LIPID COMPOSITION ANALYSIS BY
THIN LAYER CHROMATOGRAPHY

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INTRODUCTION

Fish lipids are generally composed of neutral fat (triglyceride, wax, cholesterol ester, glyceryl ether etc.) and polar lipids (phospholipids and galactolipids). Furthermore some pigments, fat-soluble vitamin and hydrocarbon are dissolved in neutral fat which is contained in hypodermal tissue, dark muscle and fatty tissue around viscera.

Lipid composition varies with fish species, and fish tissues and organs, therefore lipid composition analysis using chromatographic methods has an important role in the study of fish lipid metabolism. Silica gel column chromatography and thin layer chromatography (TLC) are available for the isolation, identification and determination of each lipid component.

TLC is able to analyze a small amount of lipids faster than column chromatography.

TLC was first reported by Izmailov & Shriber, who were scientists of USSR, in 1938. After that E. Stahl who was a pharmacologist in Germany, applied TLC to fields of medicine, pharmacy and food technology and built up the foundation of TLC method.

THIN LAYER PLATE PREPARATION[1]

1. Apply the absorbent, which is usually a mixture of 1 g of silica gel, 0.05 g of calcium sulphate and 2 ml of water, on the glass plates (20 x 20 or 10 x 20 cm) in the form of an aqueous slurry by means of a suitably designed spreader to obtain even layers of 0.25 mm thickness.

2. Air dry and activate the plates by heating in oven at 110 - 120°C for one or two hours.

3. Store in an airtight box or in a desiccator.

SAMPLE PREPARATION

Crude lipids are extracted by modified Folch method or Bligh method (see C2 or C-12 this book). A preparation of 5-10% of this crude lipid in chloroform and methanol mixture (2:1 v/v) is put into a glass test tube with glass stopper [2], flushed with nitrogen gas and stored frozen.

REAGENTS

1. Developing Solvent Systems

   The following two kinds of developing solvent systems are generally used for separation of neutral fat components.

   a)   petroleum ether : diethyl ether : acetic acid

   (90 : 10 : 1, V/V/V)

   b)   n-hexane : diethyl ether : acetic acid

   (80 : 20 : 1, V/V/V)

   The following two developing solvent systems are usually used for separation of phospholipids.

   c)   Chloroform : methanol : water

   (65 : 25 : 4 V/V/V)

   d)   Chloroform : methanol : acetic acid : water

   (25 : 15 : 4 : 2, V/V/V/V)

2. Detecting reagents (spray)

   The following two reagents are common detecting reagents of all lipids:

   a)   50% sulphuric acid (w/v)

   b)   3% copper (II) acetate monohydrate (w/v), $(CH_3 COO)_2 Cu.H_2O$, in 8% phosphoric acid (w/v)

   These detecting reagents break down and carbonize the lipids, so that the lipid spots blacken after heating the plates.

   By means of looking at the feature and thickness of black spots on TLC, we are able to find out the lipid composition and the estimated amount of lipids. Densitometric determination method[*3] is one of the measurement methods for estimating lipid content.

If the lipid components separated on TLC are needed to be determined accurately, mild detecting reagents should be used and the developed spots recovered from the plate. The common mild detecting reagents are:

c)   Iodine vapour : TLC plate is put into an iodine vapour filled tank for 2-3 min, after which the lipid spots turn brown. If an iodine tank is not available, a 2% iodine-ethanol solution can be used instead. TLC spots coloured with iodine vapour are discolored in air.

d)   Ultraviolet irradiation : If the TLC plate is viewed under ultraviolet light, the lipid spots show fluorescence or appear as dark spots. When fluorescence reagents such as 0.2% 2', 7' - dichlorofluorescence ethanol solution or 0.05% rodamine B ethanol solution are sprayed on TLC plates, the lipid spots will appear as splendid fluorescence spots under ultra-violet light. Iodine vapor and the fluorescence materials do not break down the lipids, so the lipid spots can be recovered and the composition or quantity be determined.

Some specific detecting reagents of phospholipids, such as Dittmer-Lester reagent, ninhydrin reagent, Dragendorff reagent et. al., are quite well known. Dittmer-Lester reagent reacts with inorganic phosphorus and becomes blue spots. Ninhydrin reagent reacts with amino compounds, phosphatidyl ethanolamine & phosphatidyl serine, and becomes violet spots. Dragendorff reagent[*4] reacts with choline, phosphatidyl choline, and becomes reddish-brown spots. Dittmer-Lester reagent is suitable for detecting phospholipids.

e)   Molybdenic ammonium - perchloric acid reagent (Dittmer-Lester reagent).

3g of molybdenic ammonium is dissolved in 50 ml of water followed by 5 ml of 6M hydrochloric acid and 13 ml of 70% perchloric acid.

Spray this reagent on TLC and heat the TLC plate at 80°C for 10 min. The spots of phospholipids will develop as blue spots.

APPARATUS

1. Spreader
   Fig. 1 is a kind of spreader called "applicator" devised by Kirchner. It is very popular and convenient.

2. Developing tank
   Normally a box type tank made of glass is used (Fig. 2). The size of the tank is usually 25 x 15 x 25 cm with an air-tight glass cover.

3. Spray
   A spray as shown in Fig. 3 can be used.

4. Oven drier
   Oven drier capable of temperature control up to 200°C.

5. Micro-syringe (50 μl) and spotting capillary tube.

PROCEDURE

1. Apply sample in a solvent as discrete spots 2.0 cm from the bottom of the plate by means of a syringe or a capillary tube.

2. Place plate in developing tank containing developing solvent system.

3. Remove plates when the solvent front reaches 10 cm from the spotted bottom of the plate.

4. Dry in air or vacuum for about 30 min to remove solvent.

5. For lipid composition analysis, spray with detecting reagents.

6. When using 50% sulphuric acid or 3% copper acetate-phosphoric acid as detecting reagent, heat the plate at 150-180°C for 15-20 min after spraying. The plate will turn white and the spots black. (See Fig. 4[*5] & Fig. 5[*6]).

7. Identification of spots is done by means of comparing with the retention time (R_f) of standard samples and analysis of qualitative reactions. Each lipid has its own R_f if the same developing solvent system is used, but the R_f may vary slightly depending on the activity of TLC plates and room temperature.

8. If it is necessary to accurately determine the lipid content, a definite amount of sample has to be spotted using a syringe and then developed with the solvent system. After the spots are detected by mild detecting methods such as iodine vapor or fluorescence under ultra-violet light, the spots are raked out with a small spatula, then the lipids are extracted from the spot powder with chloroform-methanol (2:1) and determined.

9. In keeping a record of TLC condition, the following should be included: date of experiment, room temperature, sample type, absorbent type, thickness of absorbent, developing solvent system, developing time, detecting reagent, and R_f of spots and standards.

REMARKS

*1   Commercially prepared plates are available:
TLC plates silica gel G, F_{254} pre-coated, 10 x 20 cm, layer thickness 0.25 mm.

*2   Gummy stopper cannot be used as a stopper for the sample container, because gummy stopper is soluble in chloroform-methanol which is the solvent of samples.

*3   The estimated lipid content of the spot can be determined by means of measuring the concentration of spot colour and the size of spot using a densitometer.

*4   Dragendorff reagent is prepared as follows:

Basic bismuth nitrate 1.7 g is dissolved in 100 ml of 20% acetic acid solution (A solution). 40g of potassium iodide is dissolved in 100 ml of water (B solution). 20 ml of A solution, 5 ml of B solution and 70 ml of water are mixed prior to spraying.

*5   Fig. 4. shows the developing pattern of whole shark liver oil on TLC using n-hexane - diethyl ether - acetic acid developing solvent systems.

*6   Fig. 5. shows the developing pattern of shark liver phospholipids on TLC using chloroform-methanol - water developing solvent systems.

REFERENCES

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Chemical society of Japan (1978) : New handbook of chemical experiments (Biochemistry I), Maruzen.

T. Saito et.al. (1974) : Biochemistry of fisheries & food technology experimental handbook, Koseisha-Koseikaku.
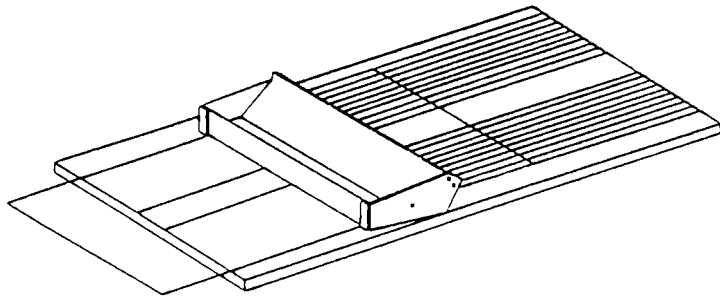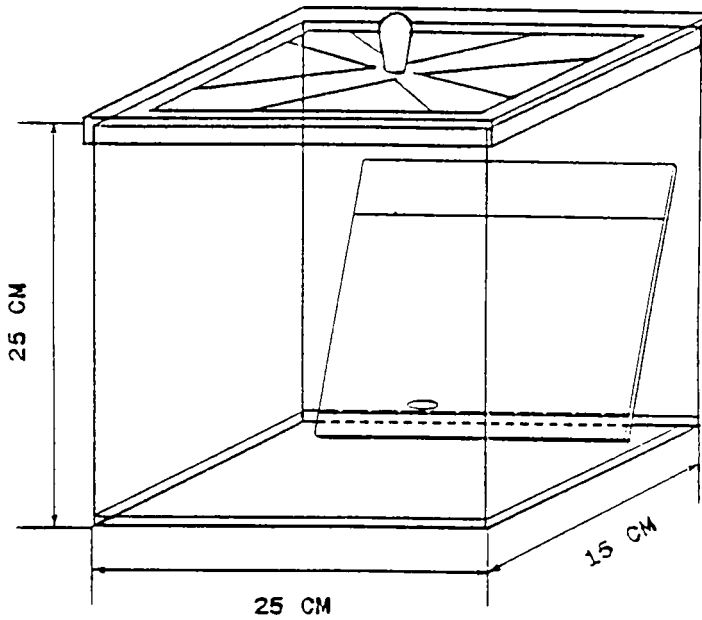
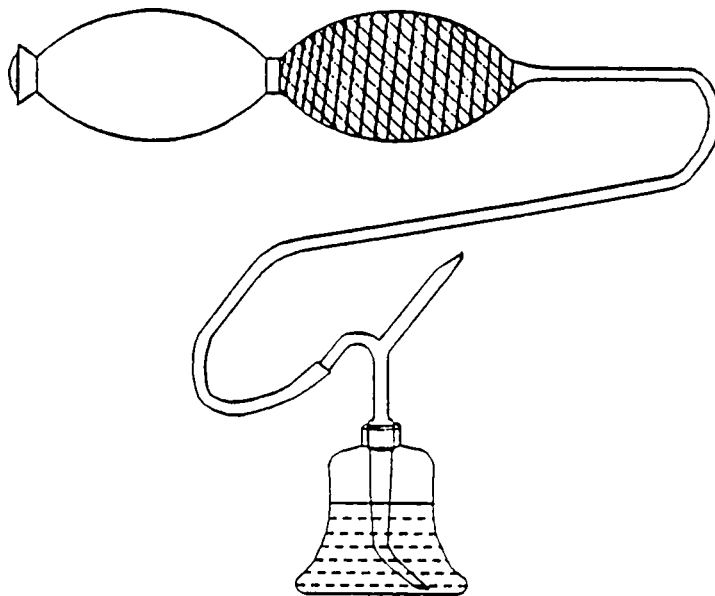Fig. 1. Kirchner's applicator

Fig. 2. Developing tank made of glass
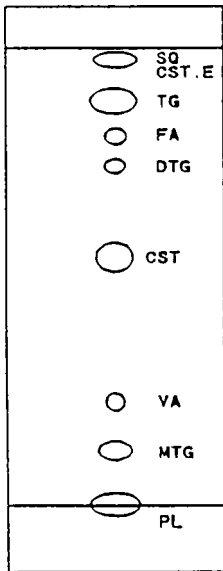

Fig. 3. Spray for detecting reagents

Fig. 4. TLC of whole shark liver oil
(developing solvent system:
n-hexane : diethyl ether :
acetic acid, 80 : 20 : 1)

SQ   : squalene

CST.E : cholesterol ester

TG   : triglyceride (triacylglyceride)

FA   : fatty acid

DTG   : diglyceride

CST   : cholesterol (free)

VA   : vitamin A
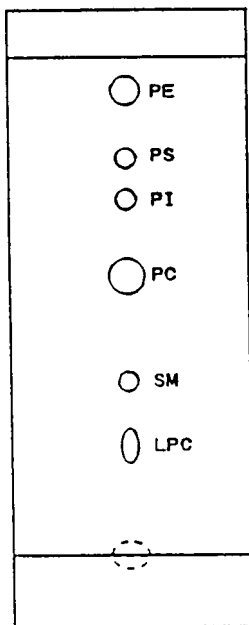
MTG   : monoglyceride

PL   : phospholipid

Fig. 5. TLC of phospholipid of shark
liver oil (developing solvent
system, chloroform:methyl
alcohol: water, 65 : 25 : 4)

PE : phosphatidyl ethanolamine

PS : phosphatidyl serine

PI : phosphatidyl inositol

PC : phosphatidyl choline

SM : sphingomyelin

LPC : Lyso-phosphatidyl choline