Chapter 2

Tracking the Glycerophospholipid Distribution of Docosahexaenoic Acid by Shotgun Lipidomics

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Summary

Docosahexaenoic acid (DHA, 22:6 n-3) is an omega-3 fatty acid with a 22 carbon acyl chain containing six *cis* double bonds and is predominantly found in membrane glycerophospholipids. Dietary consumption of DHA has been positively linked with the prevention of numerous pathologies and consequently, it has been the focus of extensive research over the last four decades. Nevertheless, our understanding of its molecular mode of action is not well understood. One likely mechanism is through DHA's influence on cell membranes and the proteins embedded within them. This influence may be altered depending on the glycerophospholipid head group DHA is esterified to and its fatty acid partner, i.e., the specific glycerophospholipid molecule. Accordingly, an understanding of the exact glycerophospholipid distribution of DHA within a tissue is important if we wish to gain further insight into its role in the prevention of disease. In this chapter a rapid, shotgun lipidomic approach for identifying the molecular glycerophospholipid distribution of DHA is described.

Key words: Docosahexaenoic acid, ESI–MS, Phospholipid, Shotgun lipidomics, Lipid

1. Introduction

Docosahexaenoic acid (DHA, 22:6 n-3) is a long-chain polyunsaturated omega-3 fatty acid with a 22 carbon acyl chain that contains six *cis* double bonds **(Fig. 1)**. It is found in most animals, particularly in glycerophospholipids (GPLs) and is abundant in fish. DHA has been the focus of a large amount of research over the last few decades with interest in this essential fatty acid initiated by the famous work of Bang and Dyerberg in the 1970s *(1)*. From this pioneering work, a link between the consumption of omega-3 fatty acids, in particular DHA and eicospentaenoic acid

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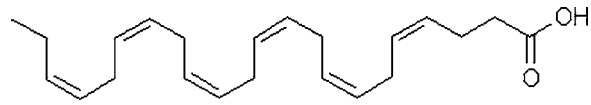

Fig. 1. Structure of dochosahexaenoic acid.

(EPA, 20:5 n-3) and a reduced risk of cardiovascular disease was identified. Since that time research has implicated DHA in the prevention of several prevalent chronic diseases affecting modern society, e.g., cardiovascular disease *(2)*, depression *(3)*, type 2 diabetes *(4, 5)*, obesity *(6)*, and cancer *(7)*. In fact, dietary consumption of this simple lipid has been associated with the prevention of a myriad of pathologies *(8)*.

Although DHA's mechanism/s of action are not well known, its high preference for phospholipids has ensured that its effect on membrane physics is of particular interest. The high number of possible structural conformations of DHA *(9, 10)* leads to a reduction in membrane packing and stability *(11)*. DHA is also known to increase membrane permeability *(12)* and propensity for fusion *(13)*. With such an extensive influence on the physical properties of membranes it is not surprising that phospholipid DHA content has been linked to the activity of numerous membrane proteins, e.g., Na + K + ATPase *(14)*. Nevertheless, studies on protein kinase C suggest that, at least in some cases, DHA's precise molecular glycerophospholipid distribution may be of more importance than its influence on bulk membrane properties *(15, 16)*.

In 2000, Farkas and co workers were able to identify several phosphatidylcholine (GPCho) and phosphatidylethanolamine (GPEtn) molecules containing DHA in vertebrate brains *(17)*. Although successful, the method employed for tracking the molecular GPL distribution of DHA was extremely laborious, requiring numerous chromatographic and derivatization steps *(18)*. More recently, the relatively simple approach of shotgun lipidomics has been applied to the identification of DHA-containing glycerophospholipids in various tissues from mice and naked mole rats *(19)*.

Shotgun lipidomics describes the ability to determine the lipid content, from classes to subclasses and even individual molecules directly from a crude lipid extract by electrospray-ionization mass spectrometry (ESI–MS) without need for prior separation or derivatization *(20–22)*. This technique is highly sensitive, specific, and can produce quantitative data with the addition of appropriate internal standards to the lipid extract *(23)*. In this technique, GPLs are initially separated by their charge polarity, i.e., by selecting either the positive or negative ion mode. These molecules are easily viewed using a simple MS scan, in

which all GPLs carrying a common charge are selected from a lipid extract. The power in ESI–MS is unveiled in its ability to select one molecular ion, and through collision-induced dissociation (CID), observe the compositional fragments of that ion. From these fragments the phospholipid head group as well as the carbon chain length and unsaturation of each fatty acid (FA) can be determined *(24)*. The production of these characteristic fragments forms the basis of the separation methods exploited by the shotgun lipidomics approach.

A triple quadrupole or a quadrupole time of flight mass spectrometer (with enhanced duty cycle) permits precursor ion scanning allowing the analysis of specific phospholipids based on a characteristic fragment ion. An example of such is the glycerol backbone of GPLs at *m/z* 153.0 that identifies phosphtidylserine (GPSer), Phosphatidic acid (GPA), phosphatidylglycerol (GPGro), phosphatidylinositol (GPIns), and cardiolipin in negative ions, or a FA scan to separate out GPLs based on their FA moieties, e.g., DHA *(20, 25)*. Alternatively, triple quadrupole mass spectrometers also provide the ability to perform neutral loss scans to identify a common neutral fragment, such as the loss of didehydroalanine (87 Da) from GPSer in negative ions or phosphoethanolamine (141 Da) from GPEtn in positive ions. Such scans focusing on the head group fragments reduce the incidence of isobaric interference from other GPLs and are used for the quantification of GPLs by comparing to an internal standard, and varying the collision energy depending on the headgroup *(25)*.

In this chapter, a step-by-step description of how to utilize these various mass spectrometric scan techniques to identify the molecular glycerophospholipid distribution of DHA within tissues will be presented.

2. Materials

2.1. Equipment

1. Glass–glass homogenizers.
2. Tube rotator.
3. Vortex mixer.
4. UV 1601 spectrophotometer (Shimadzu Scientific Instruments, Colombia, U.S.A.).
5. Waters QuattroMicro™ triple quadrupole mass spectrometer (Waters, Manchester, U.K.).

2.2. Reagents and Solvents

All solvents must be of minimum HPLC grade.

1. Ammonium acetate.

2. Ammonium molybdate.
3. Butylated hydroxyltoluene (BHT).
4. Chloroform.
5. Hydrochloric acid.
6. Methanol.
7. Phospholipid Standards (all purchased from Avanti Polar Lipids):
   (a) Dinonadecanoylphosphatidylcholine, GPCho (19:0/19:0).
   (b) Diheptadecanoyl phosphatidylserine, GPSer (17:0/17:0).
   (c) Diheptadecanoyl phosphatidylethanolamine, GPEtn (17:0/17:0).
   (d) Diheptadecanoyl phosphatidylglycerol, GPGro (17:0/17:0).
   (e) Diheptadecanoyl phosphatidic acid, GPA (17:0/17:0).
   (f) Heptadecanoyl eicosatetraenoyl phosphatidylinositol, GPIns (17:0/5*Z*,8*Z*,11*Z*,14*Z*-20:4).
8. Potassium dihydrogen phosphate.
9. Perchloric acid.
10. Stannous chloride.

2.3. Supplies

1. Pasteur pipettes.
2. Pyrex screw cap 15 mL test tubes.
3. Plastic cuvettes.

3. Methods

A workflow outlining the procedures required for the identification and relative quantification of glycerophospholipids containing DHA is shown in **Fig. 2**.

3.1. Total Lipid Extraction

Total lipids are extracted from tissues according to traditional methods *(26)* with slight modifications to enhance the compatibility of the extracts with mass spectrometric analysis as described previously *(27)*.

In detail:

1. Weigh tissue and homogenize in 2 mL methanol:chlorofrorm (1:2 v/v) containing 0.01% butylated hydroxytoluene (BHT) using a glass–glass homogenizer.
2. Add internal standard mixture at 4 µL /g tissue (*see* **Note 1**).

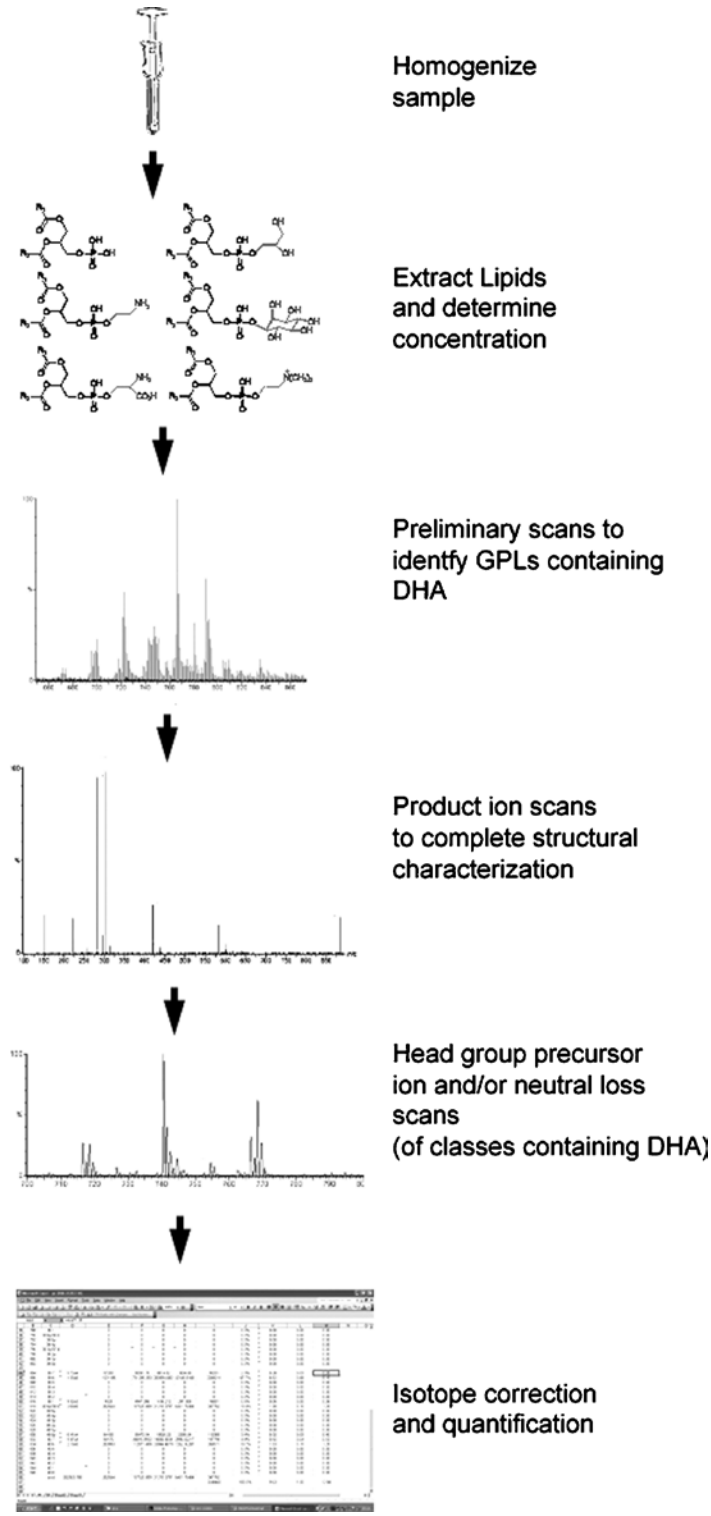

Fig. 2. Workflow for tracking the glycerophospholipid distribution of docosahexaenoic acid.

3. Add further methanol:chlorofrom (1:2 v/v) containing 0.01% BHT to ensure that the total methanol:chlorofrom volume is 20× the tissue weight.
4. Vortex and mix in a test tube rotator for a minimum of 4 h or preferably overnight at 4°C.
5. Add 500 µL 0.15 M ammonium acetate and vortex for at least 15 s.
6. Centrifuge for 5 min at 2,000 × *g*. There should be two phases with chloroform at the bottom containing the lipids. Proteins float in the water/methanol phase or interface.
7. Aspirate a glass pipette with a small amount of chloroform and expel. Insert glass pipette gently to the bottom of the tube and take out the chloroform without removing any of the water/methanol phase or proteins. Expel the chloroform/lipid mixture into a new test tube.
8. Add 2 mL methanol:chloroform (1:2 v/v) to original homogenate tube and vortex for at least 15 s.
9. Centrifuge for 5 min at 2,000 × *g*.
10. Aspirate glass pipette with a small amount of chloroform and expel. Insert glass pipette gently to the bottom of the tube and take out the chloroform without removing any of the water/methanol phase or proteins, combine with first chloroform extract.
11. Add 500 µL of 0.15 M ammonium acetate to the combined chloroform extract and vortex for at least 15 s.
12. Centrifuge for 5 min at 2,000 × *g*.
13. Insert glass pipette gently into the aqueous phase without removing any of the organic phase, and discard this aqueous phase and protein layer as waste.
14. Dry down under nitrogen at 37°C.
15. Resuspend phospholipids in 2 mL methanol:chloroform (2:1 v/v) and vortex.
16. Store in glass vial at –80°C.

3.2. Phosphorus Assay

The total phospholipid concentration of lipid extracts is determined by phosphorous assay *(28)*. The concentration of phosphorous in the lipid extract is quantified by comparison of absorbance values (680 nm) with a standard reference curve (*see* **Note 2**).

1. Take 100 µL aliquots of each lipid extract (in duplicate), dry under nitrogen and resuspend in 0.8 mL of 72% (w/v) perchloric acid.
2. Heat at 190°C for 45 min.
3. Place on ice and add 5 mL of water and 500 µL each of ammonium molybdate (8%, w/v) and stannous chloride (0.005% dilution of 40% (w/v) $SnCl_2$ in HCl).

4. Make up to 10 mL by the addition of water and allow color to develop for 10 min.
5. Measure absorbance at 680 nm.
6. Calculate phospholipid content using the following equation:

$$\text{Phospholipid content} = \frac{\mu\text{g phosphorous} \times 780}{30.97},$$

where 780 is the assumed average mass of phospholipids in grams and 30.97 is the molecular weight of phosphorous in grams.

3.3. Mass Spectrometry

3.3.1. Instrumentation

The following discussion is based on the use of a Waters Quattro-Micro™ triple quadrupole mass spectrometer (Waters, Manchester, U.K.) equipped with a z-spray electrospray ion source and controlled by Micromass Masslynx version 4.0 software.

3.3.2. Identification of Phospholipids Containing DHA

Performing this step will identify all phospholipids containing a DHA moiety, providing a targeted approach for later quantitative analysis.

To identify anionic glycerophospholipids (GPA, GPGro, GPSer, GPIns, and GPEtn; see **Note 3**) containing DHA by precursor ion scanning in negative ion mode:

1. Dilute lipid extracts to a final phospholipid concentration of 40 μM with the addition of methanol:chloroform (2:1 v/v).
2. Set capillary voltage to 3,000 V, source temperature to 80°C, desolvation temperature to 120°C and Cone voltage to 50 V. Nitrogen drying gas is used at a flow rate of 320 L/h.
3. Infuse samples into the electrospray ion source at a flow rate of 10 μL/min using the instrument's on-board syringe pump.
4. Set the argon collision gas at a pressure of 3 mTorr and accelerate the ions at a collision energy offset of 35 eV
5. Set quadrupole 3 (Q3) to *m/z* 327.3 and scan quadrupole 1 (Q1) over a mass range of *m/z* 740–920

For the identification of GPCho containing DHA by neutral loss scanning in positive ion mode:

1. Dilute lipid extracts to a final phospholipid concentration of 40 μM with the addition of methanol:chloroform (2:1 v/v) and add aqueous lithium acetate (to a final concentration of 200 μM).
2. Set capillary voltage to 3,000 V, source temperature 80 °C, desolvation temperature 120°C and Cone voltage to 35 V. Nitrogen drying gas is used at a flow rate of 320 L/h.
3. Infuse samples into the electrospray ion source at a flow rate of 10 μL/min using the instrument's on-board syringe pump.
4. Set the argon collision gas at a pressure of 3 mTorr and accelerate the ions at a collision energy offset of 35 eV

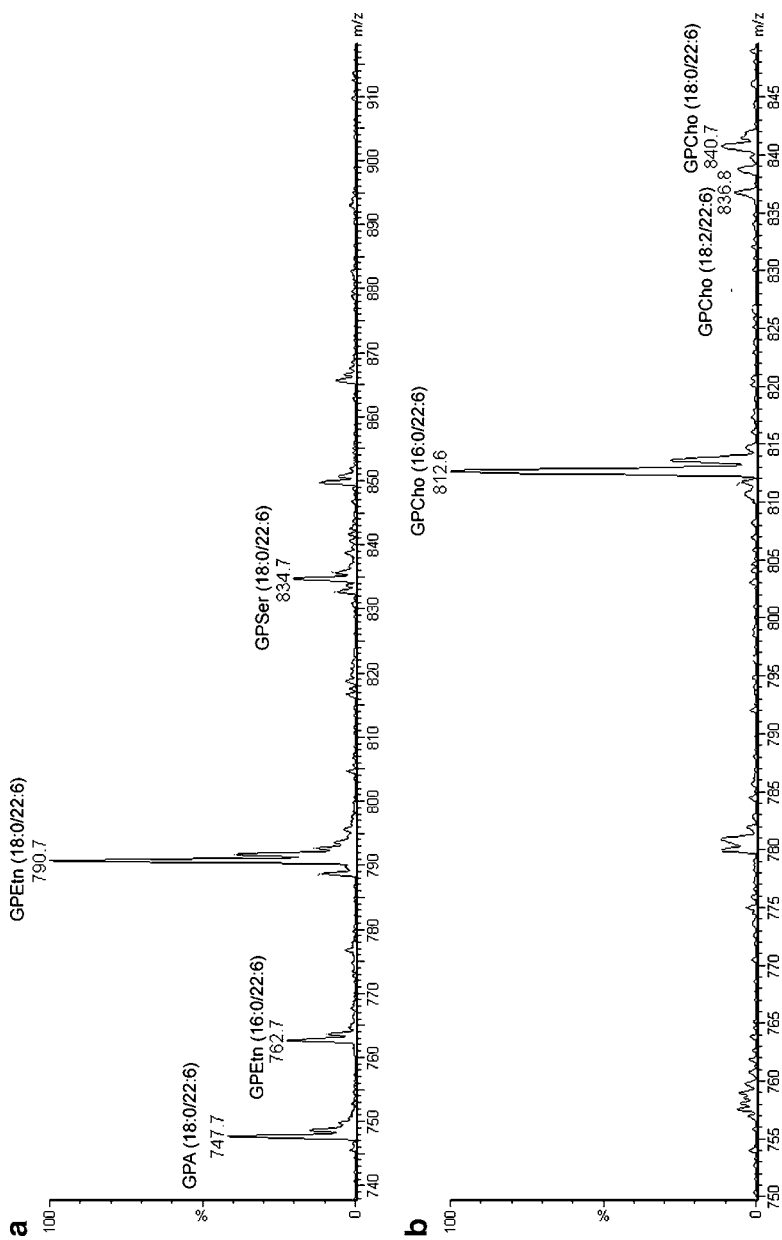

Fig. 3. (**a**) A precursor ion scan for anionic glycerophospholipids containing DHA (*m/z* 327.3) and (**b**) a neutral loss scan for GPCho containing DHA (387.3 Da; GPCho – trimethylamine – DHA) in total lipids extracts from mouse skeletal muscle. *GPA* phosphatidic acid, *GPEtn* phosphatidylglycerol, *GPSer* phosphatidylserine, *GPCho* phosphatidylcholine, *DHA* docosahexaenoic acid.

5. Scan Q1 over a mass range of *m/z* 750–850 and scan Q3 at an *m/z* offset of Q1–387.3. This offset corresponds to the loss of both trimethylamine and DHA (*see* **Note 4**).

3.4. Results

The above scans provide spectra of all anionic phospholipid and GPCho molecules containing DHA as shown in **Fig. 3**a, **b** respectively.

3.4.1. Structural Characterization of DHA Containing Glycerophospholipids

Product ion spectra can now be obtained from each of the DHA-containing phospholipid molecules to complete their structural characterization. These spectra are obtained using the same instrument settings and sample preparations as described for both positive and negative ion analysis above with the exception that:

1. Q1 is set to the *m/z* of the deprotonated or lithiated molecular ion, and
2. Q3 is scanned over an appropriate range to identify all fatty acid moieties present, i.e., *m/z* 200–350 in negative ion mode for deprotonated ions or an *m/z* range that is between 400 and 200 Da less than the lithiated molecular ion in positive ion mode.

If GPEtn or GPCho ethers are identified as containing DHA, their identity can be confirmed by the presence of ions indicative of the loss of the ether-linked acyl chain as an alcohol from lithiated ions as described by Hsu and Turk *(29, 30)*. Alternatively, the identity of ether lipids can be confirmed by ozone-induced dissociation (OzID) *(31)* that is described in detail in Chapter 21.

3.4.2. Quantification of DHA Containing Glycerophospholipids

In order to remove isobaric interference across glycerophospholipid classes, quantification of molecular phospholipids is performed using precursor ion and neutral loss scans of head group-specific fragments *(25)*. It is unlikely that all GPL classes will contain DHA and therefore the specific head group scans required are determined by the previous identification of molecular GPLs containing DHA.

GPA, GPGro, and GPIns specific scans are performed in negative ion mode:

1. Dilute lipid extracts to a final phospholipid concentration of 40 μM with the addition of methanol:chloroform (2:1 v/v).
2. Set capillary voltage to 3,000 V, source temperature 80°C, desolvation temperature 120°C and Cone voltage to 50 V. Nitrogen drying gas is used at a flow rate of 320 L/h.
3. Infuse Samples into the electrospray ion source at a flow rate of 10 μL/min using the instrument's on-board syringe pump.
4. Set the argon collision gas at a pressure of 3 mTorr.
5. Q1 and Q3 settings and collision energies are set as listed in **Table 1**.

   GPCho, GPSer, and GPEtn specific scans are performed in positive ion mode:

Table 1
Mass spectrometer scan parameters used for the relative quantification of GPA, GPGro, and GPIns

GPL class	Scan type	Q1 scan range (*m/z*)	Q2	Collision energy offset (eV)
GPA/GPGro	Precursor ion	650–830	*m/z* 153.0	50
GPIns	Precursor ion	860–920	*m/z* 241.0	45

GPL glycerophospholipid, *GPA* phosphatidic acid, *GPGro* phosphatidylglycerol, *GPIns* phosphatidylinositol, *m/z* mass-to-charge ratio

Table 2
Mass spectrometry scan parameters used for the relative quantification of GPCho, GPEtn, and GPSer

GPL class	Scan type	Q1 scan range (*m/z*)	Q2	Collision energy offset (eV)
GPCho	Precursor ion	780–840	*m/z* 184.1	35
GPEtn	Neutral loss	700–800	Q1–141.5 Da	25
GPSer	Neutral loss	750–840	Q1–185.4 Da	22

GPL glycerophospholipid, *GPCho* phosphatidylcholine, *GPEtn* phosphatidylethanolamine, *GPSer* phosphatidylserine; *m/z*, mass-to-charge ratio

1. Dilute lipid extracts to a final phospholipid concentration of 40 μM with the addition of methanol:chloroform (2:1 v/v). The formation of protonated GPSer and GPEtn ions can be enhanced by the addition aqueous ammonium acetate (to a final concentration of approximately 50 mM) *(32)*.
2. Set capillary voltage to 3,000 V, source temperature 80°C, desolvation temperature 120°C, and Cone voltage to 35 V. Nitrogen drying gas is used at a flow rate of 320 L/h.
3. Infuse samples into the electrospray ion source at a flow rate of 10 μL/min using the instrument's on-board syringe pump.
4. Set the argon collision gas at a pressure of 3 mTorr.
5. Q1 and Q3 settings and collision energies are set as listed in **Table 2**.

Glycerophospholipids are then quantified by comparing their peak areas, obtained from averaging a minimum of 100 scans with the appropriate internal standard for each class after correction

Table 3
Molecular glycerophospholipids containing DHA identified in mouse skeletal muscle using shotgun lipidomics

GPL	Proportion of Total GPL (%)
GPCho (16:0/22:6)	16.5 ± 0.4
GPCho (16:1/22:6)	0.6 ± 0.1
GPCho (18:0/22:6)	2.2 ± 0.2
GPCho (18:1/22:6)	0.9 ± 0.1
GPCho (18:2/22:6)	0.6 ± 0.1
GPEtn (16:0/22:6)	4.6 ± 0.3
GPEtn (18:0/22:6)	8.3 ± 0.5
GPEtn (18:1/22:6)	2.1 ± 0.1
GPEtn (18:2/22:6)	0.8 ± 0.1
GPSer (18:0/22:6)	3.6 ± 1.1
GPA (18:0/22:6)	0.2 ± 0.0
Total DHA-containing GPL	40.5 ± 1.3

GPL glycerophospholipid, *GPCho* phosphatidylcholine, *GPEtn* phosphatidylethanolamine, *GPSer* phosphatidylserine, *GPA* phosphatidic acid. Data are presented as mean ± SE ($n = 4$)

for isotope contributions as described by Deeley et al. *(27)* (*see* **Note 5**).

To achieve this, the isotopic ion distribution of each phospholipid can be calculated from isotope models and the area of the monoisotopic peak multiplied by the calculated correction factor. This calculation must start with the smallest observed phospholipid so that any contribution of its isotope peaks to the area of phospholipids of greater *m/z* can be subtracted before subsequent isotope calculations are performed.

Where two or more isomeric phospholipids are identified the relative abundance of each isomer can be determined from comparison of the combined abundances of the two fatty acid carboxylate ions arising from each lipid with the combined peak area of all carboxylate anions present in the product ion spectrum. Neither the relative position of the acyl chains on the glycerol backbone *(33)* (often called the *sn*-position) nor the position of double bonds *(31, 34, 35)* can be rigorously assigned from these data.

A list of DHA-containing glycerophospholipids detected in mouse skeletal muscle using this technique is shown in **Table 3**.

4. Notes

1. An internal standard mixture in methanol:chloroform (2:1, v/v) should be prepared with the concentration of each internal standard reflecting the concentration of each phospholipid class within the tissue, e.g., for skeletal muscle use GPCho (19:0/19:0), 250 μM; GPEtn (17:0/17:0), 188 μM; GPSer (17:0/17:0), 125 μM; GPA (17:0/17:0), 25 μM; GPGro (17:0/17:0), 25 μM, and GPIns (17:0/20:4), 25 μM.
2. The standard used for the phosphorous assay is potassium dihydrogen phosphate (KH_2PO_4) at 20 μg/mL and the curve constructed using 1, 2, 5, and 10 μg of phosphorous.
3. GPEtn is technically a zwitterionic phospholipid, however it can be easily deprotonated (particularly at elevated pH) to form an anion.
4. Other ions characteristic of the neutral loss of DHA are also produced by the collision-induced dissociation of lithated GPCho; however, the neutral loss described here produces the most abundant of these ions under the described conditions.
5. Isotope corrections are required to account for (a) The greater contribution of isotopic ions to the total abundance of larger phospholipids. (b) The contribution of isotope peaks of one phospholipid to the area of the monoisotopic peak of a larger one.

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