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Separation of polyunsaturated and saturated lipids from marine phytoplankton on silica gel-coated Chromarods☆

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ABSTRACT

Observations of peak splitting in Chromarod separations of extracts of marine samples led to an in-depth study of this phenomenon. By co-spotting standards with lipids from the phytoplankton *Gyrodinium aureolum* and developing in hexane-based solvent systems it was determined that triacylglycerol and free fatty acid peaks were split due to the presence of high levels of polyunsaturated species. The content of formic acid in the solvent system controlled the separation of saturated and polyunsaturated free fatty acids from each other and from triacylglycerols. The amount of diethyl ether controlled the separation of saturated and polyunsaturated triacylglycerols from each other and from more polar components. It was possible to quantify individual components of split peaks provided loads were kept below 3 μg to maximize separations between species. Iatroscan-measured calibration curves revealed a slightly lower response for polyunsaturated species when developed in hexane-based solvent systems. The proportion of polyunsaturated species determined by Iatroscan compared well with the proportion of polyunsaturated fatty acids determined by gas chromatography.

INTRODUCTION

Silica gel chromatography on Chromarods followed by flame ionization detection (FID) in an Iatroscan has been used extensively in the analysis of lipid classes in environmental samples (*e.g.* refs. 1–4). The attractiveness of this thin-layer chromatographic (TLC)–FID technique lies in the rapidity and simplicity with which samples can be prepared for analysis, the number of different sets of Chromarods that can be processed by a single Iatroscan in a day, and the synoptic type of information that it provides. In studies involving dynamic situations it is often better to obtain totals for classes in a large number of samples than it would be to obtain details of the molecular composition of each class in just a few samples. Different lipid classes may be used as indicators of different processes, *e.g.* anthropogenic inputs, energy storage in organisms, membrane synthesis, etc.

In samples from the marine environment, chromatographic peaks for a single-compound class are sometimes split [2,5–7] as a result of the partial separation of molecular species within that class [8,9]. Hydrogenation has been proposed as a means of eliminating peak splitting in marine samples [7,9]. An alternative approach that avoids this considerable increase in sample handling might be to accept that peak splitting occurs in some classes in some marine samples and to calibrate the components of such classes as individual entities. Indeed, in many

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studies involving marine lipids it may be important to know the proportion of polyunsaturated constituents within a class. Currently, there is considerable interest in lipids containing marine polyunsaturated fatty acids in biomedical research [10], aquaculture [11,12], and most recently in the study of toxic algae [13–15]. Some algae are thought to cause fish kills by producing lipid compounds containing polyunsaturated fatty acids [13]. TLC–FID analyses may provide a rapid screening method for marine samples to evaluate their potential toxicity due to the presence of polyunsaturated lipid classes.

EXPERIMENTAL

The Chromarod–Iatroscan procedures used previously for saturated lipid classes [16] were the starting point for this investigation into the separation and calibration of lipids containing significant proportions of polyunsaturated fatty acids. Standards (Sigma, St. Louis, MO, USA) and samples were spotted on Chromarods-SIII (Iatron Labs., Tokyo, Japan) using a Hamilton syringe fitted into a Hamilton repeating dispenser (Hamilton, Reno, NV, USA). Chromarods-SIII are reusable quartz rods coated with silica gel particles of uniform shape and size [17]. Solute was applied near one end of each of ten rods held in a metal frame. The solute was focused twice using acetone to produce a narrow band of lipid near the lower end of the rods. The rods were then dried and conditioned over saturated sodium chloride at 22°C for 5 min before development in a hexane-based solvent system. After separation of classes, the rods were scanned at 0.4 cm/s in an Iatroscan MK IV analyzer (Iatron) with a hydrogen flow-rate of 160 ml/min and an air flow-rate of 2000 ml/min. The scanning direction of the rods was the opposite of the development direction in the TLC tanks. Data acquisition from the FID system and calibration were performed with BOREAL software (FLOTEC, La Queue lez Yvelines, France).

The marine phytoplankton samples were taken from cultures of the toxic dinoflagellate *Gyrodinium aureolum*. The algae were grown in batch culture as described previously [18] at 13 and 18°C [15]. The particulate material in the samples was separated from that material dissolved in the culture medium by centrifugation at 13 200 *g* (12 000 rpm) and the dissolved and particulate fractions were extracted using large-scale extraction procedures [15]. For fatty acid analysis of the neutral lipids in the extracts, a portion of each extract was placed on a Florisil column and eluted with chloroform. TLC–FID analysis of an eluate revealed only 8% contamination of the neutral lipid classes by polar lipids. Fatty acid methyl esters were analyzed in a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) containing a 60 m × 0.25 mm I.D. capillary column coated with Supelcowax-10 (Supelco, Bellefonte, PA, USA). Samples were injected in the splitless mode at 200°C.

RESULTS AND DISCUSSION

Optimization of separations of polyunsaturated and saturated lipids

Lipid class separations were attempted using various standards in various developing systems to optimize the separation between polyunsaturated and more saturated neutral lipids within classes while maintaining the basic class separations according to functional groups. Since non-polar solvent systems are more effective at separating lipids according to the degree of unsaturation [9], Chromarods were developed in solvent systems ranging in polarity from hexane–diethyl ether–formic acid (H–D–F) 95:4:1 to 100% hexane. The use of non-polar solvent systems also minimizes separations according to chain-length [9].

Multicomponent standards containing free fatty acids (FFAs) and triacylglycerols (TGs) with different degrees of unsaturation were spotted onto Chromarods-SIII so that the load of each class was 3–4 μg. We were unable to separate any pair of oleic acid, palmitic acid and lauric acid in any of our hexane-based non-polar solvent systems. In order to be able to separate FFA on Chromarods the degree of unsaturation must differ by more than one double bond [9]. Thus, palmitic acid and linolenic acid were well separated in all solvent systems, provided they contained a minimum of 0.5% formic acid.

The level of formic acid was important in determining the extent to which saturated and unsaturated FFAs were separated from each other and from TGs. In solvent systems containing H–D–F

99.6:0.2:0.2, or less polar, neither saturated nor unsaturated FFAs separated from each other nor from TG. In H–D–F 99:0.5:0.5, linolenic acid and palmitic acid separated from each other and from TGs, but TGs were not well separated from more polar components. The amount of diethyl ether was important in determining the extent to which polyunsaturated and saturated TGs were separated from each other and from more polar components. To separate TGs from all other components requires a system more polar than H–D–F 98:1:1.

Fig. 1 shows the separations obtained in four solvent systems ranging in polarity from H–D–F 97:2:1 to H–D–F 95:4:1. It can be seen that small changes (1% or less) in the proportion of diethyl ether produce large changes in the efficiency of separations between polyunsaturated and saturated species. Tripalmitin and trilinolein are not separated at this load in H–D–F 97:2:1 (Fig. 1a) but are partially separated in 97:3:1 (Fig. 1b). The best separation of all four standards was obtained in 96:3:1 (Fig. 1c). In H–D–F 95:4:1 saturated TGs (TG_s) are too mobile with respect to polyunsaturated FFAs

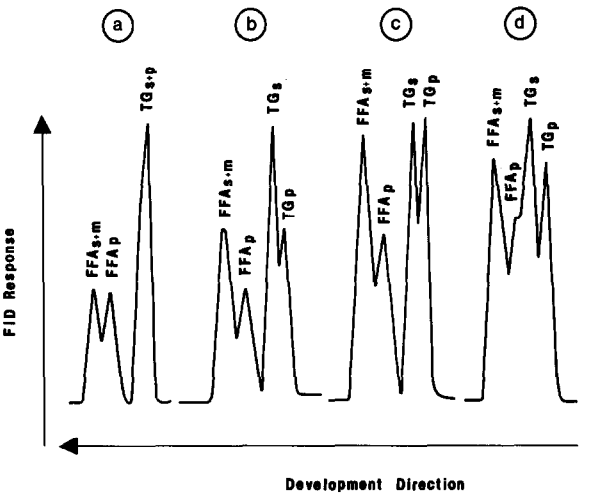

Fig. 1. Separations of saturated (s), monounsaturated (m) and polyunsaturated (p) free fatty acid (FFA) and triacylglycerol (TG) standards on Chromarods-SIII after 40-min developments in (a) hexane–diethyl ether–formic acid (H–D–F) 97:2:1, (b) H–D–F 97:3:1, (c) H–D–F 96:3:1, and (d) H–D–F 95:4:1. The attenuation in each chromatogram has been adjusted according to the size of the major peak.

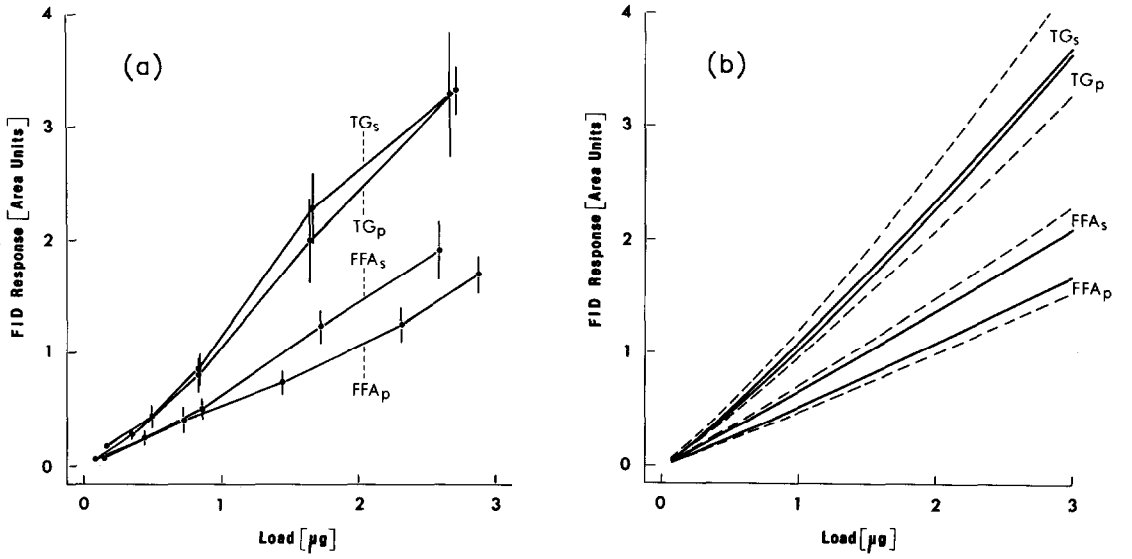

Fig. 2. Iatroscan FID responses to saturated (s) and polyunsaturated (p) triacylglycerol (TG) and free fatty acid (FFA) standards on Chromarods-SIII after 40-min developments in hexane–diethyl ether–formic acid (96:3:1, v/v/v). (a) Calibration data: error bars extend 1 standard deviation from the means. (b) Calibration curves and 95% confidence intervals. Solid lines are power law regressions through raw data points. Broken lines are the upper 95% confidence intervals for the regression lines of the saturated species and the lower 95% confidence intervals for the polyunsaturated species.

(FFA_p) and the separation of linolenic acid and tripalmitin is lost (Fig. 1d). This was also the case in another relatively polar system: H–D–F 97:1:2 (not shown). In systems more polar than this, even the separation between saturated FFAs and TGs would be lost as they run together in H–D–F 92:7:1 [1]. In systems containing more than 7% diethyl ether the elution order of FFAs and TGs is reversed [1].

Quantification of neutral acyl lipid standards

Quantitative mixtures of standard tripalmitin, trilinolenin, palmitic acid and linolenic acid dissolved in chloroform were used for calibration (Fig. 2). Calibration data were obtained at five levels between 0.08 and 2.89 μg with, on average, six analyses per level on different rods within a single set. The average coefficient of variation was 16% (Fig. 2a). These calibration curves show that polyunsaturated species give a slightly lower response as was found with a fish oil developed in a hexane-based solvent system [19].

Regression analysis with the raw calibration data suggested that linear regressions were adequate for calibration (r^2 values in the range 94.1–97.3%); however, power law regressions (Fig. 2b) gave an even better fit (r^2 = 96.6–98.2%). The power law exponents ranged from 1.05 ± 0.03 to 1.11 ± 0.05 for all regressions except that for trilinolenin where the exponent was 1.15 ± 0.03.

Linear regressions through the raw data gave intercepts that were not significantly different from zero for all except the trilinolenin data, suggesting the response for three of these standards was truly linear. This is confirmed by the proximity to 1.0 of the power law exponent for these three components. These exponents are similar to those obtained previously for tripalmitin and palmitic acid on Chromarods-SII [20].

The polyunsaturated standards were monitored for breakdown by looking for build-up of oxidized material at the origin of the Chromarods after development [7]. With normal precautions against oxidation the standards appeared quite stable: there was no evidence for breakdown in a 1-month period.

Separation of neutral acyl lipids in marine phytoplankton samples

G. aureolum is thought to produce polyunsaturated lipids which cause lysis of mouse and trout red blood cells [13,18] and which retard growth in other marine phytoplankton [18,21]. Fig. 3 shows the lipid class composition in extracts of extracellular (dissolved) and intracellular (particulate) lipids of this

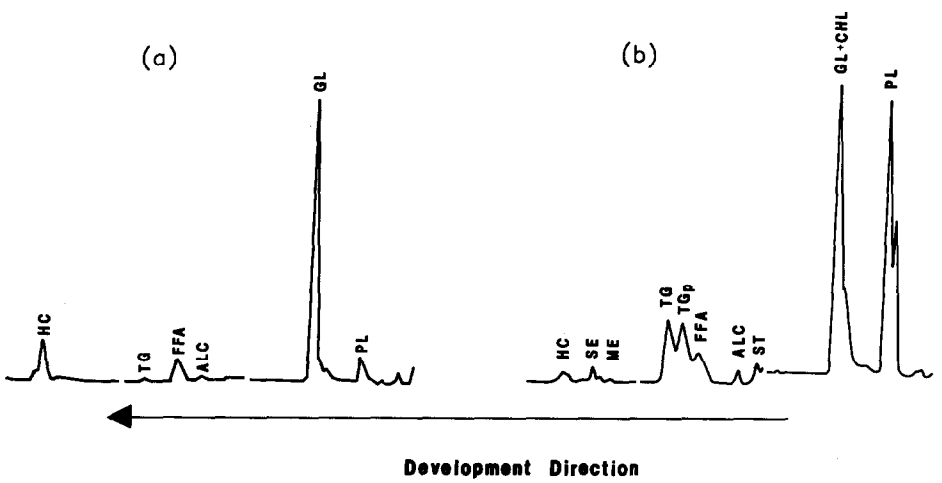

Fig. 3. Multi-step separations of extracts of (a) extracellular lipids and (b) intracellular lipids in a batch culture of *Gyrodinium aureolum* grown at 13°C. Polarity increases from left to right: HC (hydrocarbon), SE (sterol ester), ME (methyl ester), TG (triacylglycerol), TG_p (polyunsaturated triacylglycerol), FFA (free fatty acid), ALC (free alcohol), ST (free sterol), GL (glycolipid), CHL (chlorophyll), PL (phospholipid). Neutral lipid classes were separated by developing twice in hexane–diethyl ether–formic acid (99:1:0.05, v/v/v) partially scanning, and then developing for 40 min in H–D–F (80:20:0.1, v/v/v).

toxic marine dinoflagellate. These chromatograms are composites of three scans of the same sample on the same rod obtained in a multi-step procedure involving several developments with partial scanning in between [15]. It can be seen that glycolipids (identified by co-spotting with galactosyl diglyceride) are the major components in the extracellular lipids (Fig. 3a) and that TGs and phospholipids are proportionally more important within the cells (Fig. 3b). It can also be seen that the TG peak is split into two suggesting that there is a significant proportion of polyunsaturated species (TG_p) in this class. Note that TG runs ahead of FFA in the multi-step procedure because these acyl lipids are eluted in a system containing 20% diethyl ether (Fig. 3).

Figs. 4 and 5 show single-step separations of dissolved and particulate lipid samples from an 18°C culture of *G. aureolum*. The lower two panels of

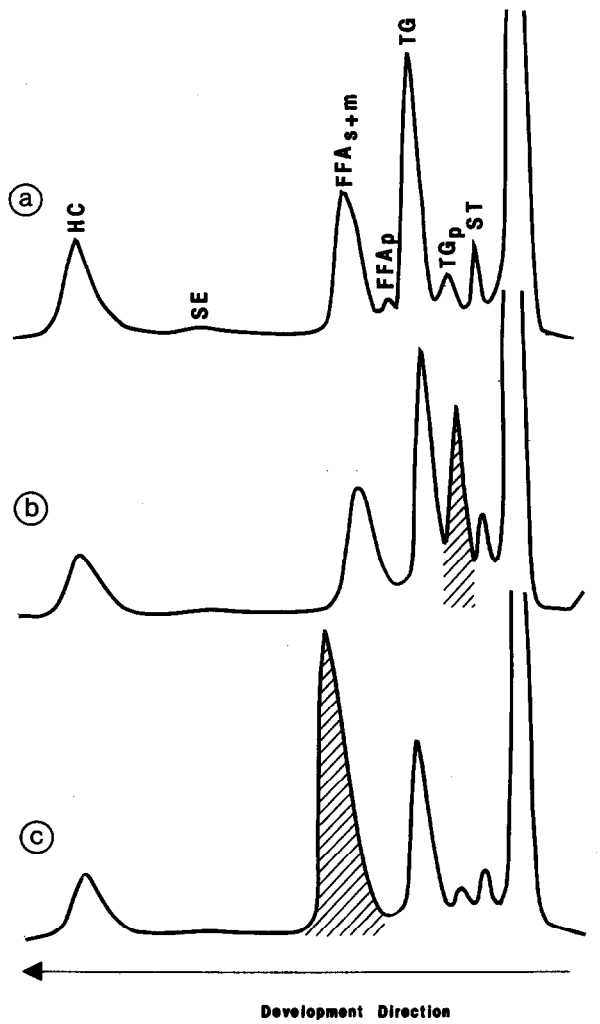

Fig. 4. Separations of extracellular lipids from an 18°C culture of *Gyrodinium aureolum* after a single 40-min development in hexane–diethyl ether–formic acid (97:2:1, v/v/v). (a) Water extract alone. (b) Water extract co-spotted with trilinolein (hatched peak). (c) Water extract co-spotted with palmitic acid and oleic acid (hatched peak).

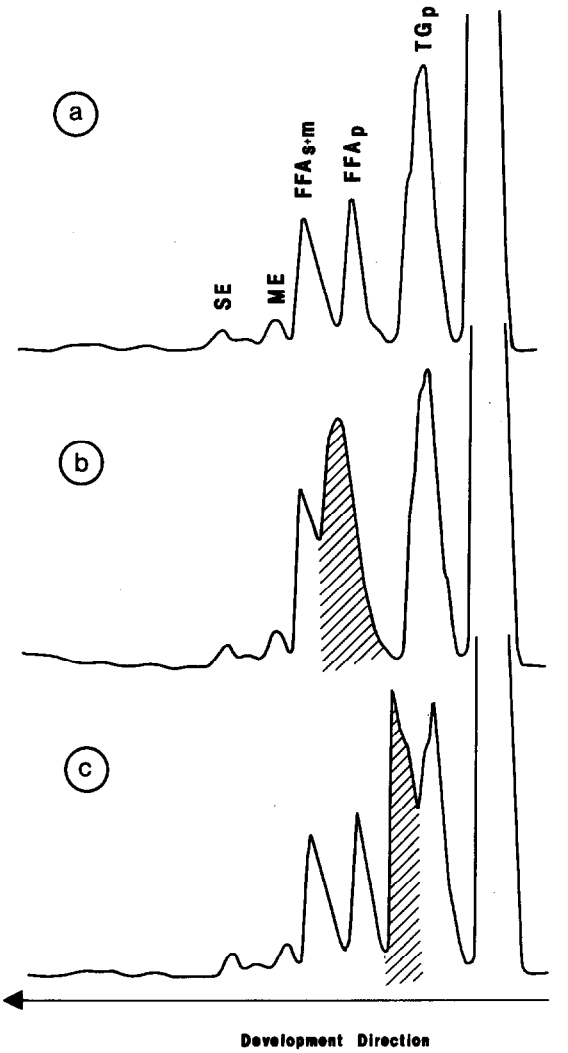

Fig. 5. Separations of intracellular lipids from an 18°C culture of *Gyrodinium aureolum* after a single 40-min development in hexane–diethyl ether–formic acid (97:2:1, v/v/v). (a) Cell extract alone. (b) Cell extract co-spotted with linolenic acid (hatched peak). (c) Cell extract co-spotted with tripalmitin (hatched peak).

these figures show how peak identities were confirmed. The 13°C particulate samples were similar to the 18°C particulate samples in that baseline separations were obtained between an FFA peak containing predominately polyunsaturated fatty acids and a peak containing more saturated species. Likewise for intracellular TGs, separations were obtained between polyunsaturated species and more saturated species in the 13°C samples. However, the 18°C samples appeared to be more strongly dominated by less saturated components (Table I, Fig. 5).

While the particulate samples clearly had a high proportion of polyunsaturated fatty acids in the neutral lipids, the dissolved samples were strongly dominated by saturated species as evidenced by the lack of TG peak splitting in Fig. 3a and the very small peaks for polyunsaturated FFAs and TGs in Fig. 4. Co-spotting with authentic standards showed that the major FFA peak in the dissolved samples resulted from more saturated species.

A single-step separation in H–D–F 96:3:1 is preferable to a multi-step separation in the determination of polyunsaturated neutral lipids as a more strongly bimodal distribution in the degree of unsaturation is necessary for separation in the multi-step procedure. Polyunsaturated and saturated FFA hardly separate in the multi-step procedure and they run close to polyunsaturated TGs so that it was difficult to separate FFAs and polyunsaturated TGs at loads higher than 2 μg using this procedure. Fewer species appear to be included in the TG_p peak as the proportion of the total TGs in this peak was lower when measured in a multi-step procedure. With six analyses, the 13°C culture gave a proportion of 38 ± 5% for TG_p and the 18°C culture gave a value of 68 ± 10% (*cf.* Table I).

While the optimum separation of standards was obtained in H–D–F 96:3:1 (Fig. 1), it should be noted that excellent separations were also obtained with these samples in 97:3:1 and even 97:2:1 (Figs. 4

TABLE I

RELATIVE PROPORTIONS OF SATURATED AND POLYUNSATURATED SPECIES WITHIN THE TRIACYLGLYCEROLS AND FREE FATTY ACIDS IN EXTRACTS OF *GYRODINIUM AUREOLUM* CELLS

Intracellular lipids were separated in a single 40-min development in hexane–diethyl ether–formic acid (96:3:1, v/v/v); data are means ± S.D., n = 6.

	Relative proportion (%)	
	13°C Culture	18°C Culture
Polyunsaturated species in TGs	50 ± 5	88 ± 10
Polyunsaturated species in FFAs	45 ± 8	46 ± 6

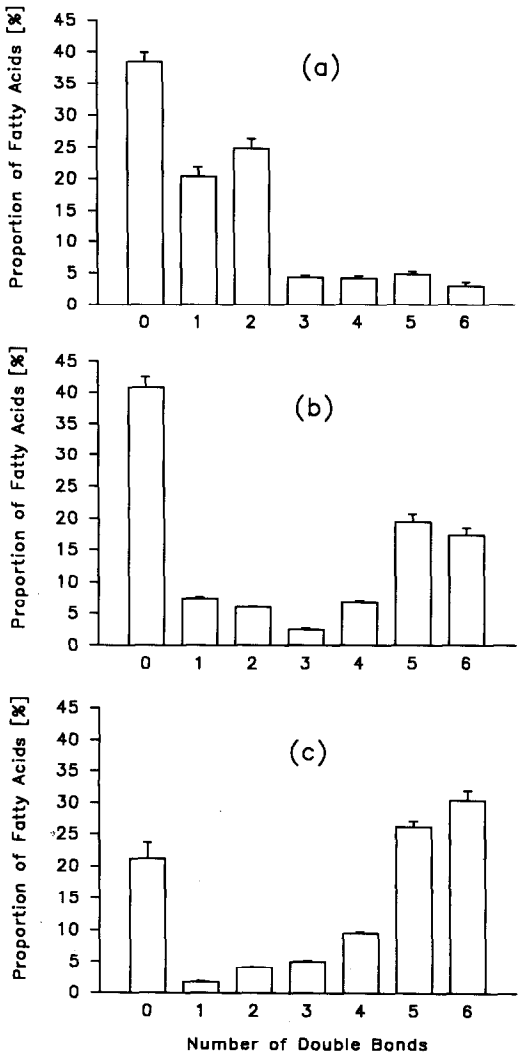

Fig. 6. Distribution of fatty acids with respect to unsaturation in dissolved and particulate matter from an 18°C culture of *Gyrodinium aureolum* sampled after 41 days. (a) Dissolved total lipids. (b) Particulate total lipids. (c) Particulate neutral lipids. Fatty acids were measured as their methyl esters by capillary gas chromatography.

and 5), suggesting the differences in unsaturation between peaks was greater in the samples than in the standards. For quantitation, however, samples and standards were routinely developed in H–D–F 96:3:1 (Table I) to maximize the range over which calibration curves could be obtained (Fig. 2). Nonetheless, overlapping of peaks made it difficult to perform analyses beyond loads of 3 μg. Accurate integration of peak areas in shouldering peaks depends on the integrator's ability to recognize the exact location of the trough between the peaks. The more the peaks overlap, the greater is the effect on accuracy and precision of any error in determining the base of the trough. As loads were lowered the degree of overlapping was reduced but the signal-to-noise ratio decreased so that, although it was possible to detect peaks of both saturated and polyunsaturated lipid classes below 100-ng load, calibration below 100 ng was unreliable.

The comparison between TLC–FID (Figs. 4 and 5) and gas chromatographic (Fig. 6) analyses of extracts of *G. aureolum* further validates the use of Chromarod separations for determining polyunsaturated lipids. The distribution of fatty acids in the total dissolved lipid extracts was biased towards the less unsaturated fatty acids with over half of the fatty acids being saturated or monounsaturated and less than 20% having three or more double bonds (Fig. 6a). The distribution of fatty acids in the total particulate lipid extracts was more bimodal with most of the fatty acids being saturated or highly unsaturated and less than a quarter containing one to four double bonds (Fig. 6b). The low levels of dienoic and trienoic fatty acids would also permit separation of the methyl esters on silica gel coated plates [22]. The distribution of fatty acids in the neutral lipids was biased towards polyunsaturation (Fig. 6c): 77% of the fatty acids in the neutral lipids contained two or more double bonds. This figure compares well with the value of 88 ± 10% obtained in TLC–FID analyses of TG (Table I) which were the major neutral lipid class present in the extracts (Fig. 5).

CONCLUSIONS

It is possible to calibrate split peaks in Chromarod analyses of marine phytoplankton lipids provided loads are kept low. Polyunsaturated standards gave lower responses but the difference in response was small by comparison with the error of repeat analyses at any one level or the error in regression equations obtained from calibration data. This is especially true in the case of TG standards where a single TG calibration curve would suffice for most applications in which a non-polar hexane-based solvent system was used. Thus the components of a split TG peak may be calibrated with a single standard and the sum of these two components would be close to the total TG load on the rod. Quantification of split chromatographic TG peaks in multi-step developments and of TG and FFA peaks in single-step developments may be a very useful way to rapidly determine the degree of polyunsaturation within these marine lipid classes.

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