Comparative studies on analyses of total human epidermal lipids by HPTLC with and without prior separation of lipid classes on LC-NH2 columns

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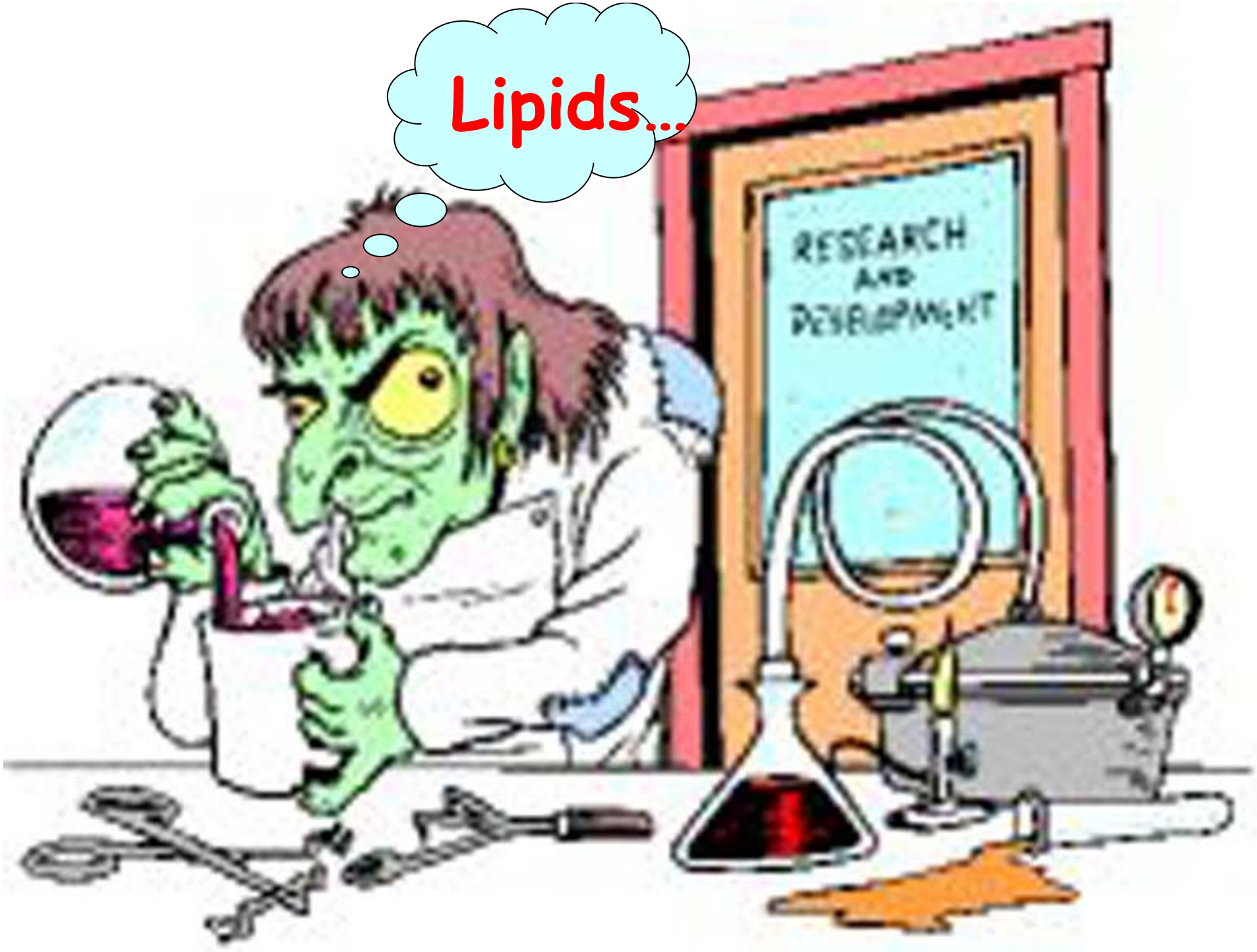

Lipids...
RESEARCH
AND
DEVELOPMENT

Basic methods of extraction with chloroform-methanol followed by partition with water to remove hydrophilic contaminants

- Folch, J., Lees, M. and Sloane-Stanley, G.H.S. A simple method for the isolation and purification of total lipides from animal tissues.

Summary: 1 g tissue homogenized in 20 ml of chloroform- methanol 2:1 for a 10 min. before filtration, then addition to the filtrate of 0.2 volume of salts solution and thorough shaking. The mixture is centrifuged to yield a biphasic system with lipids in the organic phase.

J. Biol. Chem. 1957, **226** : 497-509.

- Bligh, E.G. and Dyer, W.J. A rapid method of total lipid extraction and purification.

Summary: 100 g tissues homogenized in 100 ml chloroform+ 200 ml methanol for 2 min., then addition of 100 ml chloroform +100 ml water for 1 min. before filtration. This yields a biphasic system with lipids in the chloroform layer.

Can. J. Biochem. Physiol. 1959, **37**: 911-917.

The first method is more suitable for extraction of lipids from fat-rich tissues.

The second one can be applied to tissues containing less than 1% lipids.

- **Additional problems of purification occurred with the discovery in the 60's of hydrophilic glycolipids that are found in the aqueous layer of the biphasic system.**

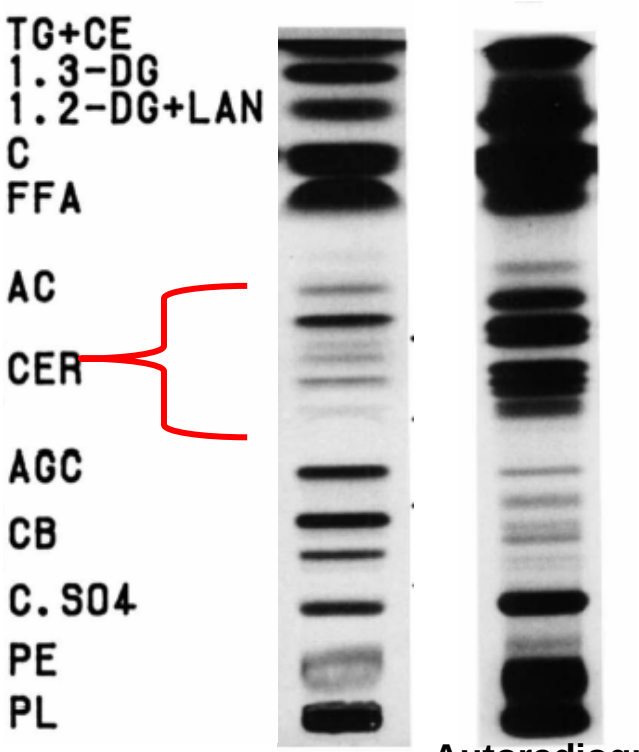
- **The first investigators used dialysis to remove salts and sugars from the aqueous layer followed by lyophilization and chromatographic analysis of the lipid residue.**

- **Later, reverse-phase chromatography on C18-bonded silica gel was used to recover the lipids from aqueous solutions.**

(Williams MA and McCluer RH, J.Neurochem.1980,35:266-269.)

Thin-layer analysis of total lipids versus isolated lipid fractions

Total lipids



Autoradiogram

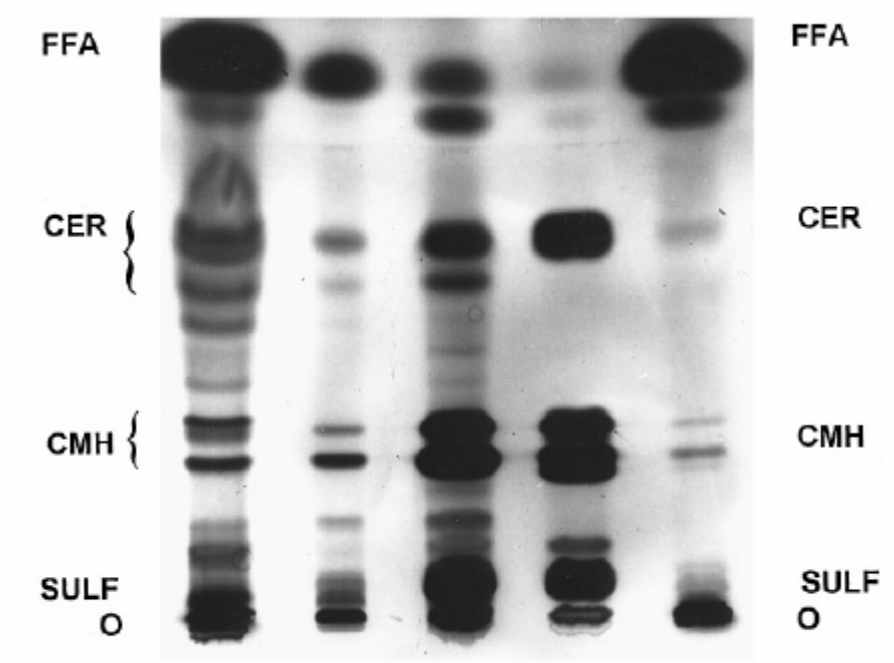
TLC (10 cm height) of total keratinocyte lipids

Sequential migration in :

- chloroform-acetone-methanol 76:4:20 (1 cm)
- chloroform-acetone-methanol 80:10:10 (7,5 cm)
- chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).

Ponec M. et al. J. Lipid Res. 1988, 29:949-961

First approach to lipid fractionation



HPTLC plate of NSL isolated from cultured rat RMC(lane 1), pig retina (lane 2) and rat brain (lane 3). About 3µg lipid P was spotted by lane. Standards were run on lane 4 (CER, CMH and SULF) and 5 (FFA and SM (at the origin)).

Sequetial migration in:

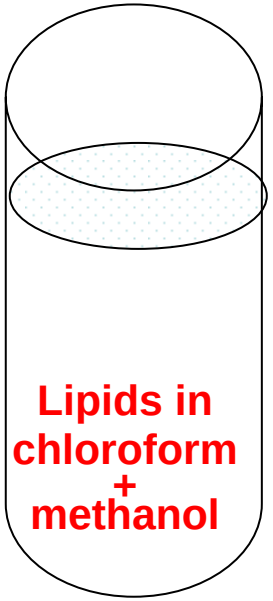
-chloroform-methanol-water 95:20:2 (V/V/V)

-hexane- diethylether-acetic acid 80:20:2 v/v/v

FFA, CER and CMH were well separated.

Dreyfus H. et al. Anal. Biochem.1997, 249:67-78

Global procedure of lipid purification



+ PBS

aqueous phase contains hydrophilic glycolipids (gangliosides)

recovery on copolymer styrene -divinylbenzene columns

Popa et al., J. Lipid Res. 2002, 43:1335-1340

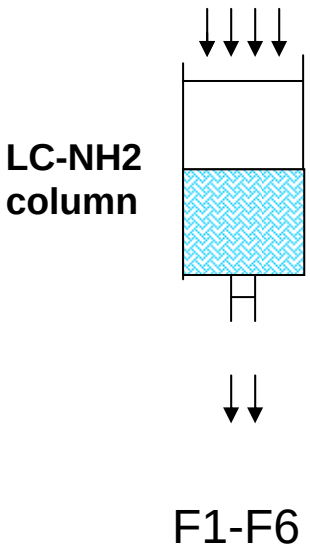
organic phase contains other lipids

separation on LC-NH2 aminopropyl-bonded silica gel columns

Bodennec et al., J. Lipid Res. 2000, 41:1524-1531

PBS: phosphate buffered saline pH 7.4

Sequential elution of lipids into 6 fractions from LC-NH2 columns (Supelco*)



F1 diethylether

F2 chloroform-methanol 23:1 v/v

F3 diisopropylether-acetic acid 98:4 v/v

F4 acetone-methanol 9:1.2 v/v

F5 chloroform-methanol 2:1 v/v

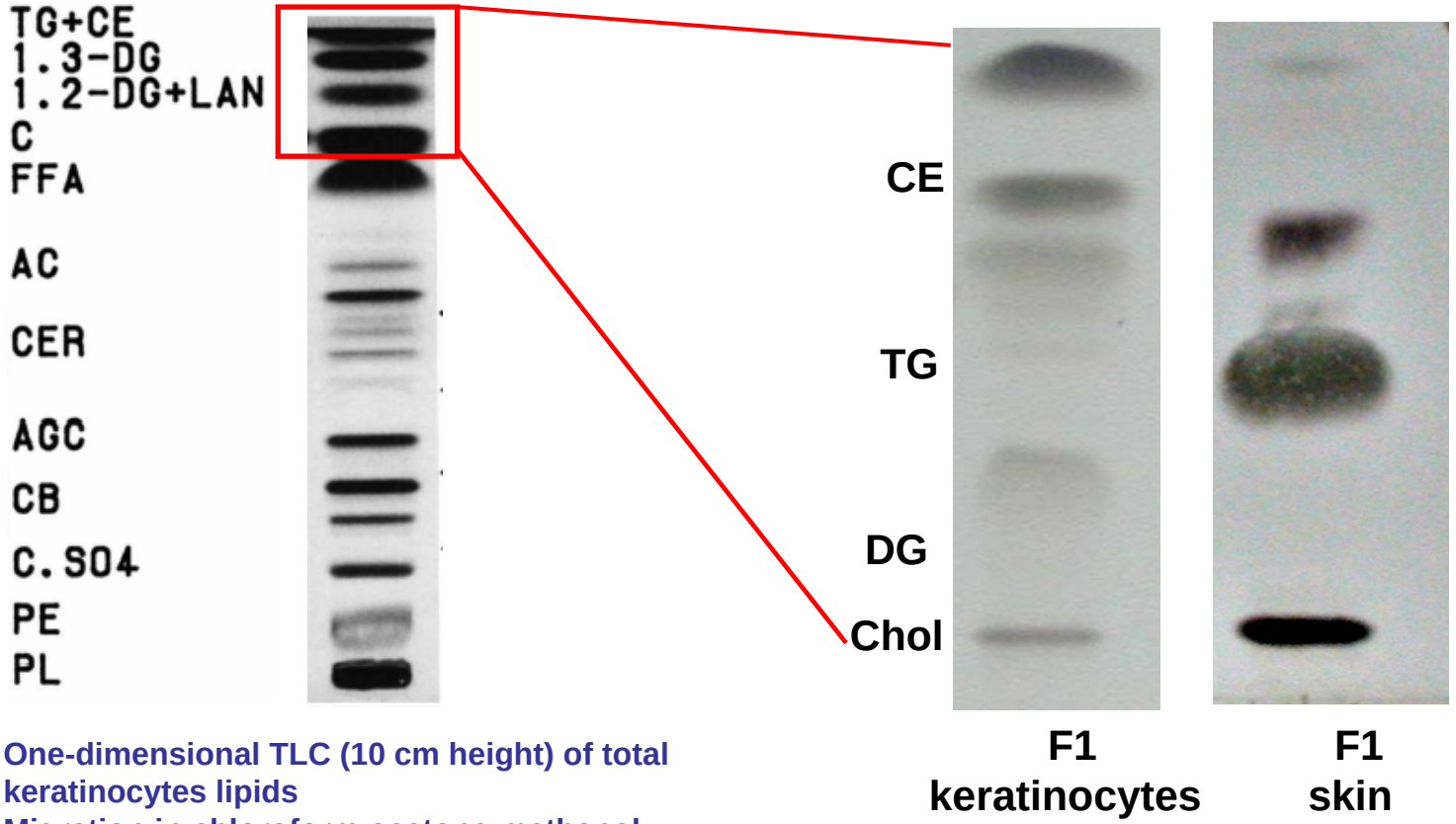
F6 potassium acetate 0.2 M in methanol

**These solvents do not give satisfactory results using LC-NH2 columns from other suppliers.*

HPTLC analysis of the lipid fractions separated on LC-NH2 columns

Fractions eluted from LC-NH2 columns	Solvent system for TLC development	Spray reagents for visualization of the TLC
F1 Free cholesterol, diglycerides, triglycerides, cholesterol esters	Hexane/diethylether/acetic acid 70:30:1 (v/v/v)	Cu acetate 3 % in H3PO4 8% reagent
F2 Free ceramides and monoglycerides	Chloroform/methanol 50:4 (v/v)	Cu acetate 3 % in H3PO4 8% reagent
F3 Free fatty acids Free hydroxy fatty acids	Hexane/ diethyl ether/acetic acid 70:30:1 (v/v/v)	Cu acetate 3 % in H3PO4 8% reagent
F4 Neutral glycolipids Free sphingoid bases	Chloroform/methanol/water 65:25:4 (v/v/v)	Orcinol –H2SO4 reagent Ninhydrin reagent
F5 Neutral phospholipids	Chloroform/methanol/water 65:25:4 (v/v/v)	Dittmer and Lester reagent
F6 Acidic phospholipids gangliosides, sulfatides	Chloroform/methanol/water 65:25:4 (v/v/v)	Dittmer and Lester reagent Orcinol-H2SO4 reagent

Comparative migration of fraction 1 versus total lipids

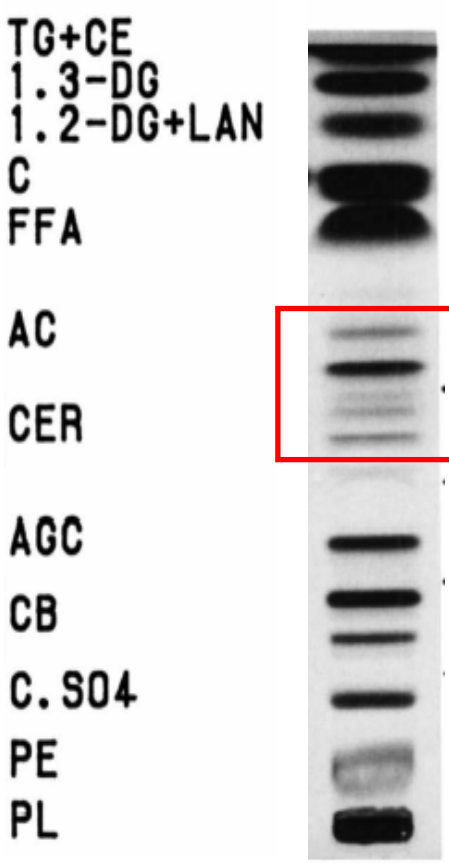


One-dimensional TLC (10 cm height) of total keratinocytes lipids
Migration in chloroform-acetone-methanol 76:4:20 (1cm);
chloroform-acetone-methanol 80:10:10 (7,5 cm);
Chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).

Ponec et al. J. Lipid Res. 1988

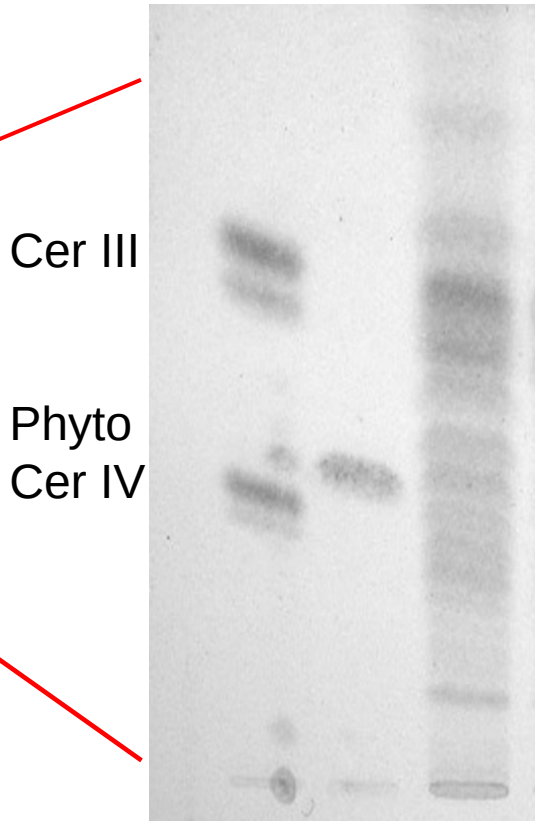
Cholestérol (Ch),diglycerides (DG), triglycérides(TG), cholestéryl-esters (CE)
Migration in Hexane / diethyl ether / acetic acid 70:30:1 (by volume)
Visualisation with copper acetate 3 % in H_3PO_4 8%

Comparative migration of fraction 2 versus total lipids



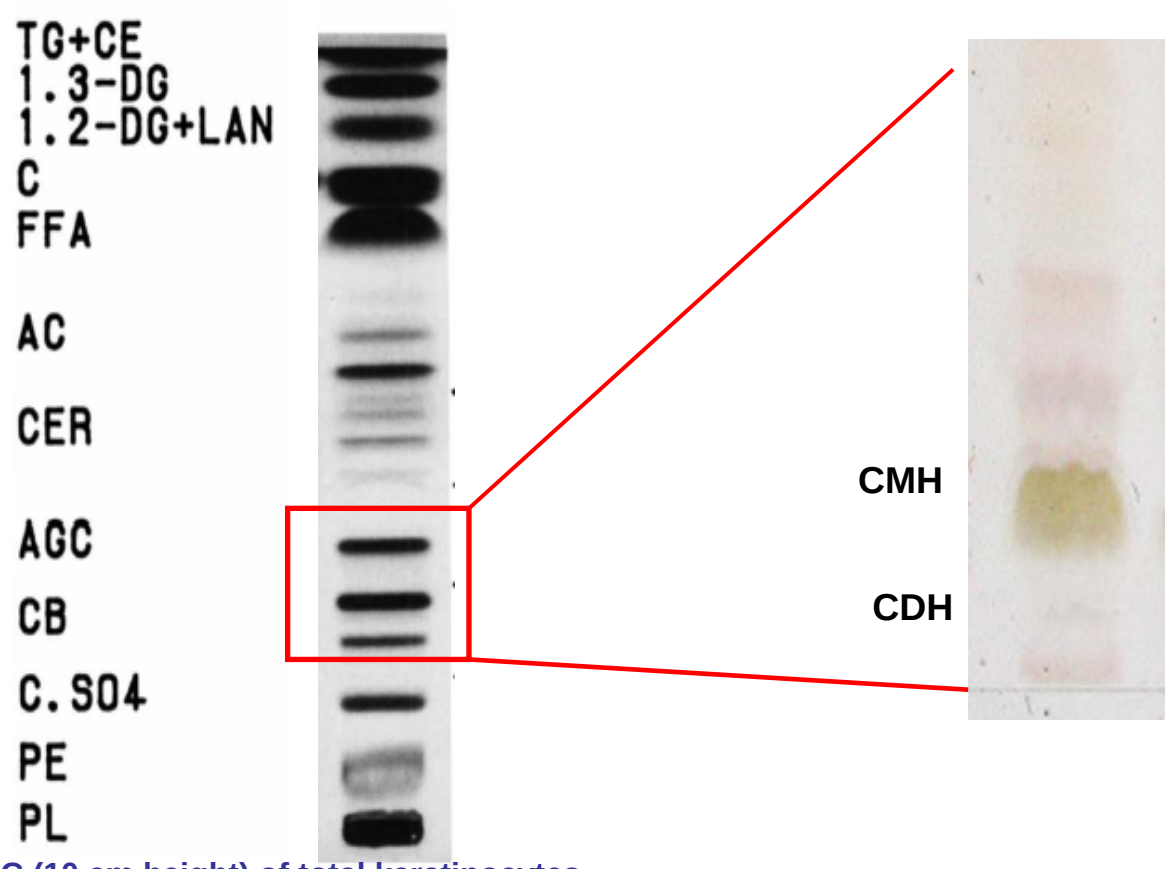
One-dimensional TLC (10 cm height) of total keratinocytes lipids
Migration in chloroform-acetone-methanol 76:4:20 (1cm); chloroform-acetone-methanol 80:10:10 (7,5 cm); Chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).

Ponec et al. J. Lipid Res. 1988



One-dimensional TLC of skin ceramides fraction
Migration in chloroform-methanol 50:3
Visualisation with Cu acetate 3 % in H3PO4 8%, at 120°C

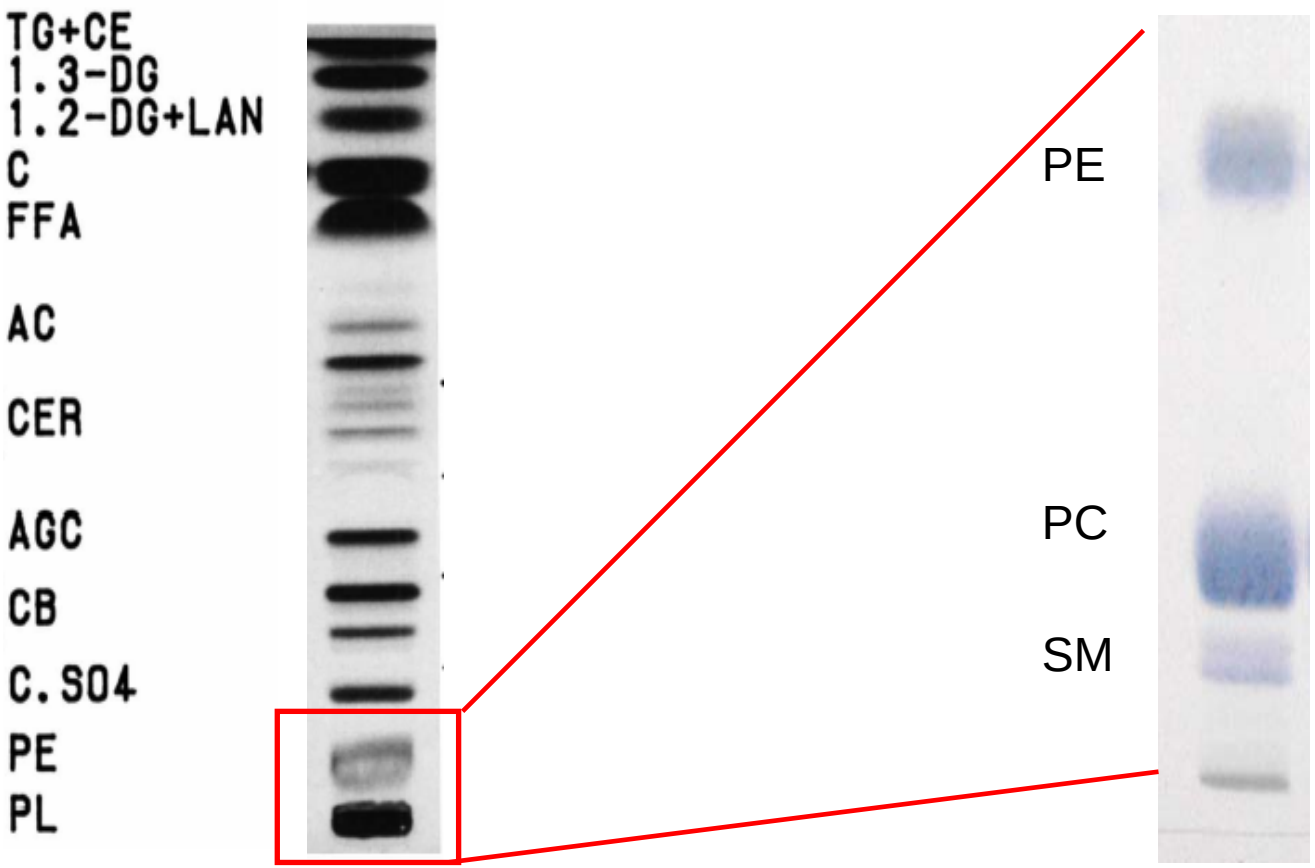
Comparative migration of fraction 4 versus total lipids



One-dimensional TLC (10 cm height) of total keratinocytes lipids
Migration in chloroform-acetone-methanol 76:4:20 (1cm); chloroform-acetone-methanol 80:10:10 (7,5 cm); Chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).
Ponec et al. J. Lipid Res. 1988

One-dimensional TLC n of skin neutral glycolipids
Migration in chloroform—methanol-water 65:25:4 (V/V/V)
Visualised in orcinol-H2SO4 reagent

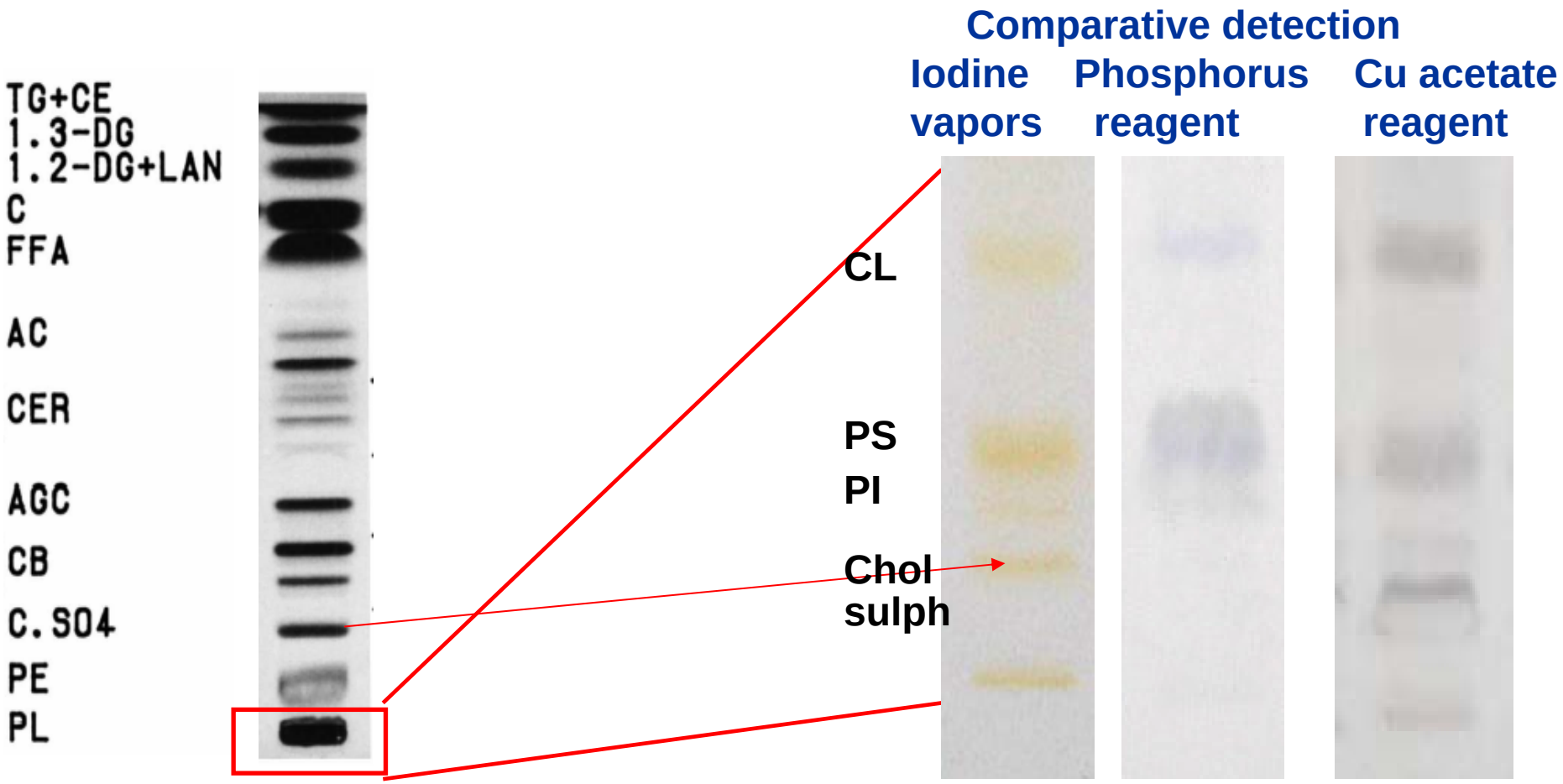
Comparative migration of fraction 5 versus total lipids



One-dimensional TLC (10 cm height) of total keratinocytes lipids
Migration in chloroform-acetone-methanol 76:4:20 (1cm); chloroform-acetone-methanol 80:10:10 (7,5 cm); Chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).
Ponec et al. J. Lipid Res. 1988

One-dimensional TLC of F5- Neutral Phospholipids PC, PE and SM
Migration in chloroform-acetone-methanol 65:25:4 (v/v/v)
Visualised with Dittmer and Lester reagent

Comparative migration of fraction 6 versus total lipids

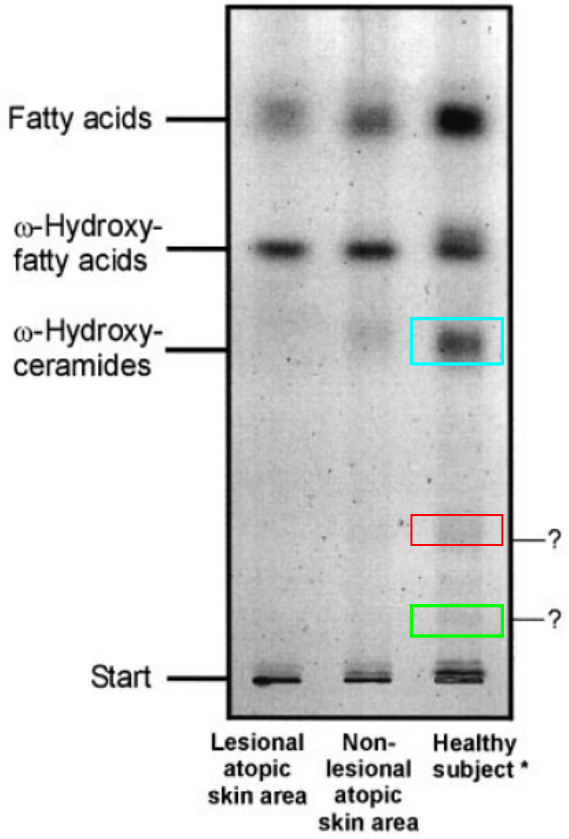


One-dimensional TLC (10 cm height) of total keratinocytes lipids
Migration in chloroform-acetone-methanol 76:4:20 (1cm);
chloroform-acetone-methanol 80:10:10 (7,5 cm);
Chloroform-diethyl ether-ethyl acetate-methanol 76:6:20:2 (9 cm).

Ponec et al. J. Lipid Res. 1988

F6 acidic phospholipids PI, PS and cardiolipin (CL), sulphatides (sulph).
Migrated in chloroform/methanol/water 65:25:4 (v/v/v)

HPTLC of total protein-bound lipids from human atopic skin



Macheleidt et al. J. Invest. Dermatol. 2002

Example of using HPTLC in identifying specific markers for atopic dermatitis in human skin

HPTLC Immunostaining

Migrate samples on 2 HPTLC plates ⟶ Plate for visualisation with orcinol / H_2SO_4 reagent.

↓

Plate Dip in 0.4% PIBM (polyisobutyl methacrylate) in hexane for 30 sec.

↓

Blocking with 1% BSA/PBS for 60 min at RT.

↓

Incubate the plate with 1st Ab or with the serum diluted in PBS at RT for 60 min.

↓

Wash the plate with 0.1%PBS 3min×5times.

↓

Incubate the plate with biotinylated second antibody diluted in PBS at RT for 60 min

↓

Wash the plate with PBS several times.

↓

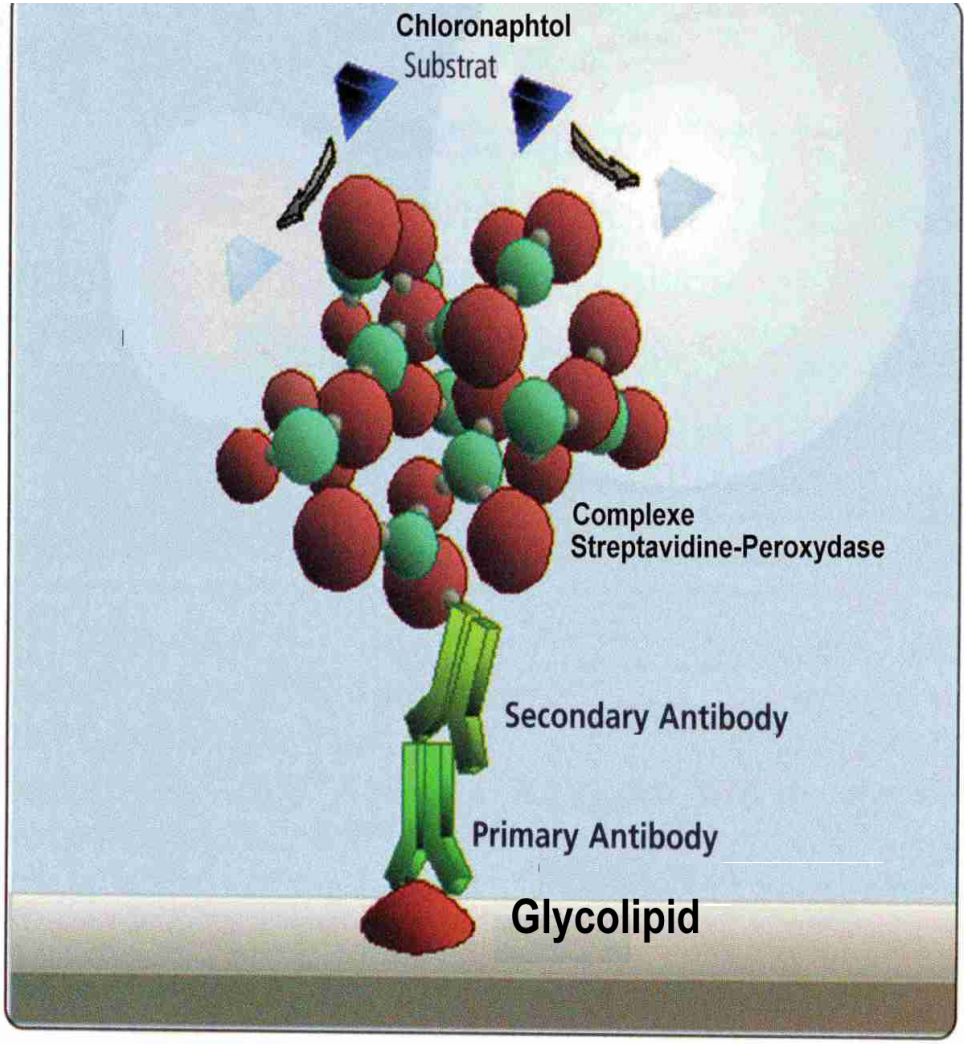
Incubate the plate with the complex streptavidin-peroxidase at RT for 60 min

↓

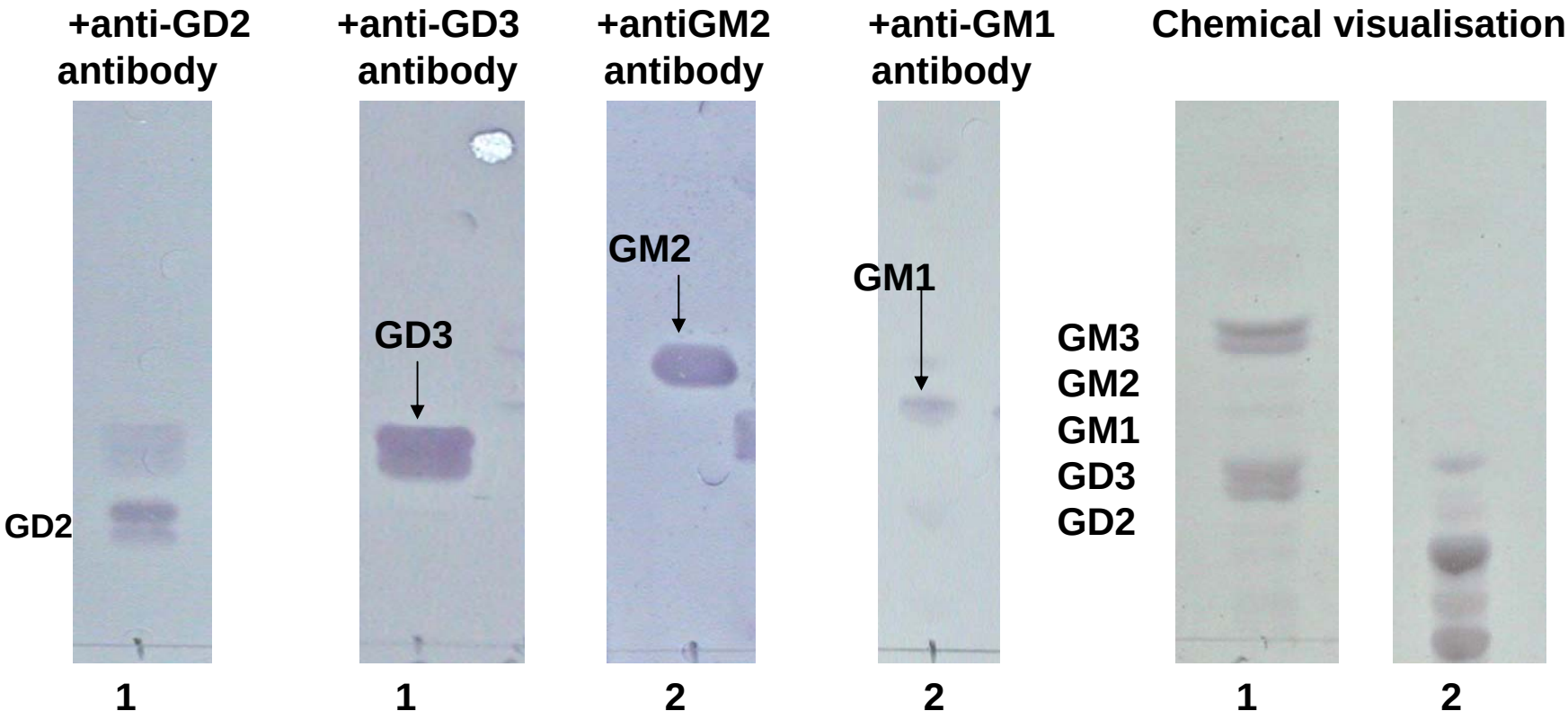
Wash the plate with PBS several times.

↓

Incubate with 4-Chloronaphtol solution to visualise the bound peroxidase in blue



Examples of Immuno-HPTLC



1-melanoma gangliosides
2-brain gangliosides

- Epitopes reactive with antibodies may be shared by several antibody-reactive molecules

Methods of detection of a minor component with a specific antibody in a total ganglioside sample

ELISA

Detection of a molecule after adsorption to a plastic well

Advantage

- many samples can be tested simultaneously

Problems

- a component in low amount is difficult to detect in a mixture
- if the reaction with the antibody is positive, the detected component is not visualized

Immuno-HPTLC

Detection of a molecule after migration of the sample on the HPTLC plate

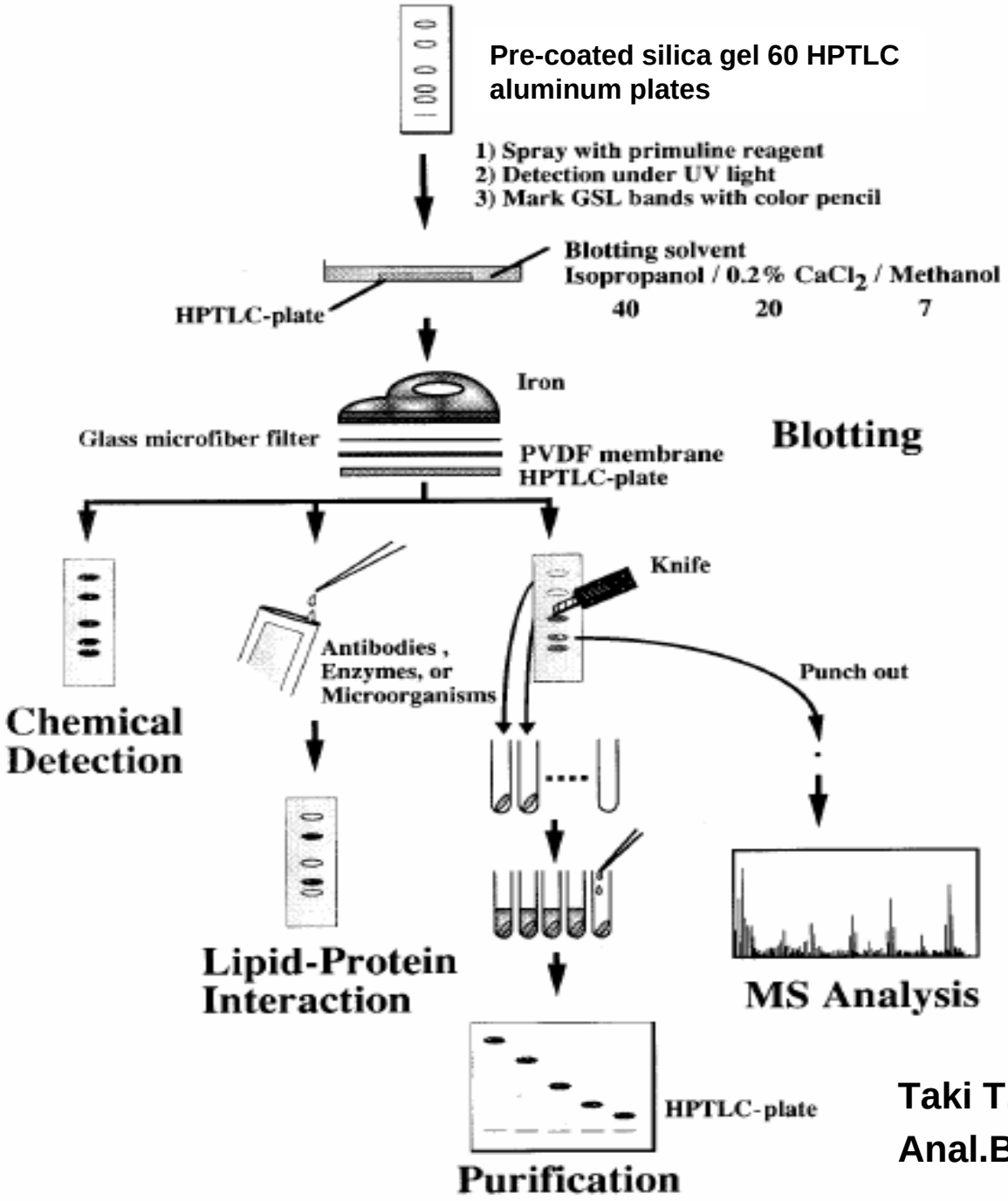
Advantages

- most components are separated by migration
- the antibody-positive component is visualized

Problem

- the amount of antibody to be used is much higher than with ELISA

Procedure of thermal blotting from HPTLC plates onto PVDF membranes



Taki T. and Ishikawa D.,
Anal.Biochem.(1997) 251:135-143

Conclusions

- The separation of total lipids into several fractions enriched in defined lipid classes has many advantages.

- It allows the use of solvent systems specific for each class of lipids to obtain a much clearer picture upon migration on HPTLC.

- It gives the possibility to visualize by chemical or serological means the minor components after concentration of the applied sample without the disturbance of migration on HPTLC plates that would result from the presence of major components in a total lipid sample.

- It is a help for the investigators to use radioactive precursors to study lipid metabolism without overlapping weak radioactive spots by highly radioactive ones.

Thank you for your attention !

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Engelhard- France

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