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Liquid Extraction: Folch

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Abbreviations

DAG	Diacylglycerol
GC-MS	Gas chromatography/mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
MTBE	Methyl-*tert*-butyl ether
PA	Phosphatidyl acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
TAG	Triacylglycerol

Definition

Folch's extraction procedure is one of the most popular methods for isolating lipids from biological samples. It takes advantage of the biphasic solvent system consisting of chloroform/methanol/water in a volumetric ratio of 8:4:3 (v/v/v).

Background

Folch's method is one of the major contributions to the field of lipid biochemistry by the Catalan biochemist and prominent scientist in the field of neurochemistry Jordi Folch-Pi (1911–1979) (Lees and Pope 2001). Prior to Folch's method there was no effective way to quantitatively isolate lipids from biological tissue samples. The standard method for lipid extraction from tissues until Folch was Bloor's method that applied a number of successive extractions using ethanol, ether, and chloroform and/or petrolether (Bloor 1928). This method was very time consuming and the resulting lipid extracts were not free from non-lipid contaminants. Folch, driven by his interest in neurochemistry, developed his famous extraction method in the course to characterize all structural components in the brain. With the limited methods available to him, he was able to identify the structure of phosphatidylserine (Folch and Schneider 1941; Folch 1941, 1948), isolate mono-, di-, and triphosphoinositides (Folch 1949; Folch and Woolley 1942), characterize gangliosides (Folch et al. 1951a), and describe myelin (Folch and Lees 1951). In 1951 Folch et al. published the first protocol based on the chloroform/methanol solvent system that

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included an excessive washing step with 10 volume equivalents of water (Folch et al. 1951b). In 1957 Folch and coworkers showed that only 0.2 volume equivalents of water are sufficient for washing the lipid-containing organic phase from non-lipid contaminants (Folch et al. 1957). Folch's approach enabled, for the first time, the ability to generate pure lipid extracts from tissue in a direct extraction step that also could be performed at 0 °C to preserve labile lipid molecules from degradation. The final protocol published in 1957 became very popular and was cited approximately 45,000 times by 2014 making it the 9th most cited research paper of all time and a golden standard for lipid extraction methods (Noorden et al. 2014).

The basic idea behind this extraction procedure was to homogenize a tissue sample (Folch and Lees 1951; Sperry and Brand 1955) in a mixture of chloroform and methanol in volumetric ratios of 2:1. The initial extraction step results in non-extractable residue and one liquid phase containing a wide range of small biomolecules. Important for the success of the method is the usage of methanol as polar component in the extraction mixture to improve the solubilization of lipid molecules from cell membranes and cell compartments (Iverson et al. 2001). When water (or buffer) is added to the chloroform/methanol mixture, a new aqueous phase is formed. The final volumetric ratio of chloroform, methanol, and water of Folch's method is 8:4:3 (v/v/v). In a state of equilibrium, the upper phase has the composition 3:48:47 (v/v/v) and the lower phase comprises 86:14:1 (v/v/v) (Folch et al. 1957). Because the upper phase is predominantly composed of water and methanol, hydrophilic components and salts are enriched here, whereas the lower phase, being predominantly chloroform, retains lipids. Afterward, the crude lipid-containing phase is washed with water (or buffer) to minimize the amount of salts, non-lipid and hydrophilic components.

The physicochemical principle of Folch's liquid/liquid extraction is Walther Nernst's distribution law for biphasic systems, which was published in 1891 (Elias et al. 1992). The distribution law states that at equilibrium, the ratio of concentrations of a solubilized compound in a biphasic system is constant at a given temperature (Eq. 1). Thus it follows that the distribution is not affected by the initial concentrations of the component 1 in either of the non-miscible solvents, and if $K \gg 1$, one can selectively enrich a component into phase A. In an ideal physical solution, Nernst's law can be independently applied for n components. In real-life applications, the tendency of lipids to form micelles and aggregates in polar as well as nonpolar solvents limits the direct applicability of Nernst's law. In such case kinetics and thermodynamics to form aggregates from lipid monomers have to be considered, which results in a much more complicated relationship than given in Eq. 1:

$$K(T) = \frac{c_1^A}{c_1^B} = \text{const; when } T = \text{const} \quad (1)$$

K: Nernst's distribution coefficient; at given temperature
c_1^A: Concentration of component 1 in phase A
c_1^B: Concentration of component 1 in phase B

A part of Folch's success (and derived approaches) lies in the phase behavior of the chloroform/methanol/water mixture that has a low aggregate-forming capacity in the initial extraction step and after phase separation. In return, diffusion processes, mostly defined by the speed to reach equilibrium state, are optimal. However, it is obvious that biological samples are inhomogeneous and several interactions within the sample suspension will hinder free diffusion.

From this perspective one has to consider adjusting the homogenization procedure (e.g., mechanical degradation of dried tissue (Hansen et al. 2015), mechanical homogenization of wet tissue in buffer (Ragab et al. 2015), or digestion of tissue (Ruiz-López et al. 2003) to minimize the diffusion distances and enable large interaction surfaces at the smallest possible particle size. In this regard, the specific biological matrix has to be considered in order to effectively adjust homogenisation procedure, volume ratios, incubation time, and number of extraction repetition and/or washings steps.

Procedure

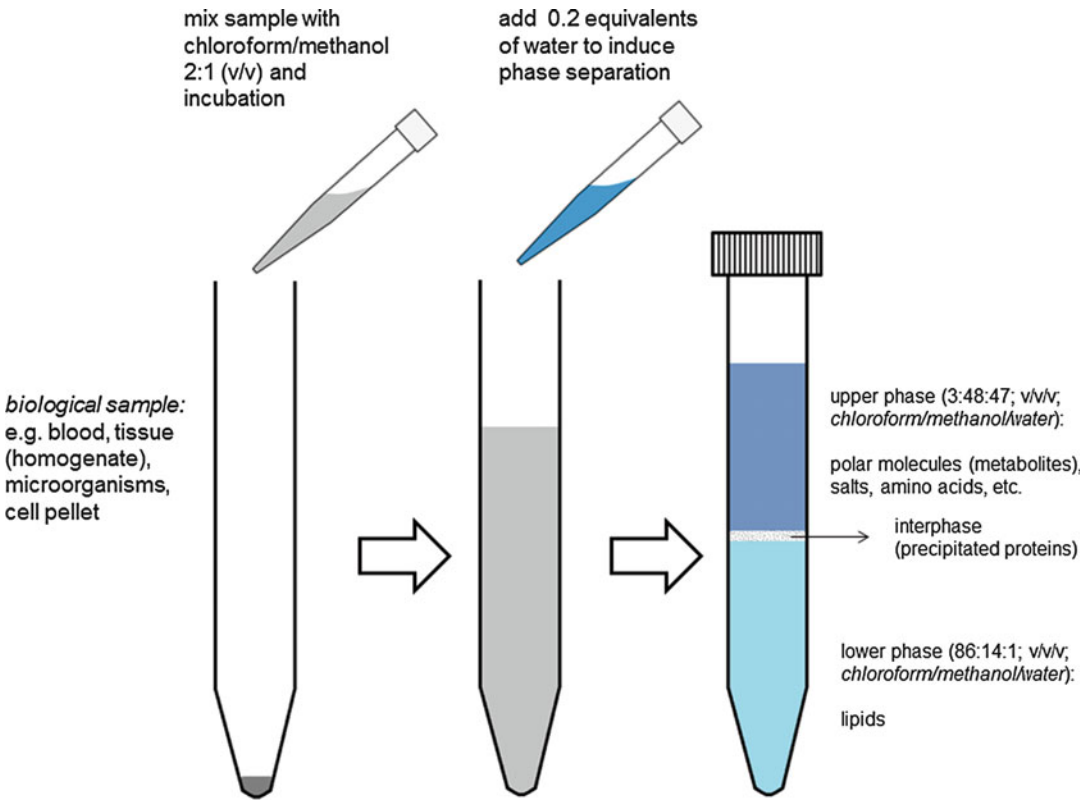
Workflow according to the original protocol (Folch et al. 1957): A biological sample (e.g., tissue, plasma, homogenate, etc.) is diluted 1:20 (v/v) with chloroform/methanol (2:1; v/v). Under the assumption that the biological material has a specific weight of 1 g/mL, one would mix, for example, 100 mg tissue with 1.9 mL chloroform/methanol. After this step the sample is homogenized. The original protocol performs tissue homogenization mechanically in chloroform. Sample homogenization before adding organic solvents should be the preferred way, specifically when working in the microscale and high-throughput setting (Hansen et al. 2015; Ragab et al. 2015; Segura et al. 2015). Mechanical procedures to disrupt tissues and solid biomaterials are hindered when chloroform/methanol mixtures are added to the sample directly. Often sticky, adhesive-like material precipitates, which is hard to shear into small debris optimal for a reproducible extraction. However, homogenization procedures should be performed at low temperature and if possible, within short time periods. In order to preserve the lipid profile of a biological system at the time of sampling, the addition of extraction solvents that denature all proteins and stop lipid enzymatic activities has to be done fast and in a standardized manner.

In Folch's original method, the crude extract is filtered to separate precipitated non-extractable residues. In modern lipidomics workflows, this step is often omitted because of practical aspects in the microscale extraction as well as throughput and to avoid contamination, which interfere with highly sensitive LC-MS instruments. Afterward, 0.2 equivalents (of the total volume of the chloroform/methanol mixture) of water are added to the suspension to induce phase separation. An upper water/methanol phase separates from the lower chloroform phase in a volume ratio 40:60 (v/v) (Fig. 1). The most popular way to support phase separation is centrifugation at 1000 g for 5–10 min (Folch et al. 1957; Matyash et al. 2008; Patterson et al. 2015). The lower (organic) phase has to be carefully collected by penetrating the non-extractable residues at the interphase to minimize the collection of particular material. That is especially important for performing subsequent analysis by LC-MS to avoid clogging capillaries and valves.

Only high-quality chloroform should be used because degradation of chloroform under air yields phosgene and hydrochloric acid, which represents a potential health risk and chemically degrades lipids. Utilizing only chloroform with ethanol stabilizer is highly recommended (Maudens et al. 2007; Turk 1998). A simple quality check is to measure the pH of the water phase above chloroform, which should be neutral or mildly acidic.

Applications

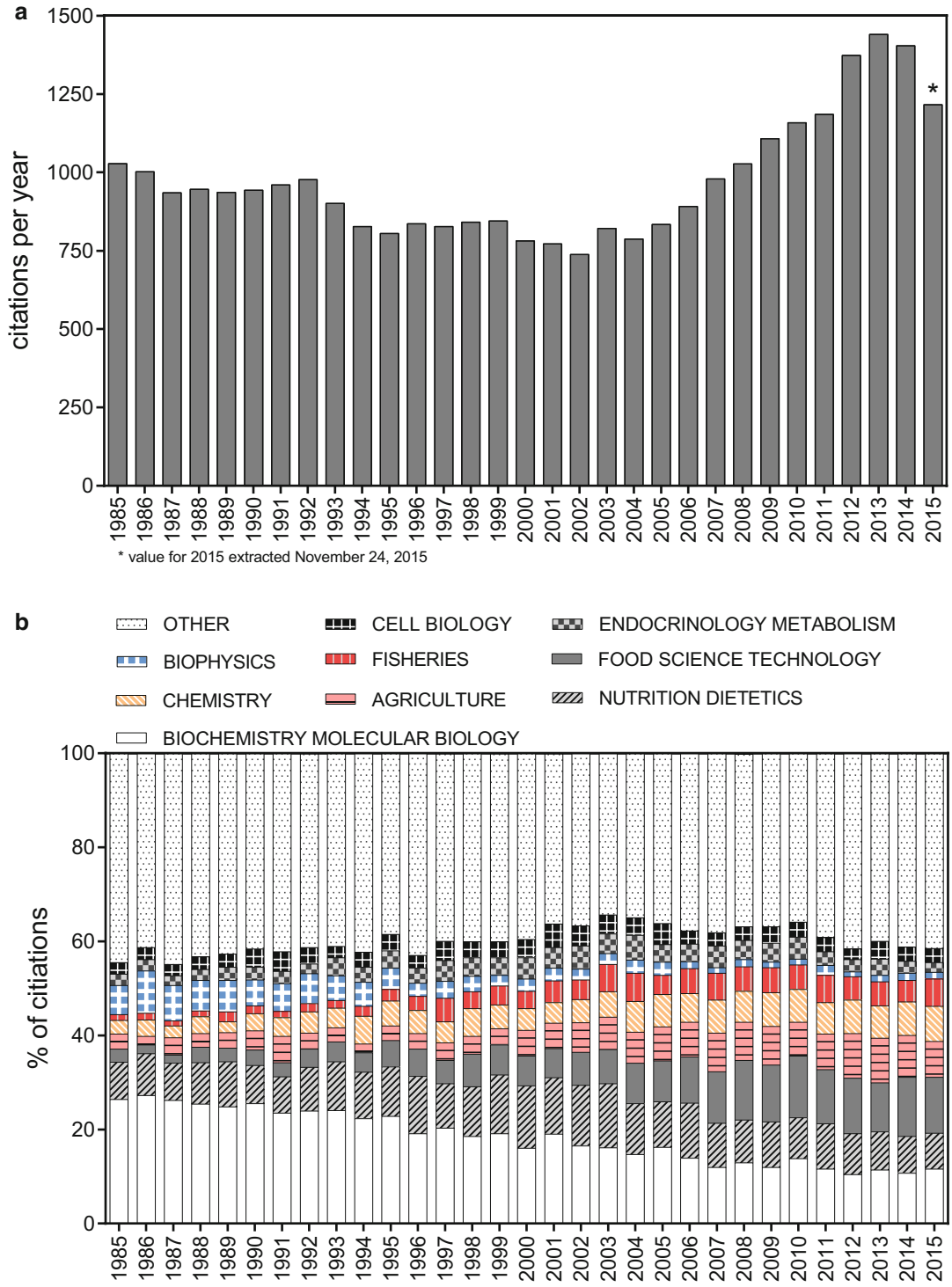
Folch's lipid extraction method is very effective for isolating a broad range of phospholipids and neutral lipids from biological specimen. The



Liquid Extraction: Folch, Fig. 1 Workflow according to Folch's extraction method

recovery for most prominent lipid classes like PC, SM, PE, TAG, and DAG is reported above 90 %. Lipid classes with more polar and partially negative charged headgroups (at physiological pH), like PI, PG, and PA, as well as lyso-lipids, have lower recoveries of around 60–70 % (Matyash et al. 2008; Patterson et al. 2015; Byeon et al. 2012). The most famous derivative method is the Bligh and Dyer procedure (Bligh and Dyer 1959) see Chapter Liquid Extraction: Bligh and Dyer.

Up until now, there are more than 1000 citations per year for the original publication as shown in Fig. 2a. Since 2005, the number of citations per year is increasing, most likely due to the increased interest in lipid analysis following the advent of lipidomics and its application in nutrition and food sciences. Folch's method has reached universal applicability in the life sciences within the last 30 years (Fig. 2b). From the basic sciences through to understanding cell biological processes, biochemistry, and pathways to the application in food sciences, nutrition, and biofuels, it is the most common method used to isolate lipid fractions of high quality and to quantify a broad range of lipid classes.



Liquid Extraction: Folch, Fig. 2 (**a**) Number of citations per year for Folch's method. Data was extracted from Web of Science, Thomson Reuters. (**b**) Distribution of citations per research area according to categorization of Web of Science and year in percent. Data was extracted November 24, 2015

Cross-References

▶ Liquid Extraction: Bligh and Dyer

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