Quantitative analysis of fatty acids in phospholipids, diacylglycerols, free fatty acids, and other lipids

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Overview

Lipids are extracted from biological tissue with chloroform:methanol (2:1, v/v),[1] and separated into lipid classes by thin-layer chromatography (TLC). Fatty acid methyl esters (FAME) are made from discrete areas containing particular lipid classes scraped from uneluted silica gel[2] using BF_3-methanol.[3] Fatty acid methyl esters are then separated and quantitated by gas-liquid chromatography.

Reagents

All reagents and solvents used should be of analysis grade. TLC plates used are silica gel GHL uniplate 20 × 20 cm (Analtech Inc., Newark, DE, USA) and silica gel H with 3% magnesium acetate Redicoat 2D 20 × 20 cm (Analtech Inc., Newark, DE, USA). In the case of very small samples, high-performance thin-layer chromatography (HPTLC) plates are used. For most separations, 30 *m* GLC glass capillary columns of 0.25 mm inner diameter containing SP-2330 (90:10, w/w, biscyanopropyl:phenylcyano-propyl polysiloxane, Supelco Inc., Bellafonte, PA, USA) are used.

Procedures

Sampling

Very active phospholipases rapidly hydrolyze lipids at the time of sampling and may artificially change the polyunsaturated fatty acid profile, particularly in the free fatty acid and diacylglycerol pools. The activation of these degradative pathways is particularly rapid in the mature central nervous system at the onset of post-mortem ischemia. Rapid enzyme inactivation, therefore, is crucial. Various procedures for *in situ* fixation have been developed for the brain; these include rapid freezing in liquid N_2,[4] head-focused microwave irradiation,[5] and high-frequency (6.5 kilowatt) head-focused microwave irradiation.[6] When subcellular fractions are used, rapid perfusion *in situ* with a mixture of enzyme inhibitors helps minimize artificial changes due to ischemia and subcellular fractionation.[7]

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Lipid extraction

1. Homogenize a 100 mg sample in 2 ml chloroform:methanol (C:M, 2:1, v/v) with a glass-glass or a glass-teflon homogenizer. (We have used samples as small as 20 mg with good results). The use of a polytron is also a rapid and effective means of homogenization. When the tissue is frozen, avoid thawing before extraction by powdering the tissue with a mortar chilled in liquid nitrogen; resuspend in C:M 2:1 before thawing. Collect the homogenate into culture tubes with teflon-lined screw caps. To prevent lipid peroxidation, flush the tubes with N_2 and tightly cap them. The lips of the tubes must not be chipped and must not come into contact with fingers, since skin oils are a source of contamination.
2. For completeness of extraction, sonicate the sample for 30 minutes in a sonication bath. Next, centrifuge for 10 minutes at 800 × g. Extracts can be stored overnight at 4°C or room temperature for no more than 4 hours.
3. Collect the C:M extracts (supernatant) and wash the pellets with half the volume of the solvent mixture used for homogenization. Repeat Step 2 and pool the C:M extracts.
4. Perform a biphasic separation by adding 0.2 vol 0.05% $CaCl_2$, vortexing for 30 s, and centrifuging for 10 minutes at 800 × g. Mark the tubes at the level of the meniscus before withdrawing and discarding the upper phase. Refill the tubes up to the mark with theoretical upper phase C:M:0.05% $CaCl_2$ by vol (3:48:47).[1]
5. Vortex and centrifuge the tubes, then separate and discard the upper phase as in Step 4. Dry the remaining lower phase under N_2 and resuspend in a recorded volume (100–500 μl) of C:M (2:1) for further chromatography. Flush the samples with N_2 and store at −20°C until needed.

Bidimensional TLC separation of phospholipid classes

1. Separate phospholipid classes on silica gel H plates containing 3% magnesium acetate as binder. Plates (20 × 20 cm) can be divided into two halves or four quarters by scoring lines through the middle line. This allows the spotting of 2 or 4 samples, respectively, per each 20 × 20 cm plate. Activate in an oven at 110°C for 3 hours. Prepare two chromatography chambers and line the walls with filter paper or ready-made saturation pads. Load one chamber, labeled "Chamber I," with 100 ml chloroform:methanol:concentrated ammonia (65:25:5, v/v,[8] enough to run four plates simultaneously). Load the second chamber, labeled "Chamber II," with chloroform:acetone:methanol:acetic acid:water (3:4:1:1:0.5, v/v).[8] Cover the chambers with glass lids and allow to equilibrate. Equilibration time depends on the size of the chamber. For a 10 × 25 × 30 cm chamber, a 30 minute equilibration is adequate.
2. A plexiglass cover allows spotting the sample onto the TLC plate under N_2 atmosphere in order to minimize lipid peroxidation and deactivation of the plate.[9] This device also allows one to guide the spotting needle of a microliter syringe or micropipette and to rest the hands. Remove the plate from the oven and immediately place into the chamber. Allow the plate to cool before spotting.
3. A microliter syringe or calibrated capillary micropipettes can be used to spot samples and standards onto plates, about one-half inch from the edge. Application volumes of 50-250 μl are optimal, combining accuracy and rapid deployment of the sample. Four samples can be spotted if the plate is divided into four quadrants. The standards for a bidimensional plate should be run in separate plates. Individual phospholipid standards such as phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SPH) are spotted in 20-40 μg quantities.
4. Place the plate into Chamber I to run the first dimension. Replace the lid

immediately to prevent chamber desaturation. Allow the plate to run until the solvent front reaches the top of the plate or sector, whichever is applicable. In the case of 20 × 20 cm plates with four samples spotted, remove the plate, dry with a hair dryer, rotate 180°, and place into Chamber I for the first-dimension run of the top sector. When the run is completed, rotate the plate 180° again and repeat Step 4. In this way, both top and bottom sectors are run twice in the first dimension to maximize resolution.

5. Ammonia residues must be completely eliminated before starting the second dimension by carefully ventilating the plate with a hair dryer until no ammonia odor is detectable.
6. Place the plate into Chamber II at a 90° angle from the first-dimension run. When the solvent front reaches the top, dry the plates and rotate 180° to run the left sectors.
7. When the run is over, remove the plate, dry with a hair dryer, and spray uniformly with a 0.005% methanolic solution of 2′7′-dichlorofluorescein. Place the plates under UV light and identify the spots by relative R_f, using known standards. The samples are now ready for fatty acid derivatization and extraction. An example of bidimensional TLC separation of phospholipids can be seen in *Figure 1*.

TLC separation of neutral lipids

1. Separate neutral lipids on silica gel G or GHL plates, activating the plates as described previously (Step 1, Bidimensional TLC). The plates can be scored for division into as many as 7 lanes, always leaving 1 lane to run a standard. Prepare a chromatography chamber. Because of its

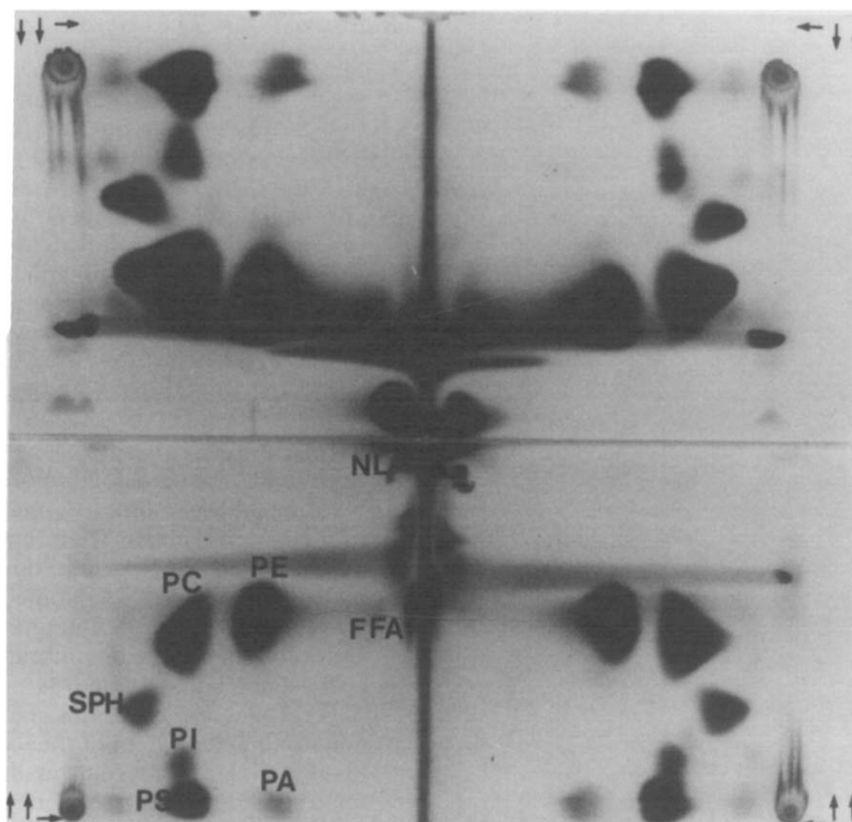

Figure 1 Bidimensional TLC plate for phospholipid separation. Two arrows indicate the first dimension. One arrow indicates the second dimension. This plate was intentionally overloaded to show the minor components that otherwise are too difficult to see on a photograph of the plate.

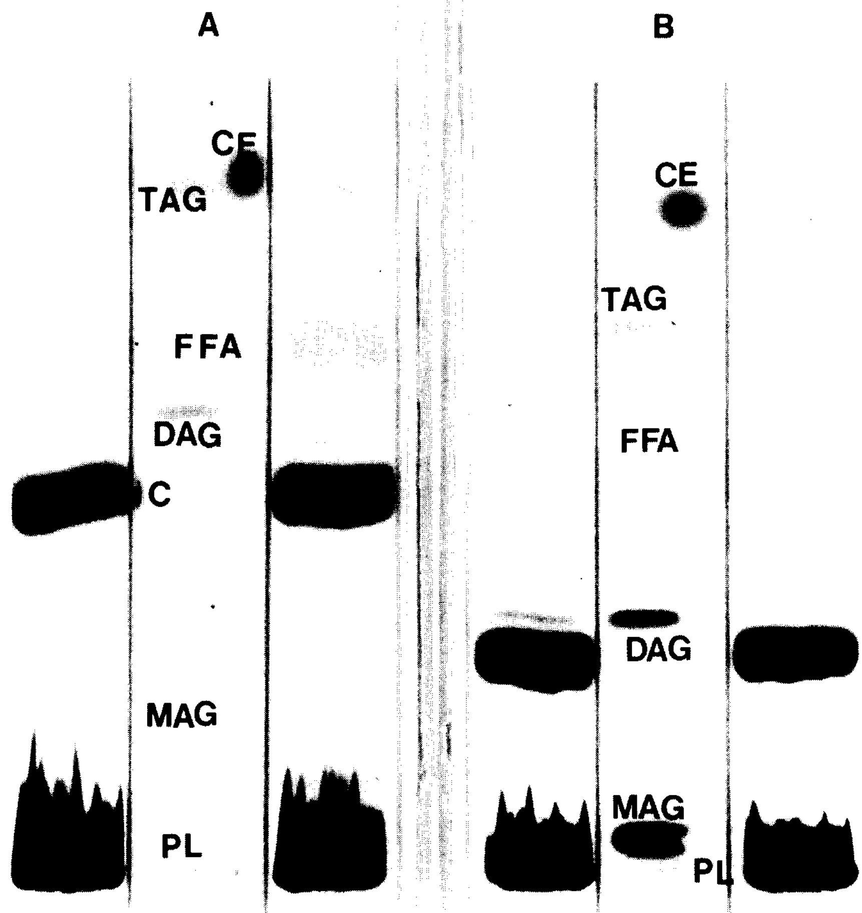

Figure 2 Neutral lipid TLC plate *A* shows the resolution obtained from a brain extract (right and left lanes) and standard (middle lane) using a hexane:diethyl ether:acetic acid (30:40:1.3) solvent system. Plate *B* was run in hexane:diethyl ether:acetic acid (70:30:2.3). The samples show the characteristic two-band separation for DAG. The standard was spotted in two-band fashion to demonstrate the different resolution for cholesterol esters (CE) and TAG. The left side of the outer lane comprises a standard mixture containing MAG, DAG, FFA, TAG; on the right side are FFA and CE. At the origin, the phospholipid is slightly displaced from the spotting area because of overloading. An excess of extract was spotted intentionally to show the FFA and DAG bands, which are usually very hard to see.

volatile characteristics, the solvent mixture should be loaded only immediately before starting the run. Two solvent mixtures are recommended; selection of the proper one depends on which lipids are involved in the analysis. Hexane:diethyl ether:acetic acid (40:60:1.3, v/v/v) is excellent for separation of free fatty acids (FFA), diacylglycerols (DAG), and monoacylglycerols (MAG), but it does not adequately separate triacylglycerols (TAG) and cholesterol esters. Hexane:diethyl ether:acetic acid (70:30:2.3, v/v/v) gives good separation of cholesterol esters (CE), TAG, and FFA, but does not separate DAG from cholesterol (C) or MAG from phospholipids (PL).

2. After spotting the samples in small bands, a standard containing 40 μg each of lipid classes such as MAG, DAG, TAG, and FFA should be incorporated into a separate lane of the plate for precise identification of the components. Place the plates into the chamber, tightly cover with the glass lid, and allow the plates to run until the solvent front reaches the top of the plate.
3. Remove the plates from the chamber immediately and dry until the acetic acid odor disappears. Spray with 0.005% 2′7′-dichlorofluorescein in methanol to identify the components of interest (as described in the

previous section, Step 7). An example of TLC separation of neutral lipids by both solvent mixtures described here can be seen in *Figure 2*.

Preparation of fatty acid methyl esters

The fatty acids present in lipids are converted to fatty acid methyl esters in uneluted silica spots[2] using BF_3 in methanol.[3] Scrape the silica gel areas off the plates immediately after the TLC procedure (Step 3, above, for neutral lipids or Step 7 for phospholipids), mix with 0.5 ml benzene and 1 ml 14% boron trifluoride in methanol (w/v), flush with N_2, and cap tightly into screw-cap culture tubes with teflon liners (13 × 100 mm is an ideal tube size). Heat the samples at 100°C for 1 hour, checking the tubes for leakage during the first 5 minutes. Allow to cool before opening. Add 3 drops of 3N HCl, 1.5 ml distilled water, and 3 ml hexane. The internal standard FAME can be added at this step (see below). Flush the tubes with N_2, cap, and vortex for 30 s. Centrifuge for 5 minutes at 2000 rpm to separate the upper phase (containing FAME). Collect the upper phase, then reextract the lower phase with an additional 3 ml hexane, as above. Combine this with the product of the first extraction. Dry the hexane extracts under N_2 and perform GLC analysis. Resuspend the FAME in about 300 μl hexane and store under N_2 at −20°C. Just before GLC analysis, dry and resuspend in a small volume of hexane or dichloromethane. The exact amount depends on the size of the sample; 20–100 μl volumes are suggested.

Gas-liquid chromatographic analysis of FAME

A GLC instrument equipped with flame ionization detector (FID) gives high sensitivity and direct relation of peak area to quantity. The injection port, set in a splitless mode, permits working with very small samples, and capillary columns give the best resolution (see Materials). Helium carrier flowing continuously at a low rate helps to preserve the column and to reduce tailing of the peaks. The flow rate of the carrier during runs is set at 1 ml/min, the gas makeup flow to the FID is set at 30 ml/min, and compressed air and H_2 to the FID are set at 300 and 25 ml/min, respectively. The FID sensitivity is set at 1×10^{-12} amp full scale (*afs*). The injector temperature is set at 220°C, and the detector temperature at 250°C.

A temperature program is set to initiate the run at 70°C, hold for 2 minutes, warm to 150°C at a rate of 20°C/min, hold for 8 minutes, warm to 210°C at a rate of 5°C/min, hold for 6 minutes, and finally warm to 230°C at a rate of 10°C/min and hold for 20 minutes (the last step eliminates all residual contamination in preparation for the next run). The program stops and returns to the initial status at a rate of 20°C/min.

Identification of the peaks is based upon relative retention times compared to commercial FAME standards (Sigma Chemical Co., St. Louis, MO, USA; Supelco Inc., Bellafonte, PA, USA; Applied Sciences Labs, State College, PA, USA; etc.). The samples should contain an internal standard, which can be one of the commercially available methyl esters of heptadecanoic acid (17:0), nonadecanoic acid (19:0), or heneicosanoic acid (21:0). The use of 17:0, which runs in an empty area of the chromatogram between 16:1 and 18:0, is recommended to minimize the risk of errors because of overlapping of the peaks. The internal standard should be a methyl ester if it is added after the methanolysis or a fatty acid if it is added at the time of lipid extraction. It is important to add enough of the standard to give rise to a peak in the same range as the major peak of the sample. For practical purposes, a sample is run first to evaluate the FAME content; then the right amount of internal standard is added to the rest of the samples. Blank samples should always be run in parallel during the whole procedure. The peaks obtained are subtracted from all the samples to eliminate contaminants collected from the solvents and the TLC plates. A prerun of the TLC plates in C:M (2:1) is a good procedure to lower the background. For quantitation purposes, the peak area is directly proportional to the amount of the FAME.

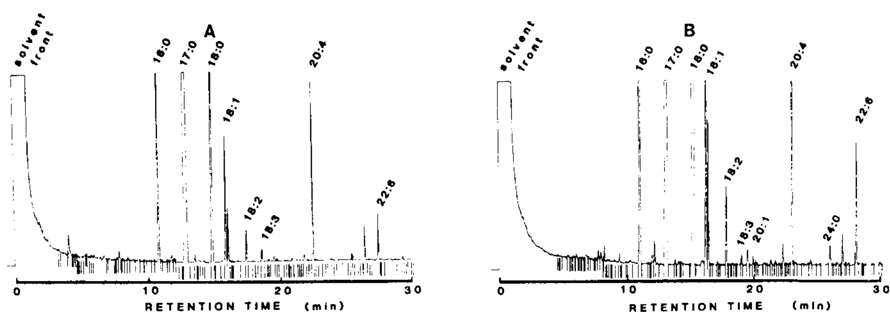

Figure 3 Gas-liquid chromatograms for *A:* FAME of free fatty acids, 1 μl injected from a sample volume of 20 μl. The internal standard 17:0 ME shown is 1 nmol. *B:* FAME of DAG. Injection volume and sample volumes are the same as in *A*. Both FFA and DAG were separated by TLC from 40 mg wet weight in rat brain cortex.

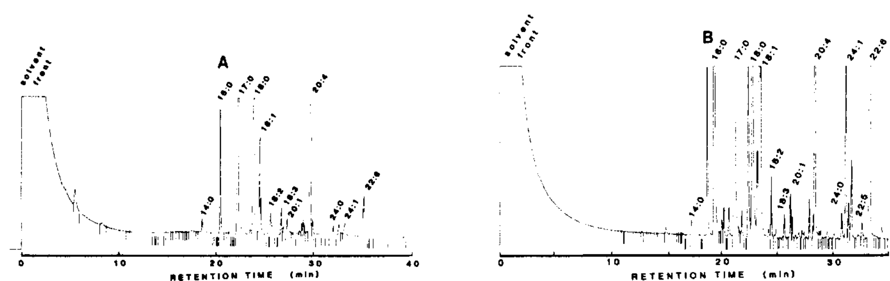

Figure 4 Gas-liquid chromatogram of *A:* FAME of phosphatidylinositol-4,5 diphosphate obtained from 200 mg brain cortex tissue. The FAME were resuspended in 10 μl and 1 μl was injected for GLC analysis. The internal standard 17:0 ME represents 1 nmol. *B:* FAME from total phospholipids obtained from the hippocampus of rat brain; 40 mg tissue were extracted, and the whole extract was used for neutral lipid TLC. The phospholipids remain at the origin. GLC was run with 0.5 μl injected from a 500-μl sample volume. Internal standard 17:0 ME represents 0.5 nmol.

Discussion

The procedure described here has been used for the analysis of polyunsaturated fatty acids in lipid classes of several tissues and biological fluids, including brain,[2] retina,[3] retinal pigment epithelium,[10] rod outer segments of photoreceptor cells,[11] brain subcellular fractions,[12] and blood lipids.[13] It should be emphasized here how important rapid enzyme inactivation is for the prevention of deacylation prior to the start of lipid extraction. The risk of lipid peroxidation can be overcome by avoiding exposure of the lipids to dryness, air, or direct sunlight in the absence of an inert atmosphere. The lipids should be stored in solution under N_2 at $-20°C$, and during procedures described here. There are three possible stopping points: 1) at the end of lipid extraction; 2) after the TLC samples are collected into the tubes in preparation for methanolysis (before boiling); and 3) after methanolysis. The FAME can then be resuspended in a large volume before GLC analysis. Once GLC analysis has been done, the identification of peaks can be obtained by one of the following procedures: comparison of parallel runs of known standards and samples; spiking of samples with known standards; log of retention time plotted as a function of carbon number;[4] and hydrogenation.[9] GLC-mass spectrometry is also an additional, powerful identification procedure.[14] Samples of 100 mg fresh brain tissue are usually adequate; however, different tissues and experimental conditions should determine adequate sample size. Fresh brain tissue of as little as 20 mg wet weight can be used for studying FFA and DAG pools. In *Figure 3*, chromatograms obtained from FAME of lipids from 40 mg cerebral cortex tissue are shown. *Figure 4* shows the gas-liquid chromatogram for FAME obtained from phosphatidylinositol-4,5 diphosphate and total phospholipids separated from 40 mg cerebral cortex. Percent distribution and total

Table 1 Relative distribution of fatty acid methyl esters in free fatty acids, diacylglycerol, phosphatidylinositol-4, 5 diphosphate, and total phospholipids of rat brain cortex

FAME	FFA	DAG	PIP_2	Total Phospholipids
		percent		
14:0	0.5	0.2	0.6	2.0
16:0	25.4	20.5	13.1	20.4
18:0	34.5	42.2	37.7	22.7
18:1	15.3	15.1	7.4	20.8
18:2	2.4	2.1	1.8	0.9
18:3	1.2	0.5	0.3	0.5
20:4	13.1	13.5	36.5	12.6
24:0	0.6	0.7	0.2	4.1
24:1	3.2	1.0	0.2	2.7
22:5	—	0.1	—	0.5
22:6	3.9	4.0	2.5	12.5
Total FAME (μmol/mg lipid P)	0.13	0.33	0.004	60.7

Data for FAME are expressed as percent distribution, and total FAME are expressed as μmol/mg lipid phosphorus (P).
The data were calculated from the chromatograms shown in *Figures 3* and *4*.
Phosphatidylinositol-4,5 diphosphate shows its characteristic composition, with equal amounts of 18:0 and 20:4.

FAME obtained from the samples shown in *Figures 3* and *4* are shown in *Table 1.*

References

1 Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226,** 497–509

2 Bazan, N.G. (1970). Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim. Biophys. Acta* **218,** 1–10

3 Aveldano, M.I. and Bazan, N.G. (1973). Fatty acid composition and level of diacylglycerols and phosphoglycerides in brain and retina. *Biochim. Biophys. Acta* **296,** 1–9

4 Ponten, V., Ratcheson, R.A., Salford, L.G., and Siesjö, BK. (1973). Optimal freezing conditions for cerebral metabolites in rats. *J. Neurochem.* **21,** 1127–1138

5 Galli, C. and Spagnuolo, C. (1976). The release of brain free fatty acids during ischemia in essential fatty acid-deficient rats. *J. Neurochem.* **26,** 401–404

6 Birkle, D.L., Kurian, P., Braquet, P., and Bazan, N.G. (1988). The platelet-activating factor antagonist BN 52021 decreases accumulation of free polyunsaturated fatty acid in mouse brain during ischemia and electroconvulsive shock. *J. Neurochem.* **51,** 1900–1905

7 Birkle, D.L. and Bazan, N.G. (1988). Cerebral perfusion of metabolic inactivators: A new method for rapid fixation of labile lipid pools in brain. *Neurochem. Res.* **13,** 849–852

8 Rouser, G., Fleischer, S., and Yamamoto, A. (1970). Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5,** 494–496

9 Cruess, R.L. and Sequin, F.W. (1965). Box for application of samples to thin-layer chromatograms under nitrogen. *J. Lipid. Res.* **6,** 441–442

10 Reddy, T.S., Birkle, D., Armstrong, D., and Bazan, N.G. (1985). Change in content, incorporation and lipoxygenation of docosahexaenoic acid in retina and retinal pigment epithelium in canine ceroid lipofuscinosis. *Neurosci. Lett.* **59,** 67–72

11 Bazan, N.G., di Fazio de Escalante, M.S., Careaga, M.M., Bazan, H.E.P.B., and Giusto, N.M. (1982). High content of 22:6 (docosahexaenoate) and active [2-^{3}H]glycerol metabolism of phosphatidic acid from photoreceptor membranes. *Biochim. Biophys. Acta* **712,** 702–706

12 Birkle, D.L. and Bazan, N.G. (1987). Effect of bicuculline-induced status epilepticus on prostaglandins and hydroxyeicosatetraenoic acids in rat brain subcellular fractions. *J. Neurochem.* **48,** 1768–1778

13 Bazan, N.G., Scott, B.L., Reddy, T.S., and Pelias M.Z. (1986). Decreased content of docosahexaenoate and arachidonate in plasma phospholipids in Usher's syndrome. *Biochem. Biophys. Res. Commun.* **141,** 600–604

14 Aveldano, M.I. and Sprecher, H. (1987). Very long chain (C_{24} to C_{36}) polyenoic fatty acids of the n-3 and n-6 series in dipolyunsaturated phosphatidylcholines from bovine retina. *J. Biol. Chem.* **262,** 1180–1186