ELSEVIER

Journal of Chromatography A, 849 (1999) 371–380

JOURNAL OF CHROMATOGRAPHY A

Improvement in the Iatroscan thin-layer chromatographic–flame ionisation detection analysis of marine lipids. Separation and quantitation of monoacylglycerols and diacylglycerols in standards and natural samples

Laurent Striby*, Raymond Lafont, Madeleine Goutx

Laboratoire de Microbiologie Marine, CNRS-INSU EP 2032, Campus de Luminy-Case 907, 70 Route Léon Lachamps, 13288 Marseille cedex 9, France

Received 15 December 1998; received in revised form 13 April 1999; accepted 28 April 1999

Abstract

Mono- and diacylglycerols are important intermediates in glycerolipid biodegradation and intracellular signalling pathways. A method for mass determination of these lipid classes in marine particles was developed using the Iatroscan, which combines thin layer chromatography (TLC) and flame ionisation detection (FID) techniques. We improved existing protocols by adding two elution steps: hexane–diethyl-ether–formic acid (70:30:0.2, v/v/v) after triacylglycerol and free fatty acid scan, and acetone 100% followed by chloroform–acetone–formic acid (99:1:0.2, v/v/v) after 1,2 diacylglycerols. Diacylglycerol isomers 1,2 and 1,3 were separated from each other, as well as from free sterols in standards and marine lipids from sediment trap particles. Monoacylglycerols were separated from pigments and galactosyl-lipids in the same trap samples and in a rich pigment phytoplankton extract of *Dunaliella viridis*. Quantitation of each class in samples was performed after calibration with 0.5 to 2 μg of standards. As many as 17 lipid classes can be identified and quantified in samples using this proposed six-step development.

Keywords: Glycerolipids; Monoacylglycerols; Diacylglycerols

1. Introduction

Monoacylglycerols (MG) and diacylglycerols (DG) are usually minor constituents in cells; they are important intermediates in anabolic and catabolic fatty acid ester pathways [1,2]. Lipases act more rapidly on triacylglycerols (TG) and less on monoacylglycerols and diacylglycerols [3]. Thus, intense degradation of lipid reserves results in an accumulation of these intermediate metabolites in cells. In addition, 1,2-diacylglycerols are physiological endogenous activators of protein kinase C involved in numerous biological processes in both animals and plants [4]. In response to many stimuli, inositol phospholipid precursors are hydrolysed, producing

*Corresponding author. Tel.: +33-491-829-385; fax: +33-491-829-051.

E-mail address: striby@luminy.univ-mrs.fr (L. Striby)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00589-0

inositol phosphates and 1,2-diacylglycerols which activate the protein kinase C [5], acting as a second messenger in the adaptive mechanism of cells to changes in environmental conditions.

Applications of the Latroscan TLC–FID technique are numerous [6], for studying of marine lipid classes as biological tracers in particular [7–11]. The combination of these two techniques allows to separate and quantify rapidly classes of lipids, giving an effectual tool for short-scale oceanologic studies. The most utilised separation scheme, in marine studies in particular [12–15], involves several steps: the first two steps to develop and quantify neutral lipids, the third developing and quantifying the more polar lipids mobile in acetone (AMPL), and the fourth developing and quantifying phospholipids. Although very informative, one limitation to this four-step protocol for understanding biological processes, in particular biodegradation, is the difficulty with which sterols (ST) and diacylglycerols (DG) separate from each other, and monoacylglycerols (MG) separate from pigments.

Separation of ST from DG is reported in Parrish et al. [10] although overlaps between the two diacylglycerol isomers and the sterol class makes it difficult to obtain reproducible quantification of the three compounds. Usually, AMPL are not fractionated by the TLC–FID technique [10,13]. This group of lipids mobile in acetone is named chloroplast lipids (containing pigments and galactosyl-lipids), although it contains monoacylglycerols. Little is known about the contribution of this latter degradation intermediate metabolite to the AMPL components. However, we expect that degraded particulate samples will contain large amount of MG after a phytoplanktonic bloom. Then, it becomes necessary to know the amount of this non-chloroplastic lipid class. Parrish et al. [16] separate galactosyl-lipids from pigments and monoacylglycerols. However, using the proposed methodology, the coelution of MG and some of the pigments didn't allow to quantify it with accuracy.

In the present paper we improved existing multistep separation schemes with the aim to achieve the complete separation of degradation metabolites such as MG and DG from other main constituents of the marine lipid spectra, compatible with the processing of large number of sample required in oceanographic studies.

2. Experimental

2.1. Material

Lipid classes were separated on chromarods SIII and quantified using a thin layer chromatography–flame ionisation detection (TLC–FID) Iatroscan TH10 apparatus model MK-IV (Iatron, Japan). The hydrogen flow rate was 160 ml min^{-1}, airflow rate was 2000 ml min^{-1} and scanning speed was 20 cm min^{-1}. Data acquisition and processing was assumed by a compatible PC (processor 486) equipped with Borwin software (JMBS Development, France). Chromarods were acid cleaned with 33% HNO_3 every ten analyses, hydrated with MilliQ water and dried with acetone before each analysis. A 1 μl Hamilton syringe was used to spot standards and a 2 μl Hamilton syringe was used to spot natural samples (4 or 6 μl). Authentic standards (Table 1) were purchased from Sigma (St. Louis, MO, USA). Organic solvents (HPLC grade) were purchased from Rathburn (Scotland).

2.2. Collection of marine samples

Large particles were sampled at a depth of 200 m in the Ligurian Sea in May 1995 at the end of a phytoplanktonic bloom (sample LIG200), and at 100 m in the Alboran Sea in January 1998 (sample ALB100) using drifting sediment traps PPS5 (Technicap, France) over 4 and 8 h respectively. A saline solution was added to the cup to minimise loss of particles by bacterial lysis and ''leaching'' [8]. After recovery of the trap, ''swimmers'' were carefully removed by hand picking. Contents of the cup were concentrated by filtering on 25mm GF/F filters previously combusted (6 h at 450°C). Filters were stored in liquid nitrogen.

Samples of *Dunaliella viridis* Teodoresco (Chlorophyceae) culture (DUN) were concentrated on GF/F filters. Filters were stored in the same conditions described above. Cells were batch cultured in a strictly inorganic medium, using air bubbling agitation (1l min^{-1}), 25°C and a continuous white photon fluence rate of 250 μmol m^{-2} s^{-1} [17].

2.3. Extraction of marine samples

Filters were extracted according to Bligh and Dyer

Table 1
Compounds representing aquatic lipid classes that can be resolved in a five-step separation[a]

Class	Abbreviation	Standard
Aliphatic hydrocarbon	HC	*n*-Nonadecane
Wax ester/Sterol ester	WE/SE	Stearic acid myristic ester
Methyl ester	ME	Palmitic acid methyl ester
Ketone	KET	1-Hexadecanone
Triacylglycerol	TG	Tristearoyl-glycerol
Free fatty acid	FFA	Stearic acid
Alcohol	ALC	1-Hexadecanol
1,3-diacylglycerol	1,3 DG	1,3-Distearoyl-glycerol
Free Sterol	ST	Cholesterol
1,2-diacylglycerol	1,2 DG	1,2-Distearoyl-glycerol
Acetone mobile polar lipids	AMPL	
Pigments	PIG	Chlorophyll *a*
Monoacylglycerol	MG	1-Monostearoyl-rac-glycerol
Monogalactosyl-diglyceride	MGDG	Monogalactosyl-diglyceride
Digalactosyl-diglyceride	DGDG	Digalactosyl-diglyceride
Phosphatidylglyceride–Diphosphatidylglyceride	PG–DPG	L-α-phosphatidyl-DL-glycerol
Phosphatidylethanolamine	PE	L-α-phosphatidylethanolamine dimyristoyl
Phosphatidylcholine	PC	L-α-phosphatidylcholine dimyristoyl

[a] Standards from Sigma (St. Louis, MO, USA).

[18]. Each filter was pulverised into a monophasic solvent mixture (methylene chloride–methanol–water, 1.25:2.5:0.8, v/v/v) with 10 μg internal standard (nonadecanone, Sigma Chemical Ltd, GC grade). After sonication and filtration to remove glass fibers, supernatant was supplemented with CH_2Cl_2 and H_2O to produce a biphasic mixture. The methylene chloride phase was recovered after complete decantation. After rinsing twice the aqueous phase with methylene chloride, the three methylene chloride phases were combined, evaporated under nitrogen, transferred into 200 μl glass insert and stored in methylene chloride under nitrogen atmosphere at −20°C until analysis (less than 3 months).

2.4. Separation and quantification of lipid classes by TLC–FID

The basic separation scheme of lipid classes on the chromarods was performed in a multi-step procedure based on Parrish et al. [12]. Neutral lipids were developed in several successive baths with hexane–diethyl ether–formic acid (HDF). Polar lipids mobile in acetone (including glycolipids, pigments and monoglycerides) were separated in a mixture which base was acetone. To obtain a good vapour saturation of the tank, each bath was prepared 60 min before development. The temperature of the room is maintained at 20°C. Amounts of spotted material on each chromarod are always comprise between 0.5 and 2 μg for each class of lipid, corresponding to the range used for calibration.

3. Results and discussion

3.1. Separation of ST/DG peak in standards

The chromatogram of neutral lipids obtained using the four-step technique (Fig. 1) shows overlap of ST and DG. The first elution system with HDF (97:3:0.2, v/v/v) was used 30 min before scanning hydrocarbons (HC), wax esters and sterol esters (WE/SE), methyl esters (ME) and ketone (KET) followed by HDF (80:20:0.2, v/v/v) for 30 min before scanning triacylglycerols (TG), free fatty acids (FFA), alcohols (ALC), free sterols (ST) and diacylglycerols (DG). The overlap of ST and DG peaks did not allow the accurate quantification of these two classes of compounds when their amount was higher than 0.2 μg on the rod.

To obtain the best separation, we increased the polarity of the third elution system used after the TG and FFA scan by adding diethyl-ether in the original HDF (80:20:0.2, v/v/v) system. A range from 25 to 50% diethyl ether in hexane was tested (Fig. 2). A

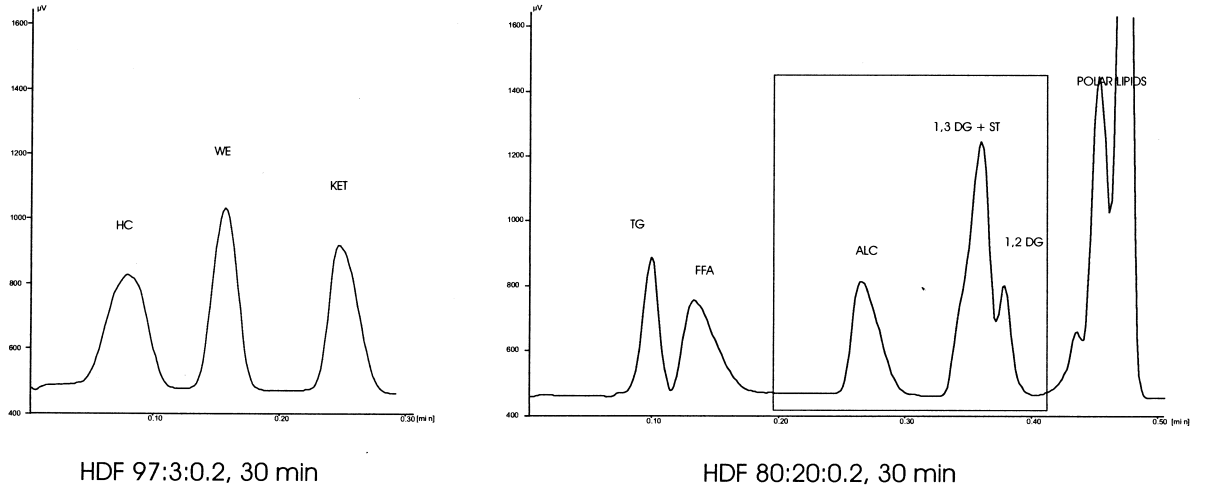

Fig. 1. Chromatogram of standard neutral lipids (1 μg each) developed with initial scheme (HDF 97:3:0.2, v/v/v, 30 min. and HDF 80:20:0.2, v/v/v, 30 min). In the frame: peak resolution to be improved. Abbreviations as in Table 1. ME are not present in standard mixture, however their Rf is known (Rf=0.22).

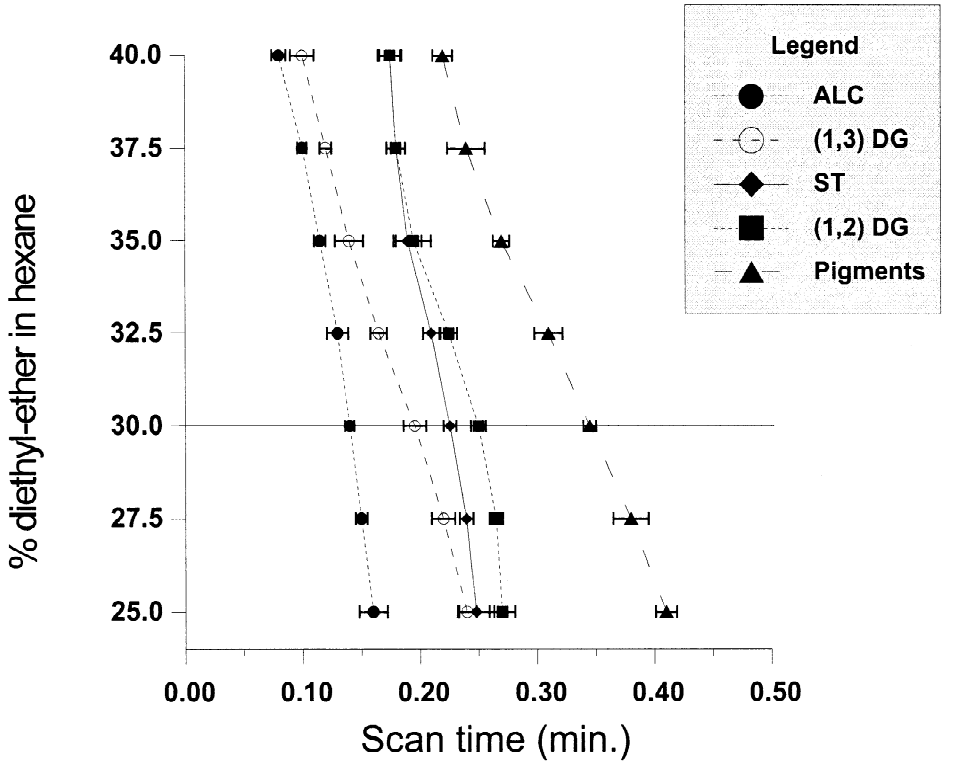

Fig. 2. Variation of ''Rf of different neutral lipid classes'' Vs ''proportion of diethyl-ether in the third bath''. Error bars=±1 SD, $n=8$. The best separation is obtained with HDF (70:30:0.2, v/v/v) in 30 min. Abbreviations as in Table 1.

development time of 30 min was chosen to avoid ALC from running out of the rod. The best separation for ALC, 1,3-DG, ST and 1,2-DG was obtained with HDF (70:30:0.2, v/v/v). The addition of increased amount of (i) cholesterol and (ii) 1,2-dipalmitin to the spot of standard mixture allowed us to confirm the relative position of these two peaks. This procedure showed a poor resolution of these two compounds when the amount of 1,2-DG was higher than the amount of ST. However, natural samples rarely contain high amount of 1,2-DG, and this peak is easily separated from the usually larger ST peak.

3.2. Separation of ST and DG in natural samples

Marine lipids are particularly complex. The same lipid class can contain molecular species, which are very different in their polarity and give several peaks on the chromarod [19]. Kramer et al. and Parrish et al. [20,21] observed that certain molecular species of TG gave different Rf values on chromarods which were developed in HDF, and that the Rf value of TG increased with the fatty acyl chain unsaturation number. In addition, Rf depends on carbon chain length [19]. We used sediment trap material (Fig. 3) because sinking particles may contain a large number of molecular species in one class of lipids due to multiple sources and degradation. In addition, we dispose of enough material in a same sample to spot it several times.

Sub-fractionation and/or Rf variations can cause the erroneous identification of a lipid class. To be sure that the separation of standards is available with natural samples of marine lipids, we used the addition method to identify peaks: natural samples were co-spotted on chromarods with standards in increased concentration. Standard was co-eluted with the peak of corresponding class, allowing the identification of this peak in the natural sample with assurance, though we observed a change in Rf values of 1,3-DG and ALC (0.187 to 0.194 and 0.136 to 0.145 min respectively).

3.3. Separation of MG from AMPL peak in standards

Solvent systems used were based on acetone and

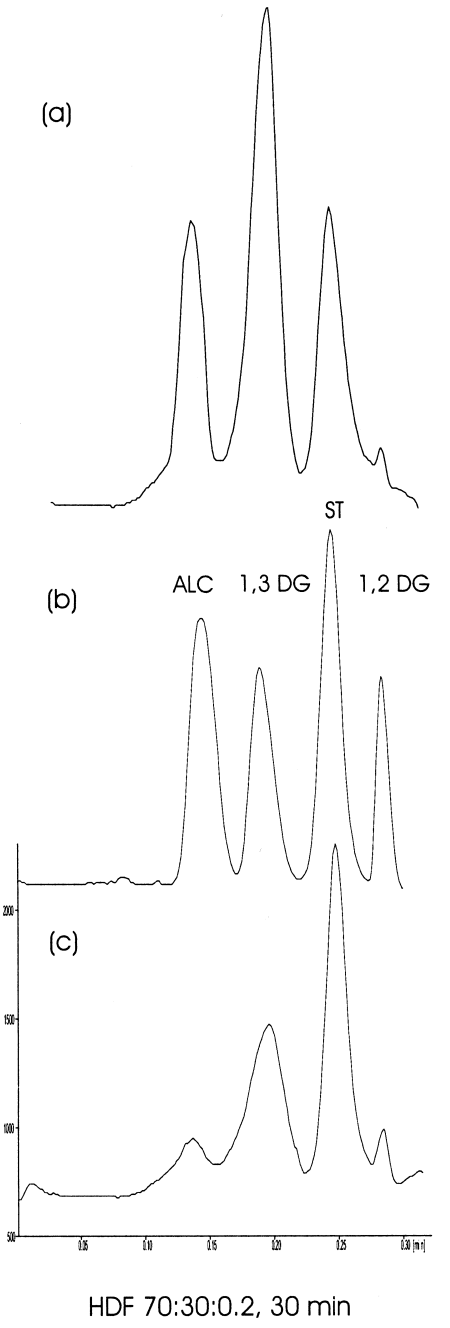

Fig. 3. Separation of DG from standard lipids (b) and trap samples (a and c) using HDF (70:30:0.2, v/v/v, 30 min), after FFA scan. Abbreviations as in Table 1. The trap sample ALB100 (a) was taken at 100 m depth in the Atlantic Jet in Alboran Sea, (winter 1997–98). The trap sample LIG200 (c) was taken at 200 m depth in Ligurian Sea, (Spring 1995).

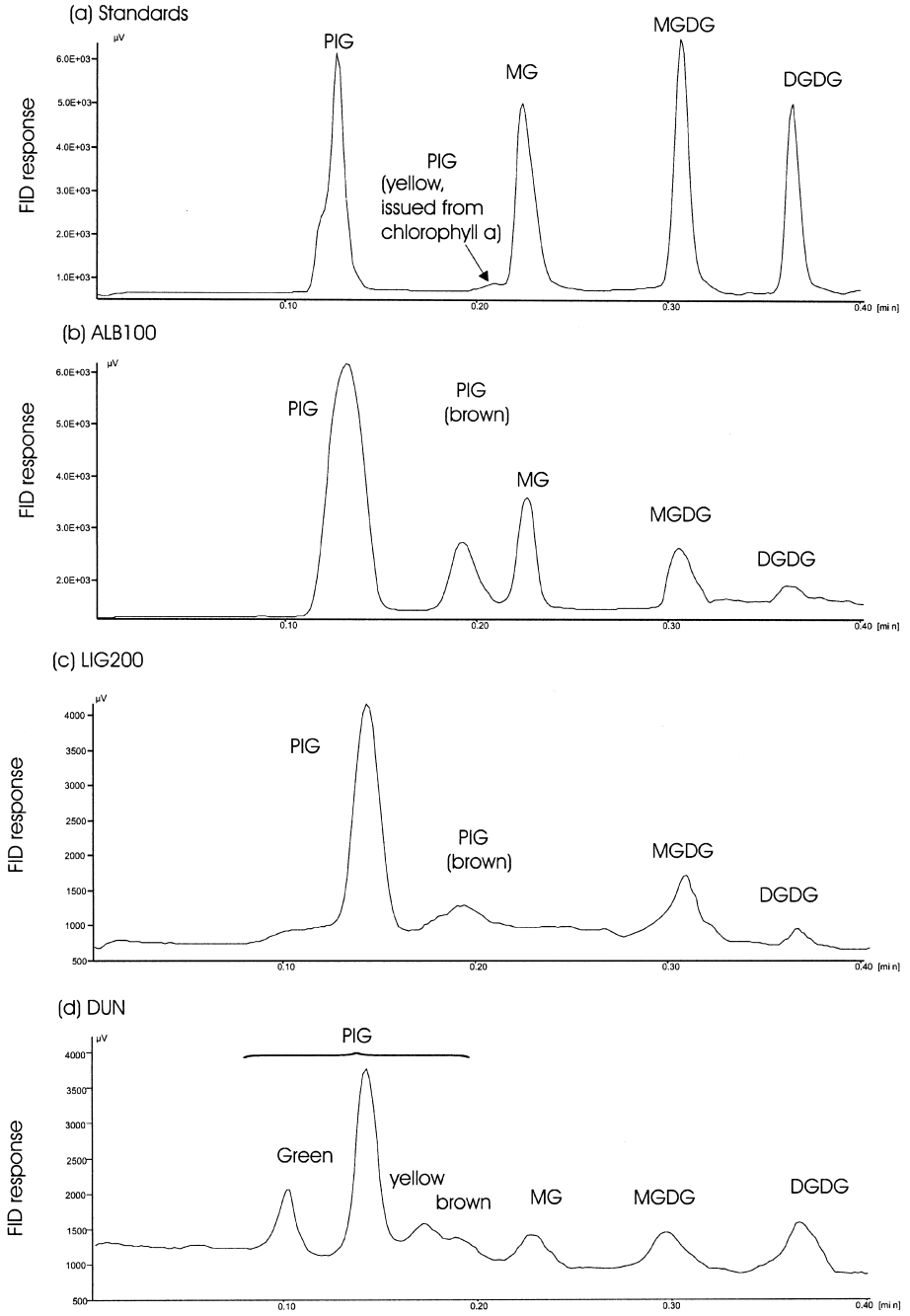

Fig. 4. Fractionation of AMPL from samples developed in chloroform/acetone/formic acid (99:1:0.2, v/v/v), 35 min after acetone 100%, 7 min. Abbreviations as in Table 1. (a) Standards, old chlorophyll a (stayed 6 months in solution exposed in light), 2 μg each. (b) Trap sample ALB100 (100 m depth, Atlantic Jet in Alboran Sea, winter 1997–98). (c) Trap sample LIG200 (200 m depth, Ligurian Sea, spring 1995). (d) Phytoplankton culture (*Dunaliella viridis*).

chloroform to decrease the polarity of the solvent. 0.2 ml of formic acid was added to the 100 ml of mixture to avoid broadening peaks [20]. The systems chosen were based on those used by Parrish et al. [16] for fractionations of algal lipids.

In the solvent systems tested with standards, the separation of CHL, MG and MGDG was better when chloroform exceeded 50% but DGDG was not separated from phospholipids. Complete separation was achieved by developing first for seven min in a 100% acetone bath. Then, the totality of AMPL migrated on chromarod at 2 cm of phospholipids peak. After drying chromarods, they were placed in a second bath containing chloroform–acetone–formic acid. Different elution times were tested. When it exceeded 38 min, CHL was out of the scanning zone.

The best separation between PIG and MG was obtained with chloroform–acetone–formic acid (99:1:0.2, v/v/v), although DGDG was closest to MGDG (Fig. 4a). We did not observe the migration of phospholipids.

3.4. Separation of MG from AMPL peak in samples

The separation of AMPL in trap samples is complicated because of the accumulation of many brown and yellow pigments of different structures and near polarity. These brown pigments stem mainly from degradation of chlorophyllic pigments, which appear in a light green band in some samples.

With 1% acetone in chloroform (plus formic acid, 0.2 ml), pigments were distributed in several well-resolved peaks (Fig. 4). MG was eluted just after yellow pigments although they were not separated in samples from micro-algal cultures of *Isochrisis galbana* [16]. The colour of each band simultaneously of the FID scan informed us about the pigment content of each band. Thus, the band preceding MG was lightly coloured, whereas the band corresponding to MG was colourless in ALB100 (Fig. 4b). The sediment trap sample LIG200 did not contain MG (Fig. 4c), as the contribution of DG to total lipids was low, suggesting a low degradation of organic matter in these sinking particles. On the contrary, the presence of MG and the high contribution of DG in the ALB100 sinking particles showed a more important degradation of this organic matter by lipases.

To verify the satisfactory separation of these compounds, we used a green extract of *Dunaliella viridis* culture, a fresher material containing other pigments. The separation procedure was successful, showing the low content of MG in the extract when pigments were more abundant and diverse (Fig. 4d).

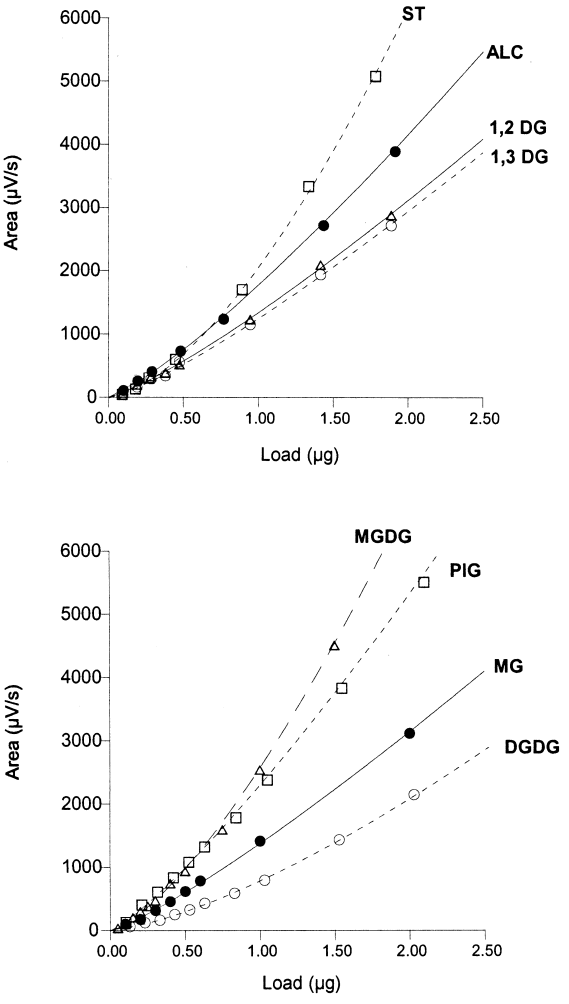

Fig. 5. Calibration curves of tested compounds, fitted by a power law equation ($y=ax^b$). Constants a and b and coefficient of determination r^2 were: ALC (1772; 1.23; 0.9937), 1,3 DG (1334; 1.22; 0.9920), ST (2072; 1.56; 0.9969), 1,2 DG (1312; 1.23; 0.9957), PIG (2335; 1.20; 0.9904), MG (960; 1.19; 0.9955), MGDG (2576; 1.40; 0.9968), DGDG (765; 1.40; 0.9988). Abbreviations as in Table 1.

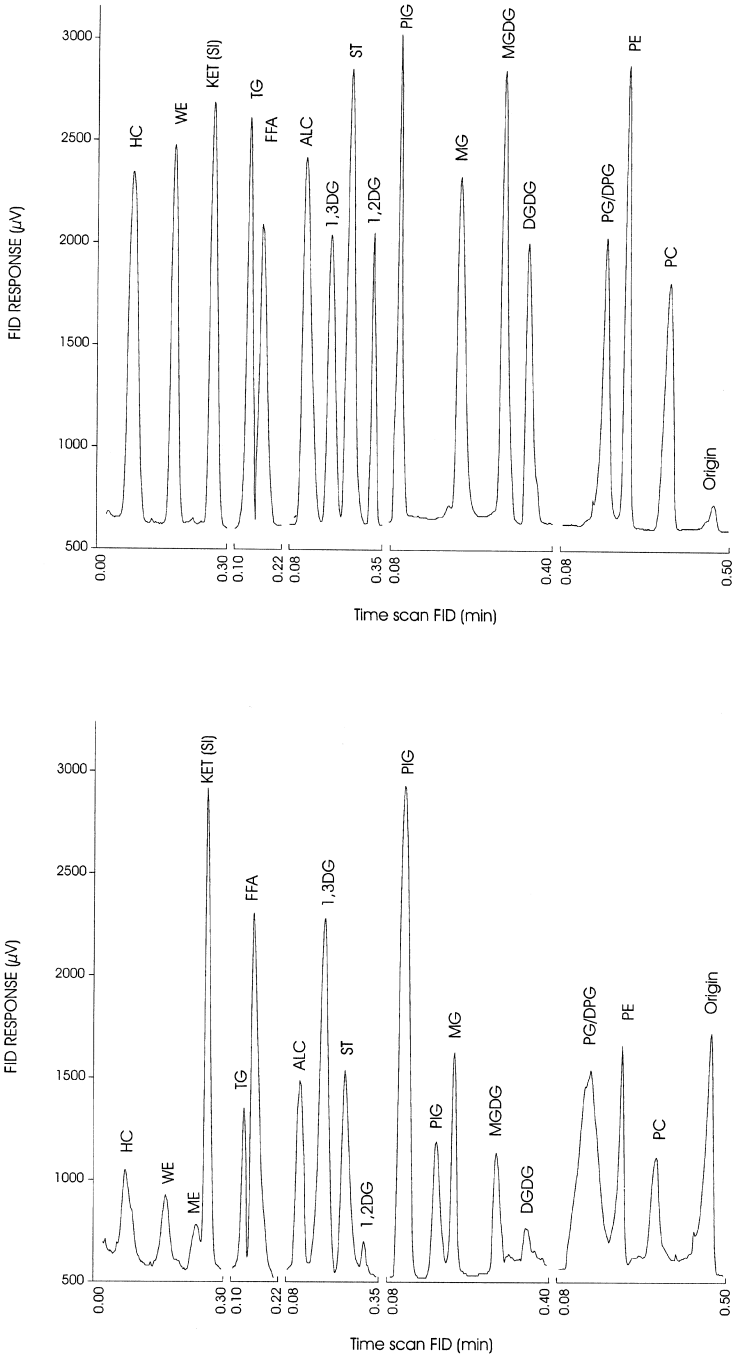

Fig. 6. Chromatogram of total lipids developed with the total procedure. (a) Standard lipids, 0.8 μg each. (b) Sediment trap sample ALMO100. Abbreviations as in Table 1. ME are not present in standard mixture, however their Rf is known (Rf=0.22).

3.5. Quantitation

Most of the calibration curves reported in the literature for the Iatroscan follow the same basic form and are fitted by a power law equation (FID response versus weight of lipid, $y=ax^b$) for loads ranging from 1 to 10 μg of standard lipid. Usually, FID response is linear, but the range of load used in TLC–FID is too large to obtain this linearity in one curve. Besides, the high sensitivity of the FID detector allows the quantification of low amounts of lipid (0.1 μg) with a low coefficient of variation (S.D.<6%, $n=3$) for each standard class. Thus, it was unnecessary to fit calibration curves in a larger range than 0.1 to 2 μg lipid. The response was again curvilinear in this range, but linear in two ranges of load from 0.1 to 0.5 μg ($y=ax+b$, $r^2=0.9953$ to 0.9999, $n=5$) and from 0.6 to 2 μg ($y=ax+b$, $r^2=0.9901$ to 0.9994, $n=5$)). The improvement of fits with the two ranges method was not significant. In addition, it is more difficult to use it in data processing because it would be necessary to test the area value before choosing the appropriate equation. Then, we considered that the power law equation was accurate enough for a satisfactory fitting ($r^2=$ 0.9905 to 0.9992, $n=10$) (Fig. 5) and easier to use in data processing.

3.6. Complete separation of lipid classes

Lipid classes in natural samples were separated using the new elution scheme described above followed by a development of phospholipids in a bath of chloroform–methanol–ammonium hydroxide (50:50:5, v/v/v) to separate PG–DPG, PE and PC. An example of complete separation in standards and natural samples is shown in Fig. 6. Results of analysis for monoacylglycerols and diacylglycerols in natural samples are presented in Table 2.

In natural samples, the coefficient of variation for triplicates did not exceed 12% for minor component classes, generally 7% for major component classes and only 5% for total lipids. Coefficients of variation are much lower than those generally observed with the TLC–FID technique [10,12,15,22–24]. This improvement of reproducibility seems to be linked directly to the improvement of the separation method.

4. Conclusion

This new separation scheme resulted in a reliable quantification of degradation metabolites of acyllipids. It improved the interpretation of fluxes of organic matter in the water column and the identification of processes involved in the degradation of particles. In addition to the development of phospholipids, it allows us to obtain a complete chromatogram of lipid classes in marine particles. With this scheme, the method remains rapid and precise for determining lipid class concentrations in marine particles sampled at high frequency: It is possible to analyse three extracted samples in triplicate in less than five h.

Acknowledgements

This work was supported by the European MAS3-CT96-0060 CANIGO program. The authors wish to thank the JGOFS programs (DYNAPROC and ALMOFRONT) for providing sediment trap samples, Francisco Gordillo for Dunaliella extracts, and Micheline Bianchi, director of the laboratory for giving them opportunity to work in the framework of the CANIGO program. Tracy Bentley is acknowledged for English corrections.

Table 2
Quantity of monoacylglycerols and diacylglycerols in marine particle extracts (mass. in μg, $n=3$) and relative proportion in total lipids

	(1,3)DG			(1,2)DG			MG		
	Mass	RSD.	%	Mass	RSD	%	Mass	RSD	%
ALB100	1.287	3.1	6.98	0.034	8.8	0.18	0.873	2.2	4.74
LIG200	5.607	0.5	2.47	1.294	0.9	0.57	0.000	0.0	0.00

References

[1] W.W. Christie, Lipid Analysis, Pergamon Press, Oxford–New York, 1982.
[2] C.C. Parrish, Mar. Chem. 23 (1988) 17.
[3] M.I. Gurr, A.R. James, Lipid Biochemistry: An Introduction, Chapman and Hall, London, 1980.
[4] M.J. Berridge, Nature 361 (1993) 315.
[5] Y. Nishizuka, Nature 334 (1988) 661.
[6] N.C. Shanta, J. Chromatogr. 624 (1992) 21.
[7] M. Goutx, Mar. Env. Res. 26 (1988) 83.
[8] W.S. Gardner, B.J. Eadie, J.F. Chandler, C.C. Parrish, J.M. Malczyk, Can. J. Fish. Aquat. Sci. 46 (1989) 1118.
[9] A. Saliot, J. Laureillard, P. Scribe, M.A. Sicre, Mar. Chem. 36 (1991) 233.
[10] C.C. Parrish, C.H. McKenzie, B.A. MacDonald, E.A. Hatfield, Mar. Ecol. Prog. Ser. 129 (1995) 151.
[11] J.H. Skerratt, P.D. Nichols, T.A. McMeekin, H. Burton, Mar. Chem. 51 (1995) 93.
[12] C.C. Parrish, Can. J. Fish. Aquat. Sci. 44 (1987) 722.
[13] M. Goutx, M. Acquaviva, J.C. Bertrand, Mar. Ecol. Prog. Ser. 61 (1990) 291.
[14] A.T. Lombardi, P.J. Wangersky, Hydrobiology 306 (1995) 1.
[15] R.G. Ackman, C.A. McLeod, A.K. Barnerjee, J. Planar Chromatogr. 3 (1990) 450.
[16] C.C. Parrish, G. Bodennec, P. Gentien, J. Chromatogr. A 741 (1996) 91.
[17] F.J.L. Gordillo, M. Goutx, F.L. Figueroa, F.X. Niell, J. Appl. Phycol. 10 (1998) 135.
[18] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[19] T. Ohshima, R.G. Ackman, J. Planar Chromatogr. 4 (1991) 27.
[20] J.K.G. Kramer, R.C. Fouchard, E.R. Farnworth, J. Chromatogr. 198 (1980) 279.
[21] C.C. Parrish, G. Bodennec, P. Gentien, J. Chromatogr. 607 (1992) 97.
[22] T. Terabayashi, T. Ogawa, Y. Kawanishi, M. Tanaka, K. Takase, J. Ishii, J. Chromatogr. 367 (1986) 280.
[23] C.C. Parrish, A.S.W. De Freitas, G. Bodennec, E.J. Macpherson, R.G. Ackman, Phytochemistry 30 (1991) 113.
[24] Q. Liu, C.C. Parrish, R. Helleur, Mar. Chem. 60 (1998) 177.