal muscle: PC, lysophosphatidyl choline (LPC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI), CL, and sphingomyelin (SM). Ideally, they should all contain the heptadecanoyl group in order to be used as internal standards (just like 17:0 PC, already listed as Subheading 2.2, item 12). However, 17:0 LPC, PE, PS, PI, CL,
and SM are either not commercially available or too expensive. Therefore, obtain LPC, PE, PS, PI, CL, and SM without regard to their acyl groups for use only in establishing the separation pattern of muscle phospholipids under 2D TLC (quantitation without internal standards will be described below). Make a 7 μg/μL solution of each in chloroform just like you did with 17:0 PC, although you do not need to be accurate with these. Store indefinitely at −20°C.

3. Methyl pentadecanoate (15:0 ME) to be used as external standard. Make a solution of approximately 2 mg/mL (no need to be accurate) by weighing 4–8 mg inside a 4-mL screw top vial and dissolving in 1–2 mL of hexane. Cap and store indefinitely at −20°C.

3. Methods

3.1. Gas Chromatography

In order to be able to go directly from the separation of lipid classes (by either 1D or 2D TLC) to the preparation and separation of MEs and DMAs by GC, you need to establish the operating parameters of the gas chromatograph (i.e., temperature program of the column, head pressure or flow rate of the carrier gas, and split ratio) in advance. Since GC is a demanding technique, we assume that the person who will carry out this analysis is familiar with the basic theory of GC and the operation of a gas chromatograph. Therefore, we will not provide instructions on, for example, how to install a column, how to set up gas flow rates, how to set up a temperature program, or how to inject a sample. Besides, these functions depend greatly on the particular instrument available.

1. You may begin with the operating parameters suggested by the manufacturer of the column you have obtained for the specific application (fatty acid ME analysis) or with the following parameters that we use with columns fitting the description under Materials (see Subheading 2.1, item 2): column temperature, 160–250°C at 5°C/min, then isothermic at 250°C for 10 min; flow rate of carrier gas, 1 mL/min; split ratio, 1:10.

2. Prepare a series of ME and DMA working solutions by diluting 3 μL of each ME and DMA stock solution with 200 μL of hexane in a 2-mL screw top vial (see Note 9). Cap and store at −20°C (see Note 3).

3. Inject 1 μL of each ME and DMA working solution separately with a 10-μL syringe with pointed needle either manually or through an autosampler.

4. Note the retention time of each ME and DMA. If necessary, adjust the operating parameters to achieve a distinct retention time for each component (see Note 10).
5. Confirm that all MEs and 16:0 DMA are adequately separated by mixing 3 μL of each stock solution in a 2-mL screw top vial, adding hexane to 200 μL, and injecting 1 μL. Store the mixture at −20°C for possible future use.

3.2. Analysis of Triacylglycerols and Total Phospholipids

3.2.1. Lipid Extraction and Separation

1. Obtain and store muscle tissue specimens in a way that prevents lipid modification before analysis. Major threats are triacylglycerol and phospholipid hydrolysis and fatty acid oxidation. We recommend immersing the specimen in liquid nitrogen immediately after obtaining it, pulverizing with mortar and pestle in liquid nitrogen, and storing at −80°C (see Note 11).

2. Start the analysis by placing a TLC tank, with its lid on, in a place with constant room temperature and not exposed to air drafts (not in the fume hood).

3. Prepare the developer for TLC: If using a large tank (one that accommodates 20-cm wide plates), decant 86 mL of petroleum ether, 14 mL of diethyl ether, and 1 mL of acetic acid in a 250-mL conical glass flask under the fume hood operating at full speed. If using a small tank (one that accommodates 10-cm wide plates), mix 43 mL of petroleum ether, 7 mL of diethyl ether, and 0.5 mL of acetic acid in a 100-mL flask (see Note 12).

4. Mix well with a swirling motion and promptly pour off into the TLC tank after sliding the lid sideways just enough for the liquid to enter. Replace the lid immediately and turn the fume hood’s motor off. Grasp the tank with both hands and, while holding the lid in place with your index fingers, shake the tank from one side to the other for a few seconds to facilitate saturation of its atmosphere with developer vapors. We do not recommend lining the tank with filter paper. Let the tank stand no less than 1 h and no more than 2 h before chromatography to ensure reproducible separations (see Note 13). In the meantime, proceed to lipid extraction from the samples (see Note 14).

5. Prepare a mixture of the 17:0 TG and 17:0 PC standard solutions by mixing one volume of the former with four volumes of the latter in a 2-mL screw top vial kept on ice and capping immediately and tightly. You will need 5 μL of this mixture per sample, but, in any case, make at least 100 μL to protect the mixture’s composition against evaporation in the vial if the volume is too low. Keep on ice and discard at the end of the day.

6. Using a 10-μL syringe with pointed needle, pierce the septum of the vial and dispense 5 μL of the mixture (in effect, 1 μL of the 17:0 TG standard solution plus 4 μL of the 17:0 PC standard solution) at the bottom of as many small glass test tubes as the muscle samples you are going to analyze. Wait a minute for
the solvent to evaporate before proceeding to the next step. Label the test tubes according to the muscle samples.

7. Take a muscle specimen out of the ultrafreezer, place the corresponding test tube in the analytical balance, tare, and promptly weigh out approximately 30 mg. Note weight to the third or, preferably, fourth decimal point of the gram in order to be able to express the lipid content per gram tissue with high accuracy (see Note 15). Return any remaining part of the specimen to the ultrafreezer.

8. Immediately add 570 µL of lipid extraction solvent [chloroform-methanol 2:1 (v/v) with 0.005% (w/v) BHT]. Vortex briefly and let stand for 5 min or longer with occasional vortexing (see Note 16). In the meantime, you may proceed with other muscle specimens.

9. Add 120 µL of distilled water and vortex vigorously for 1 min.

10. Briefly spin the test tube in a centrifuge to produce two clear phases with the muscle debris at the interphase. Lipids are contained in the lower phase. Handle the test tube gently so as not to disturb the separation of phases.

11. Attach a rubber bulb to a glass Pasteur pipette and squeeze the bulb almost completely. Immerse the tip of the pipette to the bottom of the test tube, taking care not to disturb the upper phase and interphase. Squeeze the bulb gently until you release one air bubble in order to push out any upper phase that has entered the tip of the pipette during immersion. Then gently release the pressure on the bulb to aspirate the lower phase. Take up as much of the lower phase as possible, but do not aspirate any of the muscle debris or upper phase.

12. Transfer the aspirate to another small glass test tube and evaporate the solvent under a stream of nitrogen (see Note 17). Discard the test tube containing the muscle debris and upper phase. This ends the extraction process.

13. If using a 20 × 20-cm silica gel plate with soft support, cut it in half with scissors carefully, taking care not to chip off too much of the silica gel layer. Use the cut sides of the two resulting plates as the far ends in the subsequent chromatography, that is, the ends toward which the developer migrates (see Note 18). If using any other kind of TLC plate, skip this step. If using a 20 × 20-cm plate with glass support, draw a line with ruler and pencil in the middle to mark the end of development.

14. You will need 10 cm along one side of the TLC plate(s) for the developer to migrate and \((n+1) \times 1.5\) cm along the other side, \(n\) being the number of samples to be spotted 1.5 cm apart and 1.5 cm from the sides. Accordingly, you may wish to (further) cut a plate for economy.
15. Place the spotting guide on the bench. If using a plate with transparent support (that is, glass or plastic), place the plate on the spotting guide so that the sides of the two coincide. Now you can see the dots on the spotting guide under the plate. If using a plate with aluminum support, place the plate on the spotting guide so that the left sides of the two are aligned and the dots on the spotting guide barely appear along the bottom side of the plate. Then mark 1.5 cm from the bottom side on the left and right sides of the plate with a pencil. Thus, you will have the coordinates of where to spot.

16. Dissolve the dried lipid extract of the first sample in 30 μL of lipid extraction solvent. Make sure you retrieve all of the extract from the wall of the test tube by vortexing and rotating the test tube in your hands almost horizontally.

17. Draw 10 μL of the dissolved extract in a 10-μL syringe with blunt needle and bring the syringe over the first spotting position, the tip of the needle being a few millimeters above the plate.

18. Gently squeeze the plunger to create a medium-sized drop (of about 0.7 μL) and carefully lower the tip of the needle until the drop (not the needle) touches the plate and is absorbed by the silica gel.

19. Wait a few seconds until all of the solvent evaporates from the plate, then repeat the previous step until all of the syringe’s contents are spotted. Spot each drop on top of the previous one so that the spot formed does not exceed a few millimeters in diameter. You will find that it takes longer and longer for each drop to evaporate. You may speed up the evaporation by setting up a hair dryer next to the plate and directing the air at the spot (see Note 19).

20. By the end of spotting, a yellow-brownish spot will have formed on the plate. Then proceed to dissolving and spotting the next extract(s) by repeating steps 16–19 until you fill all available spotting positions on the plate. The first time you perform this analysis, spot 2 μL of each 17:0 TG and 17:0 PC standard solutions on separate positions in the middle of the plate in order to identify the triacylglycerol and phospholipid spots after chromatography. During subsequent analyses, you may spot only the 17:0 TG standard, since, as you will find out, phospholipids remain at the origin and are thus easily located.

21. Save the remaining extract in each test tube by covering the test tube with Parafilm and storing at 4°C, just in case you need to repeat the analysis. Stable for 1 month.

22. If you spot many samples, keep a record of which sample lies at which spot on the plate to avoid mix-up.
23. If you have so many samples that you need additional plates, repeat steps 15–22. Unless you use a multiplate rack (see Note 20), you may develop up to two plates in a TLC tank. Mark each plate lightly with pencil by alphabet letters at the top to avoid mix-up.

24. Once spotting is over, bring the plate(s) next to the TLC tank. Hold the (first) plate by the middle of its far end with one hand and raise it so that it hovers horizontal above the TLC tank. Open the lid and lower the plate into the tank at a distance of 1–2 cm from one of its two long walls. When the plate touches the surface of the developer lower it a bit more until it lands on the bottom and let it rest gently against the wall, making sure it does not tip over (see Note 21).

25. If you have another plate to develop, repeat the previous step on the opposite wall of the tank immediately. Do not turn the tank around! Then promptly replace the lid. It is important not to let too much of the developer’s vapor escape the tank. You may wear a mask while the tank is open to avoid inhaling the fumes. However, do not turn the fume hood’s motor on!

26. The developer will rise (initially fast, then slower) on the plate, carrying the sample spots with it. When the developer reaches about one-half centimeter from the top of the plate(s) or from the pencil line you drew on the 20×20-cm plate(s) with glass support (in about 20 min), turn the fume hood’s motor on, open the lid of the tank, and pull out the plate(s). Place each plate, face up, under the fume hood and let it dry for about 15 min. In the meantime, dispose of the developer in the tank properly and let the tank dry under the fume hood.

27. Turn the fume hood’s motor off. Place the spray box inside the fume hood and place the plate nearly vertical (silica gel facing you) inside the spray box. Fill the spray bottle with the dichrofluorescein spray reagent (see Note 22) and spray the plate evenly from a distance of about 25 cm, making sure it acquires a faint orange color without getting overly wet (see Note 23).

28. Turn the fume hood’s motor back on and let the plate dry completely (another 15 min). Bring the plate to the darkroom (or to a fairly dark place), put protective eye goggles on, and view the plate under a UV lamp. Bright yellow fluorescent spots will appear on a dark background, corresponding to the lipids present in each sample. Phospholipids will have remained at the origin, while triacylglycerols will have migrated halfway to the top.

29. Mark the contour of the phospholipid and triacylglycerol spots in each muscle sample with pencil, giving a slack of about 1 mm all around, and take the plate back to the lab.
30. Prepare and label two screw top test tubes per muscle sample, one for triacylglycerols and another for phospholipids.

31. Fit a glass funnel on top of the first test tube and, using a scalpel with round blade, carefully scrape the phospholipid spot of the first muscle sample into the funnel. Shake the funnel lightly and then apply a gentle stream of nitrogen from all around the rim down to the neck to make sure that all silica gel flakes get to the bottom of the test tube.

32. Remove the funnel from the first test tube and clean it with a stronger stream of nitrogen to make sure no silica gel is carried over to the next test tube (see Note 24). Wipe the scalpel thoroughly with tissue paper for the same purpose.

33. Fit the funnel onto the next test tube and proceed with the phospholipid spot of the second muscle sample by repeating the previous two steps. Continue with the remaining samples. Then scrape off the triacylglycerol spots of all samples into their respective test tubes in the same way (see Note 25). Discard the TLC plate.

1. To each screw top test tube containing triacylglycerols or phospholipids, add 0.5 mL of methanolic sodium methoxide and cap tightly. The liquid turns yellow, as it extracts the dichlorofluorescein from the silica gel. Vortex and heat at 50°C for 10 min (see Note 26).

2. Let all test tubes cool. To the ones containing phospholipids, add 0.5 mL of methanolic boron trifluoride, cap tightly, and heat at 100°C for 75 min (see Note 27).

3. Let the test tubes containing phospholipids cool.

4. Open all test tubes, taking care not to mix up their caps. To each test tube, add 1.5 mL of hexane, cap tightly, and vortex at full speed or shake vigorously for 1 min to extract the MEs and DMAs.

5. Let the test tubes stand for a few minutes and watch a sharp interphase form between the lower methanol phase (containing the silica gel and dichlorofluorescein) and the upper hexane phase containing the MEs and DMAs. Spin the test tubes briefly in a centrifuge if you are in a hurry.

6. Using a Pasteur pipette, remove as much of the upper phase as possible into a small glass test tube. Do not take any of the lower phase! Evaporate under a stream of nitrogen as in Subheading 3.2.1, step 12. Alternatively, this may be a good time to call it a day, especially if you are not in a hurry to start the ME and DMA analysis. In this case, you may just leave the test tubes overnight under the fume hood and let the hexane evaporate effortlessly.
7. Contrary to plain test tubes, screw top test tubes and their caps are too expensive to be used only once. Therefore, rinse the tubes containing the lower methanol phase and silica gel thoroughly with tap water and then with chloroform-methanol 2:1. Let dry for future use. Treat the screw caps likewise.

8. Turn the gas chromatograph on. If you use manual injection, dissolve the dry residue in each test tube containing MEs from triacylglycerols in 50 μL of hexane just before injection. Mix thoroughly and inject 1 μL.

9. If you use an autosampler, dissolve the dry residue in each test tube containing MEs from triacylglycerols in 50 μL of hexane. Mix thoroughly and, using an automatic pipette, transfer the solution into a microvolume insert sitting inside a 2-mL screw top vial. Close the vial with septum and screw cap, place it in the autosampler and inject 1 μL (see Note 28).

10. Dissolve the dry residue in each test tube containing MEs and DMAs from phospholipids in 200 μL of hexane and inject 1 μL as above (see Note 29).

11. Use an appropriate software to acquire the gas chromatogram. Based on the retention times you have established in Subheading 3.1, assign the peaks in the chromatogram to MEs and DMAs and integrate the peaks so that you get a table containing an area value (in arbitrary units) for each identified ME and DMA. Depending on the capabilities of the data acquisition software, this can be done automatically, manually, or in part automatically and in part manually.

### 3.2.3. Calculations

Conversion of the peak integration data into muscle content values (that is, μmol lipid per gram muscle) can be done through either the data acquisition software of the gas chromatograph or a spreadsheet (such as Microsoft® Excel). Described below is the reasoning that has to be followed in any case.

1. Calculations are based on the premise that peak area is proportional to ME mass. Of pivotal importance, then, are the amount and area of 17:0 ME derived from methanolysis of the 17:0 TG and 17:0 PC internal standards that were added to the muscle samples in the beginning of the analysis (see Note 30). On the basis of proportionality, if $M_{17}$ and $A_{17}$ are the amount and area, respectively, of 17:0 ME and $A$ is the area of an endogenous ME, then its amount, $M$, is given by the formula, $M = M_{17} \times A/A_{17}$. Let’s apply this calculation separately to the triacylglycerols and phospholipids of a muscle sample.

2. Begin with triacylglycerols. To calculate the amount of 17:0 ME derived from 17:0 TG that is present in the muscle triacylglycerol fraction, first multiply the 17:0 TG standard concentration,
a (in μg/μL), by the volume added to each sample, that is, 1 μL (see Subheading 3.2.1, step 6), yielding a μg. Divide by the $M_r$ of 17:0 TG (849.4) and get $a/849.4$ μmol. Since 1 mol of a triacylglycerol yields 3 mol of MEs, one gets $3a/849.4$ μmol of 17:0 ME. Finally, by multiplying by the $M_r$ of 17:0 ME (284.5), one gets 1.005 $a$ μg. This is $M_{17}$.

3. Apply the formula, $M = 1.005a \times A/A_{17}$, to every ME identified in the chromatogram and get the amount of each ME in μg. Then divide by that ME’s $M_r$ to express its amount in μmol, which is also μmol of the corresponding acyl group (or, more commonly, fatty acid) in muscle triacylglycerols.

4. You may divide the amount of each fatty acid by the amount of muscle tissue (either wet or dry) weighed in Subheading 3.2.1, step 7 to express the fatty acid content of muscle triacylglycerols in μmol/g or μmol/mg.

5. You may add the amounts of all fatty acids and divide the sum by 3 to get the triacylglycerol content of muscle in μmol/g or μmol/mg.

6. You may divide the amount of each fatty acid by the sum of fatty acids and then multiply by 100 to express the percentage molar distribution of fatty acids in muscle triacylglycerols (see Note 31).

7. You may use partial sums to calculate the amounts or percentages of fatty acid categories, such as saturated, unsaturated, ω6, etc.

8. Continue with phospholipids. To calculate the amount of 17:0 ME derived from 17:0 PC that is present in the muscle phospholipid fraction, first multiply the 17:0 PC standard concentration, $b$ (in μg/μL), by the volume added to each sample, that is, 4 μL (see Subheading 3.2.1, step 6), yielding 4$b$ μg. Divide by the $M_r$ of 17:0 PC (762.2) and get $4b/762.2$ μmol. Since 1 mol of PC yields 2 mol of MEs, one gets $8b/762.2$ μmol of 17:0 ME. Finally, by multiplying by the $M_r$ of 17:0 ME (284.5), one gets 2.986 $b$ μg.

9. Apply the formula, $M = 2.986b \times A/A_{17}$, to every ME identified in the chromatogram and get the amount of each ME in μg. Then divide by that ME’s $M_r$ to express its amount in μmol, which is also μmol of the corresponding fatty acid in muscle phospholipids.

10. Apply the same formula to every DMA identified in the chromatogram and get the amount of each DMA in μg. Then divide by that DMA’s $M_r$ to express its amount in μmol, which is also μmol of the corresponding fatty aldehyde in the muscle phospholipids.

11. You may divide the amount of each fatty acid (and aldehyde) by the amount of muscle tissue used in the analysis to express the fatty acid (and aldehyde) content of muscle phospholipids.
12. You may add the amounts of all fatty acids and aldehydes, then divide the sum by 2 to get the approximate phospholipid content of muscle. “Approximate” refers to the fact that most, but not all, phospholipids contain two acyl groups in their structure (see Fig. 2). To get the exact phospholipid content of muscle, you need to know the amount of each individual phospholipid, which is determined in Subheading 3.3.

13. You may divide the amount of each fatty acid and aldehyde by the sum of fatty acids and aldehydes, then multiply by 100 to express the percentage molar distribution of fatty acids and aldehydes in muscle phospholipids (see Note 31).

14. You may use partial sums to calculate the amounts or percentages of fatty acid and aldehyde categories, such as saturated, unsaturated, ω6, etc.

In order to be able to go directly from lipid extraction to phospholipid separation by 2D TLC, you need to establish the migration pattern of each phospholipid in advance. Although we provide such a pattern in Fig. 4, we strongly advise that you establish your own pattern.

1. Place two TLC tanks, with their lids on, in a place with constant room temperature and not exposed to air drafts (not in the fume hood). Number the tanks, 1 and 2.

![Fig. 4. Separation pattern of muscle phospholipids by 2D TLC. First dimension, chloroform-methanol-acetic acid 10:5:1 (v/v/v); second dimension, chloroform-acetone-methanol-acetic acid-water 10:4:2:2:1 (v/v/v/v/v) (9). CL cardiolipin; LPC lysophosphatidyl choline; PC phosphatidyl choline; PE phosphatidyl ethanolamine; PI phosphatidyl inositol; PS phosphatidyl serine; SM sphingomyelin.](image-url)
2. Prepare the developer for the first dimension of 2D TLC: If using a large tank (one that accommodates 20-cm wide plates), decant 60 mL of chloroform, 30 mL of methanol, and 6 mL of acetic acid in a 250-mL conical glass flask under the fume hood operating at full speed. If using a small tank (one that accommodates 10-cm wide plates), decant 30 mL of chloroform, 15 mL of methanol, and 3 mL of acetic acid in an 100-mL flask (see Note 12).

3. Mix well with a swirling motion and promptly pour off into tank #1 after sliding the lid sideways just enough for the liquid to enter. Replace the lid immediately and turn the fume hood’s motor off. Grasp the tank with both hands and, while holding the lid in place with your index fingers, shake the tank from one side to the other for a few seconds to facilitate saturation of its atmosphere with developer vapors. We do not recommend lining the tank with filter paper. Let the tank stand no less than 1 h and no more than 2 h before chromatography to ensure reproducible separations (see Note 13).

4. Prepare the developer for the second dimension of 2D TLC: If using a large tank, decant 50 mL of chloroform, 20 mL of acetone, 10 mL of methanol, 10 mL of acetic acid, and 5 mL of distilled water in a 250-mL flask under the fume hood operating at full speed. If using a small tank, mix 25 mL of chloroform, 10 mL of acetone, 5 mL of methanol, 5 mL of acetic acid, and 2.5 mL of distilled water in an 100-mL flask (see Note 32).

5. Mix and pour off into tank #2 just like you did with the developer for the first dimension. Let the tank stand no more than 2 h before chromatography (it will certainly stand 1 h).

6. You will need eight 10 × 10-cm TLC plates, that is, one for each of the seven phospholipids (PC, LPC, PE, PS, PI, CL, and SM) and one for the mixture of all seven. If your plates are not 10 × 10 cm, cut them carefully with scissors, taking care not to chip off too much of the silica gel layer. Use the cut sides of the resulting plates as the far ends in the subsequent chromatography, that is, the ends toward which the developer migrates (see Note 18).

7. Mix 120 μL of the 17:0 PC, 5 μL of the LPC, 40 μL of the PE, 5 μL of the PS, 10 μL of the PI, 15 μL of the CL, and 5 μL of the SM standard solutions (see Note 33) in a 2-mL screw top vial and cap promptly.

8. Place the spotting guide on the bench. If using a plate with transparent support (that is, glass or plastic), place the plate on the spotting guide so that the sides of the two coincide. Now you can see dot #1 on the spotting guide under the plate at its bottom left corner. If using a plate with aluminum support, place the plate on the spotting guide a bit to the right and up,
so you can barely see the ends of the two perpendicular lines intersecting at dot #1. Thus, you will have the coordinates of where to spot.

9. Draw 10 μL of the phospholipid mixture in a 10-μL syringe with blunt needle and bring the syringe over the spotting position, the tip of the needle being a few millimeters above the plate.

10. Gently squeeze the plunger to create a medium-sized drop (of about 0.7 μL) and carefully lower the tip of the needle until the drop (not the needle) touches the plate and is absorbed by the silica gel.

11. Wait a few seconds until all of the solvent evaporates from the plate, then repeat the previous step until all of the syringe’s contents are spotted. Spot each drop on top of the previous one so that the spot formed does not exceed a few millimeters in diameter. You will find that it takes longer and longer for each drop to evaporate. You may speed up the evaporation by setting up a hair dryer next to the plate and directing the air at the spot (see Note 19).

12. Spot 5 μL of the 17:0 PC standard solution onto the next plate. If using small tanks, you may run two plates simultaneously, so stop here. If using large tanks you may run four plates simultaneously (two plates side by side on each long wall of the tank), so continue by spotting 5 μL of each LPC and PE standard solutions onto the next two plates. To avoid mix-up among plates, mark each one softly with pencil at its upper right corner.

13. Once spotting is over, bring the plates next to tank #1. Hold the first plate by the middle of its far end with one hand and raise it so that it hovers horizontal above the tank. Open the lid and lower the plate into the tank at a distance of 1–2 cm from one of its two long walls. When the plate touches the surface of the developer, lower it a bit more until it lands on the bottom and let it rest gently against the wall, making sure it does not tip over (see Note 21).

14. Place the second plate next to the first one (if you have a large tank) or opposite the first one (if you have a small tank). If you have a large tank, place the remaining plates on the opposite wall. Do not turn the tank around! Then promptly replace the lid. Work as fast as possible to minimize evaporation of the developer. You may wear a mask while the tank is open to avoid inhaling the fumes. However, do not turn the fume hood’s motor on!

15. The developer will rise (initially fast, then slower) on the plates, carrying the sample spots with it. When the developer reaches about one-half centimeter from the top of the plates
(in about 35 min), turn the fume hood’s motor on, open the lid of the tank, and pull out the plates. Place the plates, face up, under the fume hood and let them dry for 20 min. In the meantime, dispose of the developer in the tank properly and let the tank dry under the fume hood.

16. Rotate the plates 90° counterclockwise and place them in tank #2 (just like you did with tank #1) so that now the origin lies at the bottom right corner.

17. When the developer in the second dimension reaches about one-half centimeter from the top of the plates (in another 35 min), turn the fume hood’s motor on, open the lid of the tank and pull out the plates. Let the plates dry for another 20 min, dispose of the developer, and let the tank dry. Then turn the fume hood’s motor off.

18. Repeat steps 1–17 with the remaining phospholipid standard solutions.

19. Place the spray box inside the fume hood and place the plates, one by one or two by two, nearly vertical (silica gel facing you) inside the spray box. Fill the spray bottle with the dichlorofluorescein spray reagent (see Note 22) and spray the plates evenly from a distance of about 25 cm, making sure they acquire a uniform faint orange color without getting overly wet (see Note 23).

20. Turn the fume hood’s motor back on and let the plates dry completely (about 15 min). Bring the plates to the darkroom (or to a fairly dark place), put protective eye goggles on, and view the plates under a UV lamp.

21. Seven bright yellow fluorescent spots of different shapes, corresponding to the seven phospholipids in mixture, should be visible on a dark background on the first plate. Place a transparency (or other transparent material) over the plate and outline each phospholipid spot with permanent pen. Also mark the origin.

22. One fluorescent spot should be visible on each of the remaining seven plates, each spot at a different position on the plate. Place the transparency on each of these plates so that the origins coincide and identify the seven phospholipids. In the end, you should produce something like Fig. 4 (see Note 34).

This section resembles Subheading 3.2.1 in the way lipids are extracted from muscle and separated by 1D TLC. However, from that separation, you will only need the triacylglycerol spot, since the individual phospholipids will be obtained from 2D TLC. To accurately measure the minor phospholipids, you will need a higher amount of tissue than that needed for the analysis of total phospholipids.
1. Follow Subheading 3.2.1, steps 1–4.

2. Prepare a mixture of the 17:0 TG and 17:0 PC standard solutions by mixing three volumes of the former with seven volumes of the latter in a 2-mL screw top vial kept on ice and capping immediately and tightly. You will need 10 μL of this mixture per sample, but, in any case, make at least 100 μL to protect the mixture’s composition against evaporation in the vial if the volume is too low. Keep on ice and discard at the end of the day.

3. Using a 10-μL syringe with pointed needle, pierce the septum of the vial and dispense 10 μL of the mixture (in effect, 3 μL of the 17:0 TG standard solution plus 7 μL of the 17:0 PC standard solution) at the bottom of as many small glass test tubes as the muscle samples you are going to analyze. Wait a minute for the solvent to evaporate before proceeding to the next step. Label the test tubes according to the muscle samples.

4. Take a muscle specimen out of the ultrafreezer, place the corresponding test tube in the analytical balance, tare, and promptly weigh out approximately 90 mg. Note weight to the third or, preferably, fourth decimal point of the gram (see Note 15). Return any remaining part of the specimen to the ultrafreezer.

5. Immediately add 1,710 μL of lipid extraction solvent [chloroform-methanol 2:1 (v/v) with 0.005% (w/v) BHT]. Vortex briefly and let stand for 5 min or longer with occasional vortexing (see Note 16). In the meantime, you may proceed with other muscle specimens.

6. Add 360 μL of distilled water and vortex vigorously for 1 min.

7. Follow Subheading 3.2.1, steps 10–16.

8. Draw 3 μL of the dissolved extract in a 10-μL syringe with blunt needle and bring the syringe over the first spotting position.

9. Gently squeeze the plunger to create a medium-sized drop (of about 0.7 μL) and carefully lower the tip of the needle until the drop touches the plate and is absorbed by the silica gel.

10. Wait a few seconds until all of the solvent evaporates from the plate, then repeat the previous step until all of the 3 μL of extract are spotted. Spot each drop on top of the previous one so that the spot formed does not exceed a few millimeters in diameter.

11. By the end of spotting, a yellow-brownish spot will have formed on the plate. Then proceed to dissolving and spotting the next extract(s) in the same way until you fill all available spotting positions on the plate. Also spot 2 μL of the 17:0 TG standard solution on a separate position in the middle of the plate in order to identify the triacylglycerol spot in each sample after chromatography.
12. When you first try this method, we recommend that you perform the analysis of individual phospholipids by 2D TLC on the following day(s). Therefore, save the remaining extract in each test tube by covering the test tube with Parafilm and storing at 4°C. Stable for 1 month. If, later on, you feel confident to perform both procedures on the same day, just leave the extracts on the bench.


14. Mark the contour of the triacylglycerol spot in each muscle sample with pencil, giving a slack of about 1 mm all around, and take the plate back to the lab.

15. Prepare a screw top test tube for each muscle sample.

16. Apply Subheading 3.2.1, steps 31–33 to the triacylglycerol spots only.

17. Prepare for 2D TLC by following Subheading 3.3.1, steps 1–5.

18. You will need one 10 × 10-cm TLC plate per muscle sample. If your plates are not 10 × 10 cm, cut them carefully with scissors, taking care not to chip off too much of the silica gel layer. Use the cut sides as the far ends in the subsequent chromatography (see Note 18).

19. By now, the solvent of the lipid extract(s) used to isolate triacylglycerols by 1D TLC (step 8) has evaporated. So, redissolve the remaining dry extract of each muscle sample in 15 μL of lipid extraction solvent and spot 10 μL of the resulting solution on a plate by following Subheading 3.3.1, steps 8–11 (see Note 35). Spot up to two samples if using small TLC tanks and up to four samples if using large tanks. To avoid mix-up among plates, mark each one softly with pencil at its upper right corner.

20. Perform 2D TLC by following Subheading 3.3.1, steps 13–17.

21. See the phospholipid spots on each plate by following Subheading 3.3.1, steps 19 and 20. Mark the contour of each spot with pencil, giving a slack of about 1 mm all around. Identify each phospholipid by comparing the pattern on the plate to that on the transparency created under Subheading 3.3.1.

22. Prepare and label seven screw top test tubes per muscle sample, one for each phospholipid. To the bottom of each test tube, decant accurately 5 μL of the 15:0 ME standard solution using a 10-μL syringe with pointed needle (see Note 36).

23. Apply Subheading 3.2.1, steps 31–33 to each phospholipid spot.
1. To each screw top test tube containing SM, add 1 mL of methanolic boron trifluoride and cap tightly. The liquid turns yellow, as it extracts the dichlorofluorescein from the silica gel. Vortex and heat at 100°C for 75 min (see Note 37).

2. To each of the remaining screw top test tubes, that is, the ones containing triacylglycerols, PC, LPC, PE, PS, PI or CL, add 0.5 mL of methanolic sodium methoxide and cap tightly. Vortex and heat at 50°C for 10 min (see Note 38).

3. Let all test tubes cool. Then remove their caps, taking care not to mix them up. To each test tube, add 1.5 mL of hexane, cap tightly, and vortex at full speed or shake vigorously for 1 min to extract the MEs and DMAs.

4. Isolate and dry the MEs and DMAs by following Subheading 3.2.2, steps 5–7.

5. Turn the gas chromatograph on. If you use manual injection, dissolve the dry residue in each test tube in 50 μL of hexane just before injection. Mix thoroughly and, in the case of triacylglycerols and PC, inject 1 μL. In the case of PE, inject 3 μL.

6. In the case of all other phospholipids (LPC, PS, PI, CL, and SM), evaporate the solution under a stream of nitrogen. Gently direct the stream all around the wall of the test tube, forcing the solution to concentrate at the bottom. Then redissolve in 10 μL of hexane, mix, and inject 3 μL.

7. If you use an autosampler, dissolve the dry residue in each test tube in 50 μL of hexane. Mix thoroughly and, using an automatic pipette, transfer the solution into a microvolume insert sitting inside a 2-mL screw top vial.

8. In the case of triacylglycerols, PC and PE, close the vial with septum and screw cap, place it in the autosampler and inject 1 μL (in the case of triacylglycerols and PC) or 3 μL (in the case of PE).

9. In the case of all other phospholipids (LPC, PS, PI, CL, and SM), evaporate the solution in the microvolume insert under a stream of nitrogen, taking good care not to splatter the solution. Wash the corresponding test tube with another 50 μL of hexane, mix thoroughly, and transfer the solution into the microvolume insert. Reevaporate, dissolve in 10 μL of hexane, mix, and inject 3 μL (see Note 27).

10. Use an appropriate software to acquire the chromatogram. Based on the retention times you have established in Subheading 3.1, assign the peaks in the chromatogram to MEs and DMAs and integrate the peaks so that you get a table containing an area value (in arbitrary units) for each identified ME and DMA. Depending on the capabilities of the data acquisition software, this can be done automatically, manually, or in part automatically and in part manually.
Conversion of the peak integration data into muscle content values (that is, \( \mu \)mol lipid per gram muscle) can be done through either the data acquisition software of the gas chromatograph or a spreadsheet (such as Microsoft® Excel). Described below is the reasoning that has to be followed in any case.

1. Calculations are based on the premise that peak area is proportional to ME mass. Of pivotal importance, then, are the amount and area of 17:0 ME derived from methanolysis of the 17:0 TG and 17:0 PC internal standards that were added to the muscle samples in the beginning of the analysis (see Note 30). On the basis of proportionality, if \( M_{17} \) and \( A_{17} \) are the amount and area, respectively, of 17:0 ME and \( A \) is the area of an endogenous ME, then its amount, \( M \), is given by the formula, \( M = M_{17} \times A / A_{17} \). Let’s apply this calculation separately to the triacylglycerols and phospholipids of a muscle sample.

2. Begin with triacylglycerols. To calculate the amount of 17:0 ME derived from 17:0 TG that is present in the muscle triacylglycerol fraction, first multiply the 17:0 TG standard concentration, \( a \) (in \( \mu \)g/\( \mu \)L), by the volume added to each sample, that is, 3 \( \mu \)L (see Subheading 3.3.2, step 3), yielding 3\( a \)\( \mu \)g. Divide by the \( M_r \) of 17:0 TG (849.4) and get 3\( a \)/849.4 \( \mu \)mol. Since 1 mol of a triacylglycerol yields 3 mol of MEs, one gets 9\( a \)/849.4 \( \mu \)mol of the 17:0 ME. Finally, by multiplying by the \( M_r \) of 17:0 ME (284.5), one gets 3.014\( a \)µg. This is \( M_{17} \).

3. Apply the formula, \( M = 3.014a \times A / A_{17} \), to every ME identified in the chromatogram and get the amount of each ME in \( \mu \)g. Then divide by that ME’s \( M_r \) to express its amount in \( \mu \)mol, which is also \( \mu \)mol of the corresponding acyl group (or, more commonly, fatty acid) in the muscle triacylglycerols.

4. You may divide the amount of each fatty acid by the amount of muscle tissue (either wet or dry) weighed in Subheading 3.3.2, step 4 to express the fatty acid content of muscle triacylglycerols in \( \mu \)mol/g or \( \mu \)mol/mg.

5. You may add the amounts of all fatty acids and divide the sum by 3 to get the triacylglycerol content of muscle in \( \mu \)mol/g or \( \mu \)mol/mg.

6. You may divide the amount of each fatty acid by the sum of fatty acids and then multiply by 100 to express the percentage molar distribution of fatty acids in muscle triacylglycerols (see Note 31).

7. You may use partial sums to calculate the amounts or percentages of fatty acid categories, such as saturated, unsaturated, \( \omega \)6, etc.

8. Continue with PC. To calculate the amount of 17:0 ME derived from 17:0 PC that is present in the muscle PC fraction, first multiply the 17:0 PC standard concentration, \( b \) (in \( \mu \)g/\( \mu \)L),
by the volume added to each sample, that is, 7 μL (see Subheading 3.3.2, step 3), yielding 7μg. Divide by the M_r of 17:0 PC (762.2) and get 7b/762.2 μmol. Since 1 mol of PC yields 2 mol of MEs, one gets 14b/762.2 μmol of 17:0 ME. Finally, by multiplying by the M_r of 17:0 ME (284.5), one gets 5.226μg.

9. Apply the formula, \(M = \frac{5.226b \times A_{17}}{A_{17}}\), to every ME identified in the chromatogram and get the amount of each ME in μg. Then divide by that ME’s \(M_r\) to express its amount in μmol, which is also μmol of the corresponding fatty acid in muscle PC.

10. Apply the same formula to every DMA identified in the chromatogram (see Note 39) and get the amount of each DMA in μg. Then divide by that DMA’s \(M_r\) to express its amount in μmol, which is also μmol of the corresponding fatty aldehyde in muscle PC.

11. You may divide the amount of each fatty acid (and aldehyde) by the amount of muscle tissue used in the analysis to express the fatty acid (and aldehyde) content of muscle PC.

12. You may add the amounts of all fatty acids and aldehydes, then divide the sum by 2 to get the PC content of muscle.

13. You may divide the amount of each fatty acid and aldehyde by the sum of fatty acids and aldehydes, then multiply by 100 to express the percentage molar distribution of fatty acids and aldehydes in muscle PC (see Note 31).

14. You may use partial sums to calculate the amounts or percentages of fatty acid and aldehyde categories, such as saturated, unsaturated, ω6, etc.

15. To calculate the amounts of the other phospholipids, for which no internal standards were included, we will use, in addition, the area of 15:0 ME (the external standard that was added in equal amounts to all scraped phospholipid spots after 2D TLC in Subheading 3.3.2, step 22). Let \(M_{15}\) be the amount of 15:0 ME added to each phospholipid, \(A_{15,PC}\) be the area of 15:0 ME in the PC chromatogram, and \(A_{15,PE}\) be the area of 15:0 ME in the PE chromatogram (see Note 40). Then, the amount, \(M\), of an endogenous ME having an area, \(A\), in the PE chromatogram will be \(M = \frac{M_{15} \times A}{A_{15,PE}}\). However, from the PC chromatogram, \(M_{15} = \frac{M_{17} \times A_{15,PC}}{A_{17}}\). By substitution we get, \(M = \frac{M_{17} \times A_{15,PC}}{A_{17}} \times \frac{A}{A_{15,PE}}\) or \(M = \frac{M_{17} \times A}{A_{17}} \times \frac{A_{15,PC}}{A_{15,PE}}\). This formula is like the one given in step 1, except that it contains an additional term that links the PE chromatogram (from which \(A\) is taken) to the PC chromatogram (from which \(A_{17}\) is taken), thus normalizing for the different areas of the external standard in the two chromatograms.
16. Apply the formula, \( M = 5.226 \times A / A_{17} \times A_{15,PC} / A_{15,PE} \), to every ME identified in the PE chromatogram and get the amount of each ME in \( \mu g \). Then divide by that ME’s \( M_r \) to express its amount in \( \mu mol \), which is also \( \mu mol \) of the corresponding fatty acid in muscle PE.

17. Apply steps 10–14 to PE.

18. Apply the same calculations to all of the remaining phospholipids with two exceptions. First, you do not have to bother with DMAs in these. Second, when coming to step 12, you will have to divide the sum of fatty acids in CL by 4 in order to get the CL content of muscle (see Fig. 21); do nothing with the sum of fatty acids in LPC and SM, as each contains one acyl group.

### 4. Notes

1. Other lipid classes, such as (nonesterified) fatty acids and diacylglycerols, may be analyzed by the same methodology, but the amounts of tissue required will be higher than the ones dictated herein because of the low abundance of these lipid classes.

2. Fatty acids and their acyl groups are often abbreviated as [number of carbon atoms]:[number of double bonds], occasionally followed by an indication of the position of double bonds.

3. You may store the solutions indefinitely. However, certain MEs in solution, especially the polyunsaturated ones, deteriorate with time, resulting in their peaks decreasing and, eventually, vanishing from the gas chromatograms. If you notice this, discard the solution and make a fresh one.

4. In general, an 1-alkenyl group attached by ether linkage to glycerol in a plasmalogen is hydrolyzed to 1-alkenol, which is isomerized to the more stable aldehyde. A dimethyl acetal is produced by methylation of that aldehyde.

5. Based on the presence, in the gas chromatograms of the MEs derived from PC and, in particular, PE, of certain peaks that differ in retention times from the 18:0, 18:1\( \omega 9 \) and 18:1\( \omega 7 \) MEs by as much as 16:0 DMA differs in retention time from 16:0 ME, we have tentatively identified these peaks as 18:0, 18:1\( \omega 9 \) and 18:1\( \omega 7 \) DMAs (\( M_r \) 314.5, 312.5, and 312.5, respectively). Look for such peaks in your chromatograms. Unfortunately, we have not been able to find commercially available standards for these compounds.

6. Triacylglycerols and phospholipids with acyl groups bearing other odd numbers of carbon atoms, such as 15, 19, or 21, may be used as internal standards instead.
7. Keep a small bottle of chloroform in the refrigerator or coldroom to minimize evaporation during preparation of the internal standard solutions. Rinse the pipette tip once or twice with the cold chloroform before dispensing it into the vials to make sure you deliver the right volume.

8. Keep the TLC plates in their box in a dry place, as silica gel is highly hygroscopic. Plates exposed to high humidity or kept in an opened box for over a year may need to be activated by placing in an oven at 110°C for 15–30 min prior to use.

9. Because organic solvents are volatile, it is preferable to remove an aliquot of a lipid standard solution by piercing the septum of its vial with a syringe with pointed needle, rather than by opening the vial and using an automatic pipette. However, always remember to promptly replace a pierced septum with a new one before storing a vial, or the solvent will gradually evaporate through the hole in the septum, even at −20°C.

10. Inject the larger and more unsaturated MEs first, as these are more difficult to separate than the smaller and saturated ones. Thus, you may save time by making any necessary adjustments earlier than later.

11. Alternatively, you may postpone the pulverization until the day of analysis. A pulverized tissue ensures higher sample homogeneity and more efficient lipid extraction.

12. The idea is to obtain a solvent depth of about 5 mm in the tank.

13. Prepare the developer fresh and use it only once. If, for any reason, you do not use it on the day of preparation discard it, as you cannot trust its composition the next day.

14. Depending on the number of samples, experience, dexterity, and number of available hands, lipid extraction and spotting on the TLC plate(s) may take from one-half to more than 2 h. In the latter case, start the day with lipid extraction (step 5) and prepare the developer later so that it does not have to wait for over 2 h.

15. If using lyophilized tissue, weigh out approximately one-fourth the recommended amount of wet tissue and note weight to the fourth decimal point of the gram.

16. If the muscle is not pulverized, make sure that its pieces are less than 2 mm across to achieve complete extraction of their lipids. If this is not the case, break the pieces apart within the extraction solvent with a spatula. If this is not feasible, homogenize the sample.

17. We create a fine stream of nitrogen by attaching a glass Pasteur pipette to the tubing supplying the nitrogen. We then bring the tip of the pipette about 1 cm from the surface of the liquid.
and adjust the gas flow so as to agitate the surface but not splatter the liquid. Since the evaporation cools the liquid and this slows down the process, we keep the test tube in a beaker with tap water at room temperature or up to 37°C. Alternatively, you may wish to hold the test tube in your palm during the evaporation process.

18. Draw a faint line with ruler and pencil on the silica gel to be able to cut the plate straight. Make sure the ruler is clean and does not touch the silica gel extensively.

19. The hair dryer should be turned off when delivering each drop, or the air current will blow the drop away. Also take care not to blow the plate and spotting guide away.

20. A multiplate rack can hold up to six plates and lets you develop them simultaneously in one TLC tank. All you have to do is load the plates on the rack and then carefully lower the rack into the tank. However, take care not to let the side that carries the silica gel touch the margin of the rack holding the plate in place, since the developer rises there through capillary action more rapidly than on the rest of the plate, resulting in abnormal developer front and aberrant lipid separation.

21. It is good for the plate to touch the surface of the developer horizontally so that the developer forms a horizontal front as it rises and all samples migrate uniformly. However, if the plate enters the developer sideways, do not panic and, under no circumstance, raise the plate in order to attempt a better “landing.” Just let the plate sit on the bottom. All you will get is a slightly crooked developer front and a slightly uneven migration pattern. Nevertheless, most probably, you will still be able to discern the lipid spots of interest.

22. Other means of locating lipid spots on a TLC plate, such as spraying with sulfuric acid solution or exposing to iodine vapors, are unsuitable for the subsequent ME and DMA analysis. Sulfuric acid chars all lipids, while iodine reacts with double bonds, thereby altering the unsaturated fatty acids.

23. The spray reagent may remain in the spray bottle if used daily. However, if the spray bottle is left unused for several days, the spray reagent may dry in the nozzle; then dichloro-fluorescein will block the nozzle. If this happens, return the spray reagent to its container and unblock the nozzle by rinsing and spraying with ethanol. To prevent blocking from happening, remember to return the spray reagent to its container after use, rinse the spray bottle with ethanol, and spray some of it to clean the nozzle.

24. Do not wash the funnel between samples, as silica gel flakes will stick to it. However, do wash the funnel with chloroform-methanol 2:1 at the end of the day.
25. If using a plate with soft support, you may cut it with scissors during the scraping sequence in any way you find convenient in order to bring distant spots close to the funnel and prevent silica gel flakes from falling outside the funnel.

26. This base-catalyzed methanolysis is according to Kramer et al. (8) and results in the full conversion of triacylglycerols and glycerophospholipids (see Fig. 2b–i) into MEs and DMAs. However, SM requires acid-catalyzed methanolysis which is performed in the next step on the phospholipid fraction only.

27. Heating a methanol-based solution at 100°C may cause its rapid evaporation unless it is tightly closed. Therefore, at about 5 min of heating tighten the caps (wear heat-resistant gloves!), as the caps tend to get loose. Check the level of methanol inside the test tubes from time to time. If you notice a leak, take the test tube out of the heating apparatus and let it cool briefly. Unscrew the cap and inspect both the cap’s lining and the test tube’s rim. If the cap’s lining is damaged replace the cap. If the test tube’s rim is chipped, transfer the contents into another test tube with a glass Pasteur pipette. In any case, add methanol to about 1 mL and continue the incubation. Make a note of any problematic test tubes, as, despite all efforts, they may produce aberrant data; this will necessitate repetition of the analysis.

28. Microvolume inserts require meticulous cleaning before being reused. We recommend rinsing five times with chloroform-methanol 2:1.

29. The fraction of the ME and DMA solution to be injected into the gas chromatograph depends on the split ratio chosen and on the triacylglycerol and phospholipid content of the muscle sample. Thus, you may change the volume of hexane in which you dissolve the ME and DMA residue, the volume injected into the gas chromatograph or the split ratio to achieve the best possible balance between getting signals of the minor components well above the noise and not overloading the column with the major components.

30. It is good for the area of 17:0 ME to be neither too small nor too big relative to the areas of the endogenous MEs in a chromatogram. Therefore, once you get your first chromatograms, try to adjust the amounts of the 17:0 TG and PC internal standards added to subsequent similar samples so that the area of 17:0 ME is about half the area of the most abundant ME (which is usually 16:0).

31. If you only intend to determine the percentage molar distribution of fatty acids (and aldehydes) in muscle triacylglycerols and phospholipids, you may omit the internal standards from the analysis. All you need to do then is divide the area of each ME
(and DMA) in the chromatogram by that compound’s $M_r$ and use the quotients to calculate percentage distribution.

32. This 2D TLC system is according to Kester et al. (9).

33. These proportions simulate the usual phospholipid distribution in skeletal muscles and are suggested instead of equal amounts of all phospholipids in order to produce a 2D TLC pattern that is as close to the natural one as possible.

34. Slight differences in the positions of some phospholipids between the plate containing all phospholipids and the plates containing individual phospholipids should be expected, since the migration of one compound in a chromatographic system may be affected by interactions with other compounds in the mixture and since the chromatographic conditions are rarely identical between two runs.

35. If you are unable to recover 10 μL of extract, dissolve the dry residue in 20, rather than 15, μL of lipid extraction solvent.

36. Methyl pentadecanoate will be used as external standard to enable calculation of the amounts of LPC, PE, PS, PI, CL, and SM in conjunction with the 17:0 PC internal standard.

37. Boron trifluoride affords acid-catalyzed methanolysis, which is necessary to break the amide linkage of a fatty acid to sphingosine in SM. The conditions for this reaction have been optimized in our laboratory.

38. This base-catalyzed methanolysis is according to Kramer et al. (8) and results in the full conversion of triacylglycerols and glycerophospholipids (see Fig. 2b–i) into MEs and DMAs.

39. As implied by Note 5, phosphatidal choline and phosphatidal ethanolamine comigrate with PC and PE, respectively, during 2D TLC.

40. Although the same amount of 15:0 ME is present with each phospholipid, its area will differ from chromatogram to chromatogram because a different fraction of each preparation was injected into the chromatograph.

References


