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A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION[1]

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Abstract

Lipid decomposition studies in frozen fish have led to the development of a simple and rapid method for the extraction and purification of lipids from biological materials. The entire procedure can be carried out in approximately 10 minutes; it is efficient, reproducible, and free from deleterious manipulations. The wet tissue is homogenized with a mixture of chloroform and methanol in such proportions that a miscible system is formed with the water in the tissue. Dilution with chloroform and water separates the homogenate into two layers, the chloroform layer containing all the lipids and the methanolic layer containing all the non-lipids. A purified lipid extract is obtained merely by isolating the chloroform layer. The method has been applied to fish muscle and may easily be adapted to use with other tissues.

Introduction

In the course of investigations concerning the deterioration of lipids in frozen fish, the need arose for an efficient and rapid method of total lipid extraction and purification. Furthermore, due to the highly unsaturated nature of fish lipids, the method had to involve only mild treatment so as to minimize oxidative decomposition and the production of artifacts. Several existing methods were considered but none was entirely satisfactory. The methods of Dambergs (1) and Folch *et al.* (2) were too time-consuming for routine investigations and since the former method entailed heating and evaporation it was considered unsuitable for lipid composition studies. The recent method of Folch *et al.* (3), which was published while this study was in progress, was more rapid than their previous method but still had the disadvantage of employing large and inconvenient volumes of solvent. The method used by Dyer and Morton (4) was rapid but extracted only a fraction of the total lipid.

The present paper describes a method whereby the lipids of biological materials can be extracted and purified in a single operation.

Mixtures of chloroform and methanol have had wide use as lipid extractants and examination of the chloroform–methanol–water phase diagram (Fig. 1) led to the following hypothesis. Optimum lipid extraction should result when

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the tissue is homogenized with a mixture of chloroform and methanol which, when mixed with the water in the tissue, would yield a monophasic solution. The resulting homogenate could then be diluted with water and/or chloroform to produce a biphasic system, the chloroform layer of which should contain the lipids and the methanol–water layer the non-lipids. Hence, a purified lipid extract should be obtained when the chloroform layer is isolated.

Procedure

Reagents

Methanol, absolute, analytical reagent; chloroform, analytical reagent.

Lipid Extraction and Purification

The following procedure applies to tissues like cod muscle that contain $80 \pm 1\%$ water and about 1% lipid. Each 100-g sample of the fresh or frozen tissue is homogenized in a Waring Blendor for 2 minutes with a mixture of 100 ml chloroform and 200 ml methanol. To the mixture is then added 100 ml chloroform and after blending for 30 seconds, 100 ml distilled water is added and blending continued for another 30 seconds. The homogenate is filtered through Whatman No. 1 filter paper on a Coors No. 3 Büchner funnel with slight suction. Filtration is normally quite rapid and when the residue becomes dry, pressure is applied with the bottom of a beaker to ensure maximum recovery of solvent. The filtrate is transferred to a 500-ml graduated cylinder, and, after allowing a few minutes for complete separation and clarification, the volume of the chloroform layer (at least 150 ml) is recorded and the alcoholic layer removed by aspiration. A small volume of the chloroform layer is also removed to ensure complete removal of the top layer. The chloroform layer contains the purified lipid.

For quantitative lipid extraction the lipid withheld in the tissue residue is recovered by blending the residue and filter paper with 100 ml chloroform. The mixture is filtered through the original Büchner funnel and the blendor jar and residue are rinsed with a total of 50 ml chloroform. This filtrate is mixed with the original filtrate prior to removal of the alcoholic layer.

Adaptation to Other Materials

The above procedure can be applied directly to any 100-g sample containing 80 g water. Many alterations of the procedure are permissible but it is imperative that the volumes of chloroform, methanol, and water, before and after dilution, be kept in the proportions 1:2:0.8 and 2:2:1.8, respectively. These ratios represent the total volumes present in the ternary systems, including the water present in the sample. Thus, in the extraction of materials that do not contain 80% water, either the size of the samples can be adjusted so that they contain 80 g water, or 100-g samples can be used and the volumes of chloroform and methanol changed to give the correct proportions. In cases where the moisture content is much less than 80% (e.g. fish meal), it is necessary to add distilled water. When material containing a large amount of lipid is used, or where the supply of material is limited, the size of sample can be reduced and the

Determination of Lipid Content

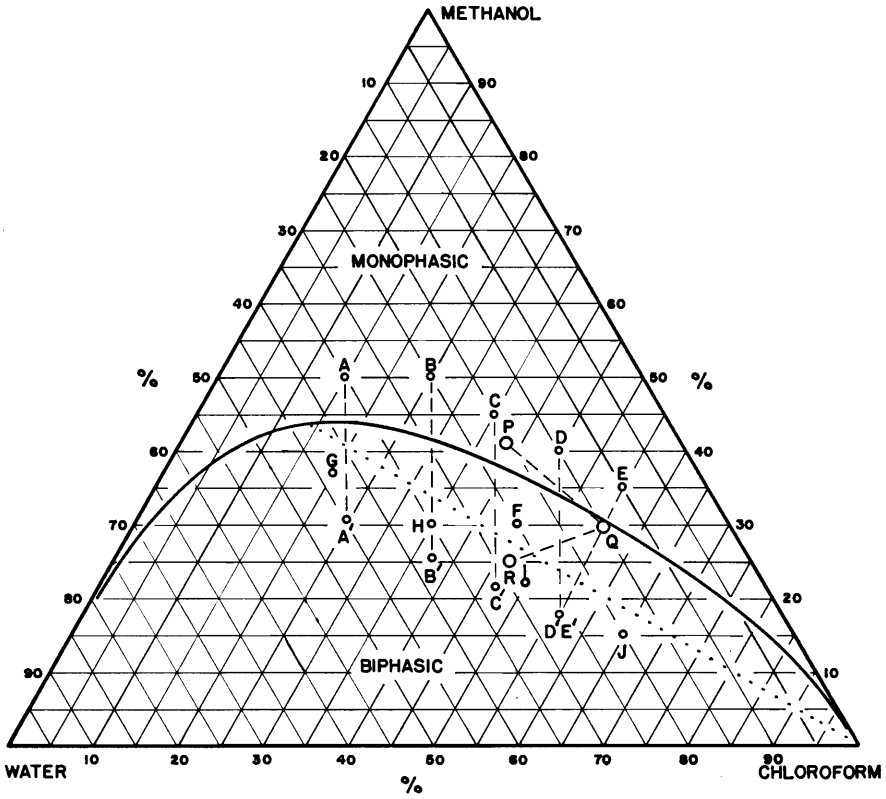
A portion of the lipid extract containing 100–200 mg lipid is evaporated to dryness in a tared flask and the weight of the lipid residue determined. Evaporation, facilitated by a stream of nitrogen, is carried out in a water bath at 40–50° C and the residue is dried over phosphoric anhydride in a vacuum desiccator. After weighing, a small volume of chloroform is added to each flask to detect the presence of non-lipid material (insoluble). If non-lipids are present, the chloroform is carefully decanted and the flask rinsed three times with chloroform. The dry weight of the residue is determined and subtracted from the initial weight. The lipid content of the sample is calculated as follows:

$$\text{Total lipid} = \frac{\text{weight of lipid in aliquot} \times \text{volume of chloroform layer}}{\text{volume of aliquot}}.$$

Experimental

Optimum Conditions for Lipid Extraction

The first objective was to find which of various mixtures of chloroform and methanol would yield quantitative extraction. The solvents were mixed in



such proportions that, with the water of the samples, ternary systems were formed having compositions as marked on the phase diagram (Fig. 1, points A–J). Samples of minced cod muscle (100 g each) were homogenized with the extractants (Table I) and filtered. To the monophasic extracts, sufficient water

TABLE I

Lipid extracted from 100 g cod muscle by ternary mixtures of chloroform–methanol–water

Initial extraction mixture				Dilution solvents			
Ref. pt. in phase diagram (Fig. 1)	Chloroform, ml	Methanol, ml	Water,* ml	Chloroform, ml	Water, ml	Total volume, ml	Lipid in chloroform layer, g
A	23	144	80	40	87	374	0.31
B	54	202	80	104	154	594	0.56
C	94	228	80	166	184	752	0.63
D	162	270	80	285	228	1025	0.64
E	296	355	80	290	325	1346	0.62
F	97	121	80	†	†	298	0.56
G	50	174	160	†	†	384	0.32
H	54	87	80	†	†	221	0.32
I	96	80	80	†	†	256	0.40
J	175	76	80	†	†	331	0.41
P	100	200	80	100	100	580	0.70

*Including 80 ml from tissue.
†No additional solvents required to render system biphasic.

and chloroform were added to render the systems biphasic. The final compositions of these ternary mixtures are given by the points A′, B′, C′, and D′E′ in Fig. 1. The lipid contents of all chloroform layers were determined as above.

The results (Table I) confirmed the initial hypothesis in that more lipid was extracted by mixtures of the monophasic area of the diagram than by those of the biphasic area. Mixtures C, D, and E gave the highest values; however, the volumes of chloroform required for D and E were large and inconvenient. Therefore, the area around point C was considered most favorable.

A tie-line has been drawn in Fig. 1 which for the purposes of this paper has been called "the maximum chloroform tie-line". This tie-line was particularly significant in this study since the lower layers of ternary systems having compositions on or below it are practically 100% chloroform (6). Systems having compositions above this line have chloroform layers contaminated with methanol and water. This explains why the chloroform layer of the system represented by point F in Fig. 1 was the only one found to contain non-lipid.

Point P in the monophasic area of Fig. 1 was chosen as a convenient starting point for further investigation since the composition was equivalent with 100 ml chloroform, 200 ml methanol, and 80 ml water. The composition after adding another 100 ml chloroform is represented by point Q in the biphasic area of the diagram. Point R, which is below the maximum chloroform tie-line, is obtained by subsequent dilution with 100 ml water. This sequence was followed with cod muscle. The tissue (100 g) was homogenized with 100 ml chloroform and 200 ml methanol, filtered, and the residue rinsed with 100 ml chloroform in three portions. The pooled filtrates were mixed with 100 ml distilled water and the mixture was allowed to separate in a 500-ml graduated

chloroform layer contained 0.70 g lipid, the highest yield obtained for this lot of cod muscle (Table I).

Further experimentation showed that the same result was obtained when dilution with chloroform and water preceded filtration. The chloroform had to be added before the water and each addition followed by blending. This resulted in a rapidity of separation of the layers and a yield of lipid which could not be obtained by adding the solvents in a different order.

The extraction of frozen samples did not significantly lower the final temperature of the extract; consequently it was unnecessary to thaw samples prior to extraction.

Substitution of glass fiber filter paper for Whatman No. 1 filter paper showed that with cod flesh there was no significant loss of lipid with the latter due to adsorption. Sintered glass filters also had no advantage over the regular filter except perhaps with materials such as fish meal.

Non-Lipid in Chloroform Layer

It was shown by three methods: (*a*) the determination of chloroform-insoluble material, (*b*) purification according to Folch *et al.* (2), and (*c*) purification according to Shorland *et al.* (7), that the lipid extracted by the above procedure contained no significant amounts of non-lipid material. The amounts of impurities, if present at all, were below the sensitivity limit of each of these methods.

Lipid in Methanol–Water Layer

The methanol–water layer was quantitatively removed and evaporated to less than 50 ml by distillation under reduced pressure at 40–50° C. The concentrate was quantitatively transferred to a separatory funnel and extracted four times with an equal volume of ethyl ether. Any emulsions were broken by centrifugation. The weight of lipid recovered from the pooled extracts was 8 mg. This loss, being only about 1% of the total lipid, was considered insignificant in most applications.

Lipid Remaining in Tissue Residue

Several procedures were employed to recover any lipid remaining in the extracted tissue. The most effective procedure involved re-blending the tissue residue and filter paper with 100 ml chloroform, followed by filtration and rinsing of the blendor jar and residue with a total of 50 ml chloroform. The recovered lipid weighed 40 mg, approximately 6% of the total extracted lipid. Re-extraction of the washed residue with chloroform–methanol–water did not yield any further amount of lipid. Thus, the initial extraction isolated approximately 94% of the extractable lipid.

The washed residue was further treated with 200 ml 6 *N* hydrochloric acid at 100° C for 90 minutes to establish whether there was lipid remaining which was bound in such a way that chloroform–methanol would not remove it. The liberated "lipids" were extracted from the digest according to A.O.A.C. specifications (8). The yield was 47 mg (Table II). At least part of this

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