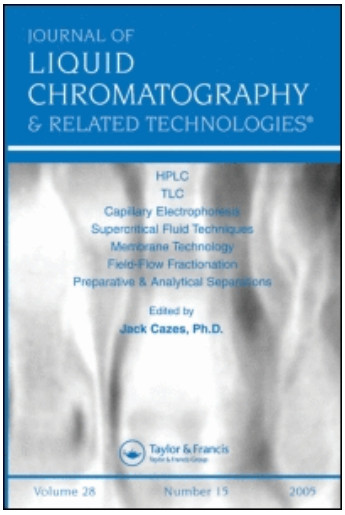


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# IMPROVED METHODS FOR THE SIMULTANEOUS SEPARATION OF FREE DIACYLGLYCEROL SPECIES FROM CERAMIDES CONTAINING PHYTO-SPHINGOSINE AND SPHINGOSINE BASES WITH NON HYDROXY AND ALPHA-HYDROXY FATTY ACIDS

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# IMPROVED METHODS FOR THE SIMULTANEOUS SEPARATION OF FREE DIACYLGLYCEROL SPECIES FROM CERAMIDES CONTAINING PHYTO-SPHINGOSINE AND SPHINGOSINE BASES WITH NON HYDROXY AND ALPHA-HYDROXY FATTY ACIDS

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## ABSTRACT

Second messenger ceramides differ in their composition according to the nature of their sphingoid bases and their amide linked fatty acids. These different species may be involved in many different biological responses, as may be the diacylglycerol species. For this reason, it is interesting to have a simple method which allows for the separation of these compounds into classes of species for further analysis.

We describe here a simple procedure which resolves ceramides containing phytosphingosine and normal fatty acids from ceramides with sphingosine and alpha-hydroxy fatty acids, and ceramides with sphingosine and normal fatty acids. Moreover, by means of a three-dimensional thin-layer chromatography, an enhanced simultaneous resolution between diacylglycerol species was obtained.

This improved method was used to obtain the separation of free ceramides extracted from fish gills where the presence of ceramides with phytosphingosine was shown. Chemical and gas liquid chromatography analyses of the separated species showed the efficiency of the described TLC procedure.



## INTRODUCTION

Studying the interrelations between the two lipid second messenger ceramides and diacylglycerols (DAGs) is emerging as an exciting research area, since they seem to be able to counterbalance their respective effects or to synergise in some physiological processes.[1] Ceramides are synthesized by linking fatty acids onto the amino group of sphingoid bases, leading to the existence of different ceramide species according to the kind of the sphingoid base and to the nature of the fatty acid linked in the amide position.

These different species may have different biological activities.[1,2] Therefore, it is interesting to have a simple procedure which allows the simultaneous separation of DAGs and ceramide species from a single extract.

With this aim in view, we have previously described a 2D-TLC procedure[3] for the separation of DAGs from ceramides with normal fatty acids or alpha-hydroxy fatty acids. With this method, the relative amount of each product or relative incorporation of radioprecursors can be rapidly obtained from the same sample simultaneously, avoiding imprecisions arising when different procedures are used to resolve each kind of molecule.

However, in this previous published method, ceramides were not separated according to their sphingoid bases. Moreover, samples containing high amounts of cholesterol can be troublesome since cholesterol migrates close to DAGs.

We describe, here, a procedure improving the previously published method, since it allows, in addition to an enhanced resolution between 1,2 and 1,3 DAGs, a separation of ceramides according to the presence of sphingosine or phytosphingosine bases and alpha-hydroxy or non-hydroxy fatty acids. This

method, coupled to a solid phase extraction procedure, is applied to separate ceramides extracted from fish gills in which we show the existence of free ceramides with phytosphingosine bases. Identification of this compound is assessed by co-migration with standards on TLC, chemical degradation, and GLC analysis of sphingoid bases.

## EXPERIMENTAL

### Chemicals

All solvents were of analytical grade and were purchased from SDS (Peypin, France), except petroleum ether 40°-70° and ethyl ether, which were from Carlo Erba (Milan, Italy). Chloroform was stabilised by 0.5% of ethanol. Standard lipid mixtures, i.e., ceramides type III, containing sphingosine with normal fatty acids, ceramides type IV, containing sphingosine with alpha-hydroxy fatty acids were purchased from Sigma Chemical (St Quentin Fallavier, France). Cholesterol, cholesteryl oleate, oleic acid methyl ester (esterified fatty acids), oleic acid (FFA), oleyl alcohol, triolein (TAG), diolein (18:1, cis-9) containing 85% 1.3-DAG and 15% 1.2-DAG isomers, $1^2$-monoolein, sphingosine, erythro-dihydrosphingosine, and phytosphingosine were also purchased from Sigma. Ceramide standard containing phytosphingosine acylated with normal non-hydroxy fatty acids was purchased from Coletica (Lyon, France). Silica gel 60 TLC plates, 10×10 cm or 10×20 cm, and aluminium-backed HPTLC plates, with a layer thickness of 0.25 mm and without fluorescence indicator, were from Merck (Darmstadt, Germany).



### Extraction of Lipids from Fish Gills

Gills were taken, in October 1997, from a Mediterannean euryhaline fish, the sea bass *Dicentrarchus labrax*. Gills were cut at their bases and treated as previously described.[3] Lipids were extracted according to the Folch procedure[4] modified by Chapelle et al.[5] An aliquot of these total lipids was directly chromatographied by liquid chromatography on a 100 mg LC-$NH_2$ pre-packed column (Supelco, Bellefonte. PA) as described elsewhere,[3] except that lipids were dissolved in hexane/diisopropyl ether (200:3, v/v) and applied to the LC-$NH_2$ column.

The column was washed with hexane/diisopropyl ether (96:4, v/v) to remove a part of neutral lipids before the chloroform/isopropanol (2:1, v/v) step, in order to obtain a chloroform/isopropanol fraction containing ceramides with a lower content of other lipids.

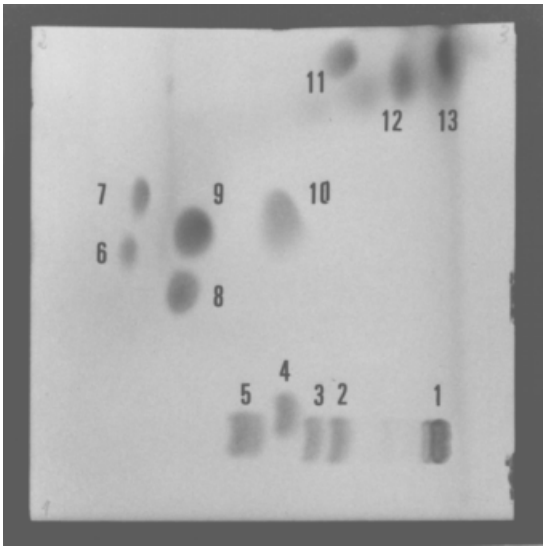


**Figure 1**. Three-dimensional thin-layer chromatography of standard lipids. 1: Origin (phospholipids plus cerebrosides plus free sphingoid bases and their methylated analogs); 2: ceramide type IV from Sigma with sphingosine linked to alpha-hydroxy fatty acids; 3: ceramides containing phytosphingosine base linked to normal fatty acids; 4: MG; 5: ceramide type III from Sigma with sphingosine linked to normal fatty acids; 6: 1,2-DAGs; 7: 1,3-DAGs; 8: cholesterol; 9: oleyl alcohol; 10: FFA; 11: TAGs; 12: esterified fatty acids; 13: cholesteryl esters plus hydrocarbons.



Another aliquot was submitted to methanolic hydrolysis in 0.6 N NaOH methanol during 1 hr at room temperature as described previously[6] to remove alkali labile lipids. The lipid mixtures obtained in this way or after LC-$NH_2$ chromatography were applied to a TLC or HPTLC plate as described below.

### Separation of Neutral Lipids by 2D- or 3D-TLC

The standard lipid mixture was applied to the lower right-hand corner of the plate as a 5 mm elongated spot with a micropipette. The plate was first developed (see Fig. 1) in the solvent system chloroform-methanol 50:3.5 (v/v), which is close to the system described by Karlsson and Pascher.[7] Migration was stopped when the solvent front reached 0.5 cm from the top of the plate. After air-drying, the plate was developed in the second direction with the second solvent system (petroleum ether-ethyl ether-acetic acid, 40:60:0.1 (v/v/v)), which is the system described by Bowyer and King.[8] This two-dimensional

migration is sufficient to resolve ceramides and DAG species. If separation of TAGs from esterified fatty acids and cholesteryl esters is required, a third-dimensional development can be performed in the solvent system (hexane-ethyl ether-acetic acid, 80:20:1 (v/v/v)). This migration is perpendicular to the direction of the second system and is made in the opposite direction of the first solvent system (see Fig. 1) until the front reached the top of the plate. At the end of migration, the plate is removed, air-dried, sprayed with Dittmer and Lester reagent[9] to detect presence of phospholipids, then heated at 180°C for 1 min to visualize all lipid spots.

### Extraction of Sphingoid Bases from Ceramides

Ceramides, separated and isolated by TLC or HPTLC, were scraped from the plate and transferred to screw-capped tubes in which acid hydrolysis was performed during 18 hr at 80°C in 1 mL of methanol-concentrated HCl-water 83:8.6:9.4 (v/v/v).[10] Methyl esters were removed by washing this mixture with hexane three times, consecutively. The methanol phase was concentrated and 2 mL of 7N NaOH was added to make the solution alkaline. The sphingoid bases were extracted three times with an equal volume of diisopropyl ether. The ether phase containing the sphingoid bases was dried down under nitrogen and re-dissolved in a minimum amount of chloroform/methanol 2:1 (v/v).

An aliquot was spotted on a 5×10 cm TLC plate which was run in chloroform-methanol-2N ammonia in water 40:10:1 (v/v/v) in order to separate sphingosine from erythro dihydrosphingosine and phytosphingosine.[11] After development, the plate was air dried and sprayed with ninhydrin reagent. Sphingosine and erythro dihydrosphingosine appeared pink, while phytosphingosine was colored orange. Another aliquot of these long chain bases was used for GLC analysis.

### GLC Analysis of Sphingoid Bases

The sphingoid bases were analysed according to Sweeley and Moscatelli.[12] The ether phase, containing the sphingoid bases, was evaporated under nitrogen and re-dissolved in 1 mL methanol. 0.2 mL of a solution of sodium periodate, 0.2 M, were added to the sample and the oxidation was performed at room temperature during 1 hr in the dark. Then, 0.6 mL of water and 2.4 mL of 1.2-dichloroethane were added. The lower phase of 1.2-dichloroethane containing the aldehydes derived from long chain bases was dried at 15 °C under nitrogen. The aldehydes were re-dissolved in a minimal amount of 1.2-dichloroethane, from which an aliquot was used for gas chromatography.

Aldehydes were separated on a Hewlett Packard gas chromatograph, type 5890 series II, equipped with a Hewlett Packard 0V 17-01 capillary column (25 m × 0.32mm), a split injector, and a flame ionization detector. Temperature programming was from 170°C to 220°C at 2°C/min. The nitrogen gas carrier flow was 15 mL/min. Data were recorded and integrated on a CR3-A integrator (Shimadzu, Kyoto, Japan). Sample runs were compared to phytosphingosine, erythro dihydrosphingosine, and sphingosine standards derivatized to their aldehyde counterparts, i.e., respectively, pentadecanal and hexadecanal.

## RESULTS AND DISCUSSION

As shown in Figure 1, the separation of all major classes of neutral lipids was achieved on a 10×10 cm pre-coated silica gel TLC plate, while the phospholipids remained at the origin with cerebrosides, free sphingoid bases, and their methyl derivatives. The procedure described in this paper improves the resolution between ceramide species and DAG species, compared to a previously published 2D-TLC procedure,[3] in which ceramide species were separated according to the presence of normal fatty acids or alpha-hydroxy fatty acids. This separation was performed in a first solvent system which was chloroform-methanol 50:5 (v/v).

In this system, ceramides were well resolved from monoacylglycerols, but ceramides containing phytosphingosine bases linked with normal fatty acids were mixed with ceramides containing sphingosine with alpha-hydroxy fatty acids. With the new procedure, ceramides containing phytosphingosine linked to normal fatty acids were well resolved from ceramides containing sphingosine bases and alpha-hydroxy fatty acids. Moreover, a good resolution between monoacylglycerols and ceramides species was preserved (Fig. 1). Since the proportion of methanol was lowered in the new first solvent system, compared to the previously published method (chloroform-methanol 50:3.5 instead of 50:5 (v/v)), the $R_f$ of all compounds was lowered. This lowest solvent polarity results, also, in a best separation between cholesterol and fatty alcohol from DAG species when running the plate in the first direction.

The separation of 1,2-DAG species and cholesterol from 1.3-DAG species and fatty alcohol, obtained in the second direction, was also enhanced, as compared to the previous procedure. The present 2D-TLC procedure was sufficient for simultaneous separation of ceramide and DAG species. However, in this second solvent system, TAGs, fatty acid methyl esters, sterol esters, and hydrocarbons mix with each other at the top of the plate, so that individualized data on these compounds could not be obtained. If one wants to separate these compounds, a 3D-TLC procedure can be applied, in which the first and second

migration are performed as for the 2D procedure. In this 3D procedure, a third migration in hexane-ethyl ether-acetic acid 80:20:1 (v/v/v) run in an opposite direction to the first one (see Fig. 1), allows a good separation between TAG, fatty acid methyl esters and sterol esters.

The first solvent system used in this procedure (chloroform-methanol 50:3.5 (v/v)) was derived from the system described by Karlsson et al (7) who used chloroform-methanol 95:5 (v/v) to separate ceramide species. In this study, ceramides were successfully separated on 20×20 cm silica gel G home-made plates. In fact, separation of ceramides into species containing sphingosine with non hydroxy fatty acids, ceramides with phytosphingosine and non hydroxy fatty acids, ceramides with sphingosine and alpha-hydroxy fatty acids can readily be obtained in solvent systems ranging from chloroform-methanol 50:1.8 (v/v) to chloroform-methanol 50:3.5 (v/v). Hence, when using Karlsson's solvent system with 20×20 cm pre-coated silica gel 60 plates, separation of ceramide species was assured. Moreover, monoacylglycerols were well resolved from ceramides containing sphingosine with normal fatty acids. Such separation could not be obtained when small 10×10 cm pre-coated silica gel 60 plates were used; monoacylglycerols overlap with ceramides type III (photo not shown).

In contrast, when using chloroform-methanol 50:3.5 (v/v), separation between monoacylglycerols and ceramides type III, but also between other ceramide species, is efficiently achieved even with 10×10 cm pre-coated TLC plates. It is important to point out that, when using 10×10 cm plates, it is necessary to apply the lipids as thin a line as possible instead of a round spot, since ceramide species migrate close to each other. The same separation was obtained with HPTLC plates. However, with such plates, resolution was enhanced, since the migrating spots were more condensed. This property is useful when using small size TLC plates since, as shown by Karlsson et al.,[7] ceramide species tend to migrate with enhanced $R_f$ when increasing the length or unsaturation of the amide linked fatty acids. This can be troublesome because, when using 10×10 cm plates (Fig. 1), ceramide species can be separated, but remain relatively close to each other. This applies for the separation of ceramides with phytosphingosine and normal fatty acids from ceramides with sphingosine and alpha-hydroxy fatty acids. Hence, overlapping of ceramides containing phytosphingosine and short chain saturated normal fatty acids with ceramide species containing sphingosine linked with long chain unsaturated alpha-hydroxy fatty acids could happen.

This phenomenon was shown by Karlsson et al.,[7] using synthetic ceramides. This may also occur with ceramides from natural sources since they are often a mixture of different species with different fatty acids and sphingoid bases.

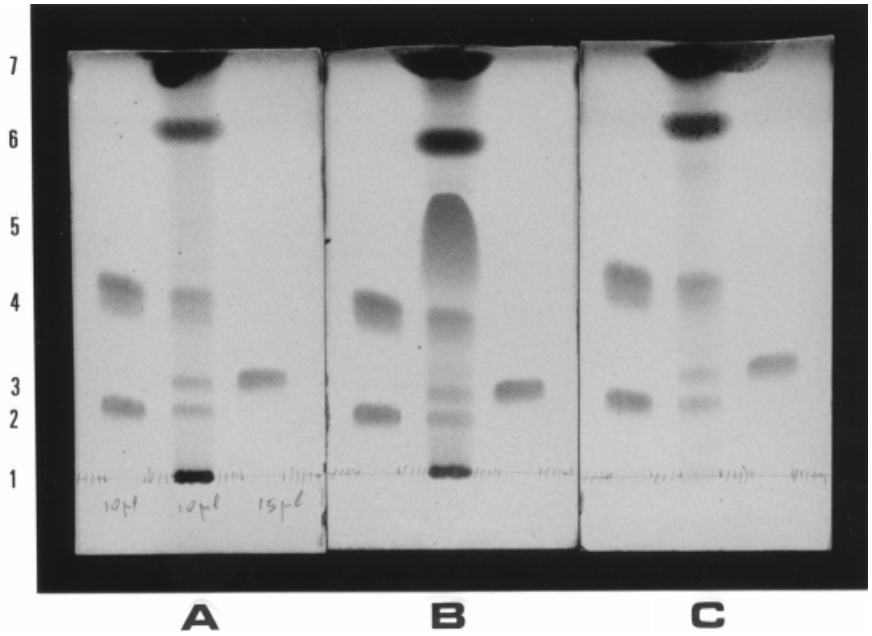


**Figure 2**. Separation of fish gill's lipids by one-dimensional TLC in the system chloroform-methanol 50:2 (v/v). Total lipids extracted from fish gills are spotted on the middle of the plates. Left and right spots of each plate are ceramide standards. Plate A: chromatogram of an aliquot of total lipid mixture before methanolysis; Plate B: chromatogram of total lipid mixture after alkaline methanolysis; Plate C: chromatogram of fraction 1 from LC-NH2 chromatography of the total lipid after alkaline methanolysis (see methods). Lines 1 to 7 are: 1: phospholipids plus cerebosides; 2: ceramides with sphingosine and alpha-hydroxy fatty acids; 3: ceramides with phytosphingosine and normal fatty acids; 4: ceramides with sphingosine and normal fatty acids; 5: FFA; 6: cholesterol and fatty alcohol; 7: TAGs plus esterified fatty acids plus sterol esters.

In order to control the occurrence of such an overlapping phenomenon during our separation procedure, ceramides obtained from fish gills were separated (see Fig 2) on 10×10 cm TLC or HPTLC plates in chloroform-methanol 50:2 (v/v) after total lipid extract has been treated as described in the Methods. When an aliquot of this total lipid fraction was chromatographed on TLC, three bands co-migrated with ceramide standards (see Fig. 2A). The upper spot co-migrated with ceramides type III while the lower one co-migrated with ceramides type IV. Just above this spot, a band ran at the position of ceramide standards containing phytosphingosine bases linked with normal fatty acids. Base treatment[6] (Fig. 2B) showed the alkali-stable nature of these spots, above which appeared a spot of free fatty acids (product of methanolysis) which could

be removed from the sample by LC-$NH_2$ chromatography (Fig. 2C) before elution of phospholipids (see Experimental). The identity of the ceramide spots was further assessed after acid hydrolysis[10] and TLC of the liberated sphingoid bases.[11] A ninhydrin-positive spot migrating as phytosphingosine standard was observed (data not shown) when running sphingoid bases obtained from the spot of ceramides with phytosphingosine and normal fatty acids, while a ninhydrin positive spot migrating as sphingosine standard was obtained when running sphingoid bases liberated from other ceramide spots.

Moreover, only pentadecanal, and not hexadecanal, could be detected by GLC when sphingoid bases obtained from the ceramides with phytosphingosine and normal fatty acids were analyzed after periodate treatment. However, when ceramide spots corresponding to ceramides with sphingosine linked to normal fatty acids or ceramides with sphingosine linked to alpha-hydroxy fatty acids were analysed by GLC, only hexadecanal corresponding to sphingosine could be detected with a trace amount of dihydrosphingosine.



These results showed that the separation of ceramide species was achieved efficiently in the TLC developing system described here. The GLC analysis also indicated that ceramides containing dihydrosphingosine mix with ceramides containing sphingosine bases, in accord with the results obtained by Karlsson et al[7] who showed co-migration of ceramides containing sphingosine linked to normal fatty acids with ceramides containing dihydrosphingosine base.

This new procedure, including 2D and 3D-TLC, allows enhanced resolution of ceramides and DAG species. Ceramide separation can be achieved according to the nature of sphingoid bases and the presence or absence of alpha-hydroxy fatty acids, improving previously published procedures. Moreover, this separation can be achieved without excluding separation of other compounds of interest. The good resolution between compounds of interest allows for the separation of molecules from samples containing high amounts of these products.

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