

# Chapter 23

## Basic Techniques for Lipid Extraction from Tissues and Cells

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**Abstract** Lipids as well as proteins, nucleic acids, and carbohydrates are essential components of animals and plants, and their chemical structures and biological functions are highly variable. However, most lipids are insoluble in aqueous solutions, and careful manipulation using organic solvents is required for the extraction and purification of lipids. In this chapter, we describe basic techniques for the extraction and purification of lipids from animal tissues and cells using the Bligh and Dyer method, solvent fractionation, and column chromatography. General methods for the extraction and purification of lipids are mentioned here; refer to other chapters for detailed information on individual lipids.

**Keywords** Lipid extraction • Bligh and Dyer • Solvent fractionation • Ion-exchange chromatography • DEAE cellulose column • Reversed-phase column

### 23.1 Handling of Lipids

Lipids are usually defined as molecules that are soluble in organic solvents such as chloroform, methanol, hexane, and ether. However, some lipids [e.g., lysophospholipids, glycolipids, and phosphoinositides (PIPs)] are readily soluble in aqueous solutions but are only slightly soluble in organic solvents. Lipid compounds should be stored in appropriate organic solvents according to their polarity. For example, polar lipids should be stored in a chloroform:methanol solution with a ratio of 2:1, whereas nonpolar lipids should be stored in pure chloroform. Organic solvents containing lipids should be stored in glass vials, and plastic lids that have Teflon welded into their top are recommended. When it is important to completely avoid contamination, such as in mass spectrometric analyses, lipid solutions should be transferred using glass pipettes, glass syringes, or autopipettors that contain Teflon and have glass surfaces. Organic solvents should always be prepared in high-quality, fresh

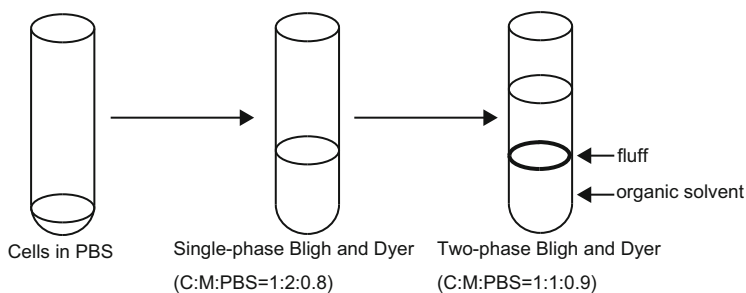
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solvents that have a low water content [high performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC-MS) grade]. In general, lipids are stored in a deep freezer (below  $-20^{\circ}\text{C}$ ) and in glass containers at relatively high concentrations. Lipid samples should be stored in containers that have a blanket of inert gas such as nitrogen or argon in their headspace. In optimal storage conditions, light and oxygen are absent to avoid isomerization and oxidation of the double bonds of lipids. When removed from the freezer, lipid samples should be allowed to reach room temperature before the cap is opened because moisture in the atmosphere may hydrolyze the ester or amide bonds of lipids.

## 23.2 Extraction of Lipids

Animal tissues should be prepared immediately before lipid extraction to prevent hydrolysis and oxidation during the extraction procedures. Some lipids are rapidly produced or degraded after the animal is killed. When animal tissues cannot be processed immediately, they should be frozen in liquid  $\text{N}_2$  as rapidly as possible and stored at  $-80^{\circ}\text{C}$ . Generally, many lipids are tightly bound with proteins *in vivo* via hydrogen bonds, ionic bonds, and hydrophobic forces. Polar solvents such as methanol are used to separate lipids from proteins, whereas nonpolar solvents such as chloroform are used to dissolve lipids. Lipids are usually extracted at low temperatures ( $4^{\circ}\text{C}$ ) as soon as possible after animal tissues are removed. Two structural features of phospholipids include the nonpolar hydrocarbon chains of the fatty acids and the polar phosphate-containing headgroups. The combination of polar and nonpolar groups within the molecule affects its solubility and extraction efficiency in organic solvents. Hence, the use of a single organic solvent is not suitable for the extraction of all lipid species. The mixture of chloroform and methanol in appropriate ratios is used for the efficient extraction of phospholipids. This method was developed by Folch et al. [1] and uses chloroform:methanol in a ratio of 2:1 and large volumes of water to wash out the nonlipid components [1]. Although this extraction procedure is extremely efficient, rapid, and conducted at room temperature (or below), the formation of emulsions is a major drawback. Despite a modification (including salt in the media) made by Folch et al. [2], this procedure has been replaced by the most widely adapted method for lipid extraction in use today. The method of Bligh and Dyer [3] was originally designed to extract lipids from fish muscle [3]. This method is advantageous for tissues containing a high percentage of water. The method is a variation of Folch's extraction and calculates the amount of water present in the sample so that the final composition of chloroform:methanol:water is 1:2:0.8, creating a single extraction phase. The entire procedure is extremely rapid and is the most efficient means to extract lipids. After the addition of equal volumes of water and chloroform, total lipids can be recovered in the chloroform-rich lower phase (Fig. 23.1). We describe an example of lipid extraction using the Bligh and Dyer method. Please also refer to the useful and detailed websites of 'Avanti Polar Lipids' and 'LIPID MAPS,' etc. [4–6].



**Fig. 23.1** Lipid extraction by the Bligh and Dyer method. *C* chloroform, *M* methanol, *PBS* phosphate-buffered saline

### 23.2.1 *Bligh and Dyer Extraction of Total Lipids from Cells*

1. Collect and suspend cells ( $\sim 10^8$  cells) in 0.8 ml phosphate-buffered saline (PBS) and transfer to a glass centrifuge tube with a Teflon cap.
2. Add 3 ml ice-cold chloroform/methanol solution (1:2) and mix to generate a single-phase Bligh and Dyer mixture (final ratio of chloroform:methanol:PBS = 1:2:0.8). Vortex vigorously and incubate for 5 min.
3. Add 1 ml chloroform and 1 ml PBS and mix to generate a two-phase solution (final ratio of chloroform:methanol:PBS = 1:1:0.9).
4. Centrifuge at 1000 *g* for 2 min. The lower phase is composed of chloroform:methanol:water in a ratio of 86:14:1 (by volume) and contains lipids; the upper phase consists of the same solvents in the ratio of 3:48:47 (by volume) and contains the majority of nonlipid contaminants.
5. Recover the lower phase carefully with a Pasteur pipette. Do not remove the white material at the interface, which contains denatured proteins.
6. (Optional) Add 1 ml chloroform, centrifuge, and recover the lower solution.
7. Concentrate the lower solution under a  $N_2$  stream, resuspend the resulting pellets in chloroform:methanol solution (2:1), and store at  $-30^\circ C$ . Generally, about 10 mg dried lipid is recovered from 0.1 g cells or tissues.

### 23.2.2 *Bligh and Dyer Extraction of Total Lipids from Tissues*

The Bligh and Dyer method is also suitable for lipid extraction from animal tissues (brain, liver, intestine, etc.). The volumes of extraction solvents should be adjusted to maintain the chloroform:methanol:water ratio at 1:2:0.8 for a single-phase Bligh and Dyer mixture and at 1:1:0.9 for the formation of a two-phase solution (3, Fig. 23.1). The exact volumes must be determined empirically for each sample type when extracting lipids from tissues. The water volume should be considered the aqueous component of the tissue. Different tissues have different water contents, and the endogenous water content contributes to the aqueous component in the

single-phase mixture. Animal tissues often require homogenization in water or PBS using a Potter-Elvehjem device or a Polytron homogenizer before and after the addition of chloroform and methanol. An example protocol to extract lipids from rat liver using the Bligh and Dyer method is provided next.

1. Add 3 ml water, 20 ml methanol, and 10 ml chloroform (ice-cold) to rat liver (6–7 g) and homogenize on ice using a Polytron homogenizer to generate a single-phase Bligh and Dyer mixture (final ratio of chloroform:methanol:water = 1:2:0.8). Homogenize the mixture thoroughly, vortex vigorously, and incubate for 10–15 min.
2. Pour the solution into a 50-ml glass centrifuge tube with a Teflon cap and centrifuge at 2000 rpm for 5 min. Collect the solution by decantation.
3. Resuspend the resulting pellets in 10 ml chloroform, 20 ml methanol, and 8 ml water. Homogenize again on ice using a Polytron homogenizer.
4. Pour the solution into a 50-ml glass centrifuge tube and centrifuge at 2000 rpm for 5 min. Collect the solution by decantation and combine with the previous solution.
5. Add 20 ml chloroform and 20 ml water to the solution and mix to generate a two-phase solution (final ratio of chloroform:methanol:water = 1:1:0.9).
6. Pour the solution into two 50-ml glass centrifuge tubes and centrifuge at 2000 rpm for 5 min.
7. Recover the lower phase carefully with a Pasteur pipette. Do not remove the white material at the interface, which contains denatured proteins.
8. (Optional) Add 10 ml chloroform to two 50-ml glass centrifuge tubes, centrifuge, and recover the lower phase with a Pasteur pipette.
9. Concentrate the solution with a rotary evaporator or under a N<sub>2</sub> stream, resuspend the resulting pellets in chloroform/methanol solution (2:1), and store at –30° C.

## 23.3 Solvent Fractionation

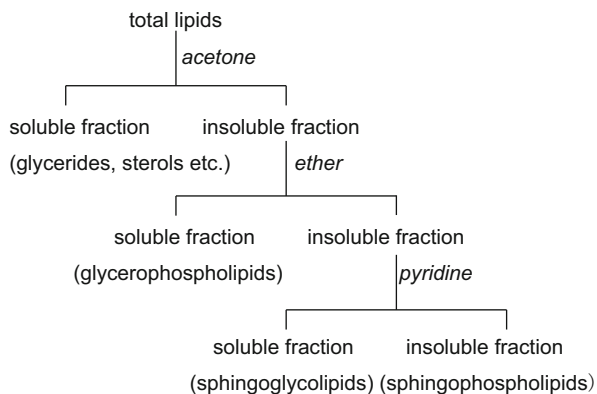
Solvent fractionation is a simple method that is sometimes the most efficient means to crudely separate lipids (Fig. 23.2). This approach depends on the differential solubility of lipids in various organic solvents.

### 23.3.1 Separation of Polar and Nonpolar Lipids

Acetone precipitation is currently used for one-step separation of polar lipids (e.g., phospholipids and glycolipids) from neutral and nonpolar lipids (e.g., triglycerides, cholesterol, and the majority of fatty acids).

1. Evaporate ~100 mg crude lipid extract in a glass centrifuge tube under a N<sub>2</sub> stream and add 20–30 volumes (~5 ml) of acetone.
2. Vortex the glass centrifuge tube for 1 min and leave on ice for 1 h.

**Fig. 23.2** Solvent fractionation of total lipids



3. Centrifuge the tube at 2000 rpm for 5 min and carefully collect the supernatant with a Pasteur pipette.
4. Repeat the procedure (steps 1–3) and combine the acetone extracts.
5. Dry the pellet, which is rich in phospholipids and glycolipids, under a  $N_2$  stream, redissolve in chloroform/methanol mixture (2:1), and store at  $-30^\circ C$  for further analysis.
6. The acetone extract contains glycerides, sterols, sterol esters, carotenoids, lipid-soluble vitamins, and fatty acids. Dry the acetone extract under a  $N_2$  stream, redissolve in chloroform:methanol mixture (2:1), and store at  $-30^\circ C$ .

## 23.4 Purification of Lipids by Chromatography

The separation of lipids by ion-exchange chromatography is based on the different ionic groups present in the molecules. Nonionic, acidic, and zwitterionic lipids are separated on several ion-exchange materials: diethylaminoethyl (DEAE) cellulose, triethylaminoethyl cellulose, or ion-exchange resins. DEAE is most frequently used to separate lipids of different classes. Carboxymethyl cellulose is also used for preparative separation of phospholipid classes.

### 23.4.1 Purification of Acidic Phospholipids by Butanol Extraction Using a DEAE Cellulose Column

1. Equilibrate the DEAE cellulose column (Wako or Sigma) with 1 ml methanol, 1 ml chloroform:methanol solution (1:1), and 1 ml chloroform three times each.
2. Homogenize  $\sim 10^6$  cells or  $\sim 100$  mg tissue in 2 ml 1-butanol.
3. Add 2 ml PBS to the homogenate and vortex for 3 min on ice.
4. Centrifuge at 2000 g for 5 min and recover the supernatant.
5. Add 1 ml methanol and 1 ml chloroform to the extract solution and apply to a pre-equilibrated DEAE cellulose column.

6. Wash the column with 1 ml chloroform:methanol solution (1:1) three times. Neutral lipids, phosphatidylcholine, and phosphatidylethanolamine are eluted.
7. Elute the column with 1 ml chloroform:methanol:28 % ammonia solution:glacial acetic acid solution (200:100:3:0.9) three times. Phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, and cardiolipin are eluted.
8. Elute the column with 1 ml chloroform:methanol:28 % ammonia solution:glacial acetic acid solution (200:100:10:6.7) three times. Lysophosphatidic acid and lysophosphatidylserine are eluted.
9. Elute the column with 1 ml chloroform:methanol/12N HCl:water solution (60:60:5:5) three times. Phosphoinositide (PIP) is eluted.

### ***23.4.2 Purification of Acidic Phospholipids by Methanol Extraction Using a Reversed-Phase Column***

1. Equilibrate the column (Oasis HLB, 3 ml/60 mg; Waters) with 3 ml methanol and 3 ml water containing formic acid (pH 2.0–3.0).
2. Homogenize  $\sim 10^6$  cells or  $\sim 100$  mg tissue in 1 ml methanol and incubate on ice for 1 h.
3. Centrifuge at 2000 g for 5 min and recover the supernatant (extract solution).
4. Add 9 ml water containing formic acid (pH 2.0–3.0) to the extract solution (final concentration of methanol is 10 %) and apply to a pre-equilibrated reversed-phase column.
5. Wash the column with 3 ml water.
6. Elute the column with 3 ml hexane. Neutral lipids are eluted.
7. Elute the column with 3 ml methylformate. Fatty acids and oxidized fatty acids are eluted.
8. Elute the column with 3 ml methanol. Phospholipids and oxidized phospholipids are eluted.

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