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CHROMAROD SEPARATIONS FOR THE ANALYSIS OF MARINE LIPID CLASSES BY IATROSCAN THIN-LAYER CHROMATOGRAPHY–FLAME IONIZATION DETECTION

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SUMMARY

A new scheme has been developed for the Chromarod separation of neutral lipids into eleven classes. An initial separation in a non-polar solvent system resolves classes whose polarity ranges from hydrocarbons to free fatty acids. After a partial scan the remaining classes are separated in more polar solvent systems. Good separations have been obtained with rods that have been used for over 150 developments. Preliminary seawater analyses identified lipids with R_F values of hydrocarbons, wax esters and sterol esters, methyl esters, free fatty acids, triglycerides, phthalate esters, alcohols, sterols, and polar lipids.

INTRODUCTION

The Iatroscan thin-layer chromatography (TLC)–flame ionization detector (FID) system combines the efficiency of TLC and the sensitivity of FID. The wide application this system has seen in various fields[1] suggests that it could be a useful tool for studying variability in the distribution of lipid classes in the oceans.

As little as 3 μg of each lipid class is optimal for development and detection on the silica gel coated quartz rods — the Chromarods-S. These rods are set in a frame and are passed through the FID for detection and quantification, a process which simultaneously reactivates the rods. Ten rods (potentially ten samples — perhaps a vertical water column profile) can be completely scanned in less than 10 min. The scan can be stopped at any point along any rod, thus allowing for multi-step separation strategies.

The Iatroscan system appears to be well suited for rapidly collecting synoptic lipid class data. Shipboard Iatroscan analyses could be used as the basis for deciding where to collect larger samples for detailed chromatographic analyses at the compound level. This TLC system would also permit the measurement of classes of lipids that have not yet received much attention from chemical oceanographers.

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The model compounds used in the separations were selected to represent lipid classes known to occur (or expected to occur) in marine dissolved or particulate matter. In addition, the final separation procedure also had to resolve a compound which could be used as an internal standard.

Kramer *et al.*[2] investigated the effect of varying the composition of hexane-based solvent systems on the Chromarod separation of five neutral lipid classes. This work was used as the basis for finding a hexane-based solvent system for the separation of neutral lipids into eleven different classes, while the work of Christie and Hunter [3] on chlorinated solvent systems suggested investigations into the use of less traditional solvents for TLC separations.

EXPERIMENTAL

Apparatus, operating conditions, and materials

The air flow on the Iatroscan TH-10 Mark III analyser (Iatron Laboraties, distributed in Canada by Technical Marketing Associates) was 2000 ml/min, the hydrogen pressure was 0.73 kg/cm^2, and the scan speed was 3.1 mm/sec. The chart recorder (Linear Instruments) was operated mainly at 20 cm/min and 10 mV attenuation. Two sets of Chromarods-S were used in this study: a well-used set that had been employed for 50 separations (more than 100 FID scans) before this study was commenced, and a new set. Most of the developmental work on separations was performed on the older Chromarods; the newer set was used for comparison. A comparison was also made between Chromarods-S (8.5 μm maximum particle size) and Chromarods-SII (5 μm particle size). Peak area determinations were performed on a Technicon Integrator/Calculator (Model AAG). The lipids were obtained from various suppliers (Aldrich, Analabs, Eastman, Fisher Scientific, K & K Labs, Mann Research Labs, Matheson, Polyscience, Supelco) and were spotted singly and taken through the various development steps to determine their purity. Standards were spotted with disposable micro-pipettes (Drummond), and most of the standard peaks in the figures in this communication represent the FID response from 3 μg of material selected mainly from the first compound column in Table I. The solvents used were "distilled in glass" grade; diethyl ether and dichloromethane were redistilled in glass under an atmosphere of purified nitrogen.

RESULTS AND DISCUSSION

One-step developments

The aim of the development procedure was to produce optimum separations of the lipid classes shown in Table I. Attemps were made at one-step separations of these classes using various hexane–diethyl ether–formic acid (HDF) solvent systems (in the proportions x:a:b, where $a + b = 100 - x$ in ml). The solvent composition of these systems ranged from 85:14:1 to 99:0:1 and to 99:1:0. Developments were performed for various times (20–55 min) in each solvent system but no single-step development gave a satisfactory separation. The classes that can be separated in a particular HDF solvent system will be resolved in 20–30 min; increasing the development time simply results in a slightly larger separation between classes. Fig. 1 shows a typical result from a one-step development of these classes using an HDF solvent system. Attempts were

TABLE I

MODEL COMPOUNDS REPRESENTING MARINE LIPID CLASSES THAT CAN BE RESOLVED IN A TWO-STEP SEPARATION

The solvent systems used contain hexane and diethyl ether as their principal components. The first is a non-polar solvent system containing a minimum of 97.5% hexane, the second is more polar and contains a maximum of 92% hexane. Representatives in the second compound column almost invariably run with those in the same class in the first column, but may be completely resolved from those in the last column.

Class	*Abbreviation*	*Principal representative compound*	*Other representative compounds*	*Special compounds*
Aliphatic hydrocarbon	HC	*n*-Nonadecane	*n*-Hexadecane, *n*-heptadecane, 1-eicosene, 1-octadecene	Squalene
Polycyclic aromatic hydrocarbon	PAH	Phenanthrene	Anthracene	
Wax ester/ sterol ester	WE	Octadecyl hexadecanoate	Dodecyl hexadecanoate, cholesteryl octadecanoate	
Methyl ester	ME	Methyl docosanoate	Methyl hexadecanoate, methyl dodecanoate	
Ketone	KET	3-Hexadecanone	3-Octadecanone	2-Pentadecanone, 8-pentadecanone
Free fatty acid	FFA	Hexadecanoic acid	Dodecanoic acid	
Acylated glyceryl ether	GE	Glyceryl-1-octadecyl ether, -2,3-dioctadecanoate		
Triglyceride	TG	Glyceryl trihexadecanoate	Glyceryl trioctadecanoate, glyceryl tridodecanoate	
Phthalate ester	PHTH	Dibutyl phthalate		Di-2-ethylhexyl phthalate
Alcohol	ALC	l-Hexadecanol	2-Hexadecanol, phytol	
Sterol	ST	Cholesterol		
Monoglyceride	MG	Glyceryl-1-monohexadecanoate		
Polar lipid	PL	Lecithin		

also made at performing separations of these classes using dichloromethane as the principal component of solvent systems. The elution order was similar to that in the HDF systems but all classes were moved further from the origin and therefore there was even more shouldering of incompletely resolved classes.

Other than the difficulty of fitting nine or more separate classes on a 15-cm long Chromarod, one of the main challenges in developing HDF separations is the large variability in the R_F values for FFA, GE and TG compared to other classes, when the ratios of the component solvents are varied (Table I shows the compounds used to represent lipid classes). In addition, the elution order of these classes varies; GE always runs ahead of TG, but FFA may run ahead of TG (Fig. 2), in between TG and GE (Fig. 1), or behind TG in the position usually found in silica gel plate TLC. Note that in all the figures the direction of development is from right to left and the scanning direction is from left to right; S and E denote the start and end of scans and O is the

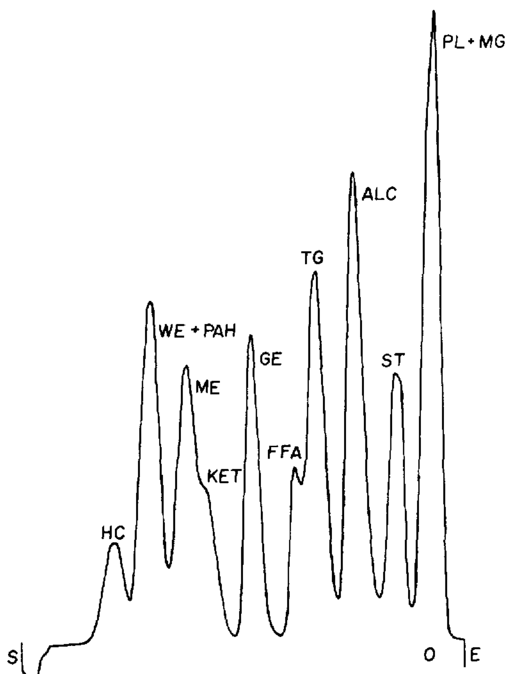

Fig. 1. One-step development and scan of twelve lipid classes; peak identifications are given in Table I. Rod handling sequence: (1) burn rod to activate it and to check that no residual material remains from a previous scan; (2) spot compounds; (3) dry rods; (4) 45-min development in hexane–diethyl ether–formic acid (95:5:0.1) (from right to left in the figure); (5) dry rods; (6) full scan (from left to right in the figure); (7) repeat scan to check that all material has been accounted for and to activate rod for next use.

point of application. TG was found to migrate faster than FFA in systems more polar than HDF 92:7:1, whereas in systems less polar than this the order was reversed. The cross-over point for GE (relative to FFA) is somewhere between HDF 95:5:0.1 (Fig. 1) and HDF 99:1:0.1 (Fig. 3b). In systems less polar than HDF 98:1:1 TG was found to remain near the origin and FFA migrated a little from it (Fig. 2a).

This single-step development work largely confirms and extends the findings of Kramer *et al.*[2]. The least polar solvent system they used was hexane–diethyl ether (97:3), and in that system TG remained quite near the origin. They also found that the mobility of TG increased compared to other classes with an increasing diethyl ether content; however, their cross-over point with FFA was at HDF (90:10:1) which they found [2] was contrary to the results of other workers. The cross-over point in this work was at HDF (92:7:1). As can be seen from the work of Kramer *et al.*, an increase in formic acid content increases the mobility of FFA more than that of other classes; however, humidity is also a factor in determining R_F values. The relative mobility of FFA differed significantly in the same solvent system at different times of the year, in spite of an almost constant laboratory temperature. At present, and in the case of Figs. 2a and 3a, the Chromarods are spotted and then equilibrated for 10 min in a constant humidity chamber prior to development so that the relative mobility of FFA remains the same. The sensitivity to humidity undoubtedly explains the slight discrepancies

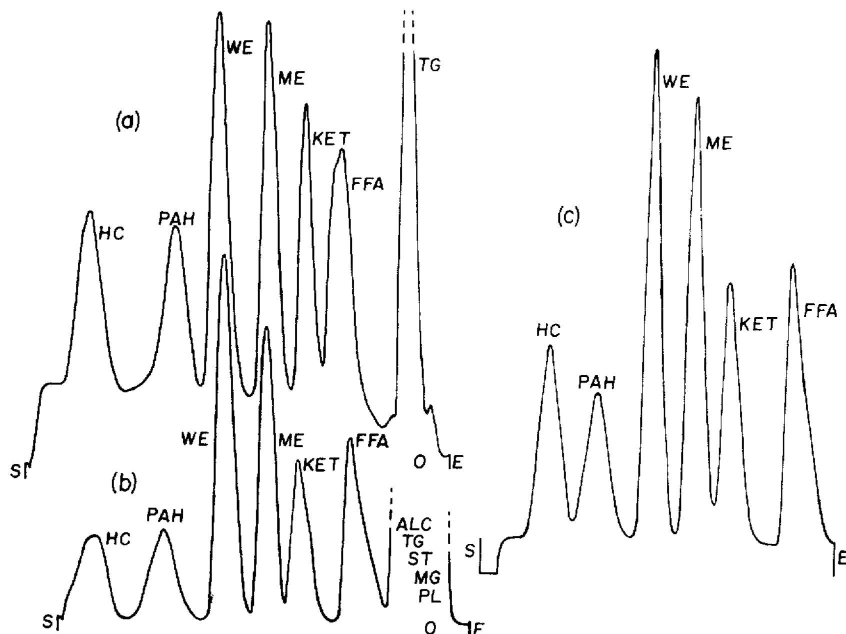

Fig. 2. Separation of neutral lipid classes on Chromarods from three different sets; peak areas are given in Table II. Each rod was developed for between 45 and 55 min in HDF (99:1:0.1). (a) Chromarod-SII (equilibrated at 65% relative humidity prior to development), full scan; TG remains near the origin; first use of this rod. (b) Chromarod-S, full scan; ALC, TG, ST, MG and PL remain near the origin; 21st separation on this rod. (c) Chromarod-S, partial scan; ALC, TG, ST, MG, and PL remain near the origin and were not scanned; 127th separation on this rod.

mentioned above for the point at which TG and FFA run together when there is almost the same amount of formic acid in the solvent systems. In this work there was little difference found between the use of acetic and formic acids in solvent systems (again confirming the findings of Kramer *et al.*[2]; however, formic acid is preferable since it is more volatile and thus more easily removed from the rods prior to scanning, and since it gives little response in the FID.

Multi-step separations

Separations with the least polar solvent systems indicated that a multiple development sequence with partial scanning would be a successful approach. Other than in the separation of neutral from polar lipids, few workers have explored the TLC separation possibilities created by the partial scan facility on the Iatroscan. The principle that led to the final development sequence is shown in Figs. 4a and b. The first development involves a low polarity solvent system, hexane–formic acid (99:1). This causes the compounds that are very mobile in this system to migrate into the upper part of the rod and they are resolved from one another and from more polar neutral lipid compounds. Fig. 4a shows a complete scan of a mixture of compounds that have been developed in this system for 25 min. The scan stop screw can then be set at a point in between the resolved and the unresolved compounds, and the remaining nine rods of the set can then be partially scanned (Fig. 4b). In this way HC and PAH are quantified

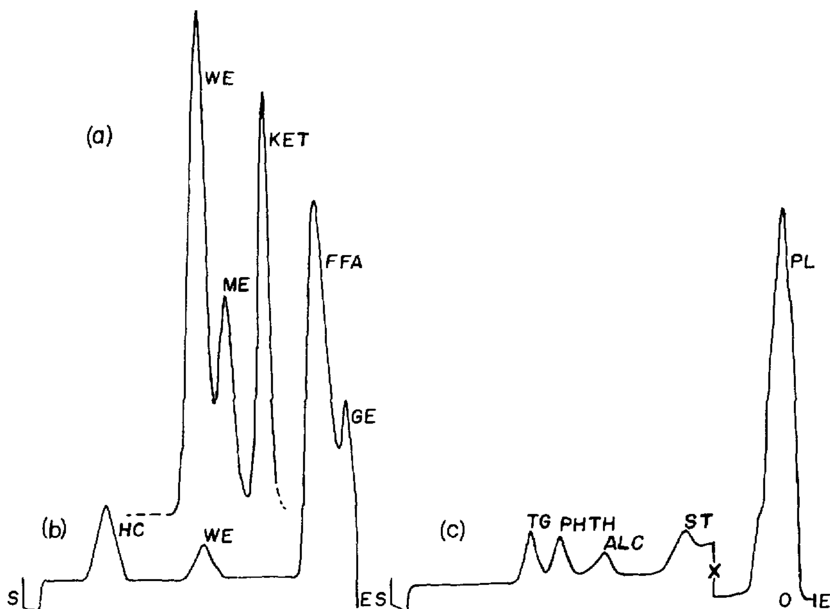

Fig. 3. Analyses of seawater lipids. (a) Part of a scan of particulate lipids on a Chromarod-SII equilibrated at 65% relative humidity prior to development in HDF (98:2:0.2). (b) First-step development of dissolved lipids in HDF (99:1:0.1), partial scan. (c) Second-step of development of (b) in hexane–diethyl ether (85:15), full scan. Increase in attenuation at X.

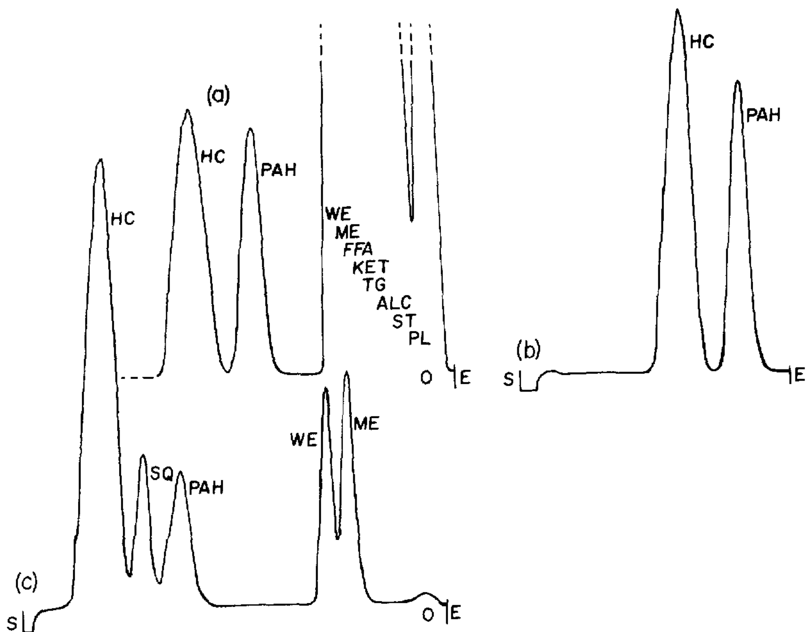

Fig. 4. Developments in non-polar solvent systems. (a) Full scan after development of ten lipid classes in hexane–formic acid (99:1). (b) Partial scan after development of ten lipid classes in hexane–formic acid (99:1). (c) Full scan after development of lipid classes in the polarity range HC to ME in hexane–diethyl ether (99.75:0.25). Squalene (SQ) is separated from other hydrocarbons.

and this part of the rod is reactivated so that a second development can be performed and other compounds can be moved into this region. A second development in a slightly more polar HDF system will move WE and ME away from the unresolved compounds and these two can also be quantified in a partial scan. This proces can be repeated using more and more polar solvent systems until all the neutral lipids have been resolved and quantified (see figures in ref. 4 for an example of this type of approach).

This multi-step development sequence necessitates several different solvent systems and is very time consuming. Thus, the next stage of this study involved optimising the number of classes that can be separated in each step. It is the first solvent system which is the most critical in this approach. Fig. 4c illustrates this point: an optimum separation of HC, squalene (SQ), and PAH is obtainable with a long development in hexane–diethyl ether (99.75:0.25). However, WE and ME are poorly resolved and are still near the origin. In hexane–diethyl ether (99:1) squalene was found to shoulder on HC, whereas in 100% hexane squalene can be separated from HC by several centimetres but it runs with PAH in this system. In HDF (99:1:0.1) squalene runs with HC. The large differences in compound mobility created by relatively small differences in solvent composition also caution against using solvent systems for too many separations and suffering the consequences of composition changes arising from the loss of the more volatile solvents.

HDF (99:1:0.1) produces the most successful first-step separation of the largest possible number of the model classes (Fig. 2). Fig. 2b shows a total scan of the complete range of classes on one of the newer S rods and Fig. 2c shows the partial scan that is normally used, this time on one of the older S rods. Fig. 5a demonstrates the development of the remaining compounds after such a partial scan. This method turns the relative variability in the R_F value of TG mentioned earlier into a distinct advantage. TG is at first kept near the origin (Fig. 2a) and in the second development it is moved well clear of the other classes (Fig. 5a). The short development in chloroform–methanol (2:1) prior to the second scan improves the resolution of MG and PL.

Preliminary analyses of seawater lipids

Fig. 3 shows some preliminary analyses of seawater lipids using these solvent systems. The lipids were extracted from local seawater samples into dichloromethane, and several microlitres of the final concentrate were spotted on the rods. In order to overcome the spreading of compounds due to repeated spotting, an initial development in a polar solvent system to just above the origin is performed. This form of solvent focusing has already been described for total lipid analyses on Chromarods [5]. After this partial development the rods are dried and developed for 35–50 min in the first solvent system.

Fig. 3b and c are the first and second scans on the same rod. The presence of a peak at the R_F of ALC means that for marine lipid analyses it is not possible to use the internal standard that was suggested for plasma lipid quantification[6]. However, PAH, ME, and KET were below the level of detection in this particular analyses. PAH may be expected to be detectable in certain samples and relatively small amounts of ME have been detected in particulate matter (Fig. 3a). However, in all the preliminary analyses performed (dissolved matter, particulate matter, cultured *Phaeodactylum tricornutum* and its exudate) there has been very little FID response in the region oc-

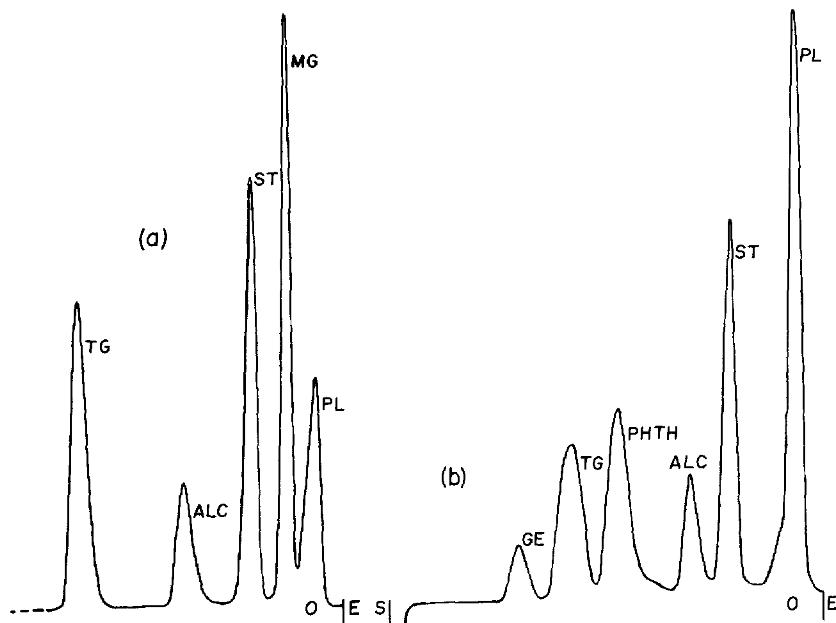

Fig. 5. Second scans after redevelopment in hexane–based solvents. (a) First-step development in HDF (99:1:0.1), second-step development in hexane–diethyl ether (85:15) followed by a 1 cm development in chloroform–methanol (2:1) prior to scanning. (b) First-step development in HDF (99:1:0.2), second-step development in hexane–diethyl ether–acetone (92:4:4).

cupied by saturated ethyl ketones. It is this compound type that is presently being used as an internal standard (Fig. 3a) — note that other ketones are separated from ethyl ketones (Table I). It should also be noted that to date the only means of identifying the peaks in seawater has been by comparing R_F values with the compounds in Table I, by doing some co-spotting, and by comparing these results with the results of seawater analyses at the compound level which have appeared in the literature. Other analyses are in progress in order to confirm the class designations given in Fig. 3.

Some natural samples have FFA peaks which show considerable tailing compared with the model compounds (Fig. 2 and Table I) and in these cases fine tuning of the first solvent system is necessary. Increasing the amount of formic acid in the first solvent system (see solvent systems used for the first-step separations in Fig. 3a and Fig. 5b) moves FFA further from the origin and so reduces the amount of carry-over into the second development. It also means that GE can be separated in the second development (Fig. 5b); however, the quality of the PAH–WE–ME separation is reduced, though the separation between the latter two is still reasonable (Fig. 3a).

Optimisation of the second-step separation

At present, experiments are being conducted on the production of separations of GE, TG, PHTH, ALC, and ST. A good separation of GE, ALC, and ST is shown in Fig. 1; however, to include TG and PHTH in the second step (following a partial scan) it was necessary to use solvent systems other than those described above. ALC runs ahead of TG in non-polar solvent systems, and so to effect a separation in a second

step it is necessary to use a considerably more polar solvent system to reverse this elution order and to move TG well ahead of the other remaining neutral lipids (especially dibutyl phthalate).

The chlorinated solvent system of Christie and Hunter [3] was designed to separate the more polar of the neutral lipid classes. This system, as well as variations of it containing dichloromethane, did not separate GE, TG, and PHTH. Thus, if there is the possibility of significant amounts of PHTH or GE in TG-containing samples which are developed in chlorinated solvent systems, an analysis in a more specific solvent system (*e.g.* those used in Fig. 1 or Fig. 5b) should be performed.

Although the acetone-containing solvent system used in Fig. 5b gives a very good separation of GE, TG, PHTH, ALC and ST, problems were encountered with its use with marine lipid samples; the presence of acetone causes the development of pigments associated with the PL peak (Fig. 3c) away from the point of application. Another problem with finding a second-step development is that the two ubiquitous phthalate esters used as model representative compounds do not run together (Table I) and, as yet, no solvent system has been found which completely resolves both of these from ALC and TG.

Conditioning and ageing of Chromarods

For most of this work Chromarods were stored in their frames in distilled water when not being used for more than an hour or so. Now that humidity chambers have become part of the development scheme (Figs. 2a and 3a) these are also used for rod storage. There has been no noticable difference in rod quality caused by this change. The use of humidity chambers is preferable since the rods do not have to be dried prior to activations scans.

The older set of rods was cleaned in 60% sulphuric acid five times during the course of this study. However, when marine samples are being regularly analysed more frequent cleaning is necessary. Each rod should be acid-cleaned in a test-tube whose dimensions are such that only the ends of the quartz rod touch the glass so that abrasion of the silica coating is minimised. For quantitative work the rods should be returned to their original position in their frame after acid cleaning and rinsing so that the history of each rod of a set is known [7].

The older set of Chromarods has given successful separations beyond the 127th analysis shown in Fig. 2c. Qualitatively (Fig. 2) and quantitatively (Table II) there appears to have been little deterioration in rod performance with ageing. Contrary to previous suggestions[2,3], it was not necessary to change the composition of the developing solvents in order to maintain the quality of the peak separations on this older set of rods.

Chromarods-S and -SII and some aspects of lipid class quantification

In spite of the narrower size range and smaller absolute particle size of Chromarod-SII coatings the separations on these rods are not notably better than the separations on Chromarods-S (Fig. 2). Chromarods-SII did not separate wax ester standards from cholesteryl octadecanoate (Table I). The separation of these two types of esters has been previously reported[8]; however, different solvent systems were used and different lipids were analysed in the latter study.

The peak areas for Chromarods-SII were found to be intermediate between the newer and the older Chromarods-S (Table II). By taking a ratio to the proposed internal standard for marine lipid analyses, the difference between the three rods becomes much smaller (Table II). However, for more quantitative work, the same rod

TABLE II

PEAK AREAS AND RATIOS FOR RODS FROM DIFFERENT SETS

The areas are those of the first six peaks for each FID trace shown in Fig. 2. Peaks were integrated three times on an integrator/calculator with an average standard deviation of ±4% of the mean. Ratios are italicized and are relative to the peak area of 3-hexadecanone.

Rod type	*Previous analyses**	*Areas and ratios*											
		HC		*PAH*		*WE*		*ME*		*KET*		*FFA*	
SI	126	88.8	*0.89*	74.3	*0.74*	169.7	*1.70*	149.1	*1.49*	100.1	*1.00*	121.3	*1.21*
SI	20	55.0	*0.88*	51.5	*0.83*	135.5	*2.17*	97.3	*1.56*	62.4	*1.00*	87.0	*1.39*
SII	none	86.0	*0.98*	75.5	*0.86*	141.4	*1.60*	127.7	*1.45*	88.2	*1.00*	115.0	*1.30*

* The number of previous developments performed on each rod.

type, the same developing times and the same rod cleaning and conditioning should be used since all of these factors are likely to affect peak areas [7].

CONCLUSION

Complex neutral lipid mixtures may be resolved in multi-step separations with partial scanning between developments. Neutral lipids in marine samples can be separated in two steps. The first step involves the use of a non-polar hexane–diethyl ether–formic acid solvent system containing a minimum of 97.5% hexane and a maximum of 0.2% formic acid. In this polarity range triglycerides remain near the origin, and less polar classes (including free fatty acids) are moved away from the point of application. The exact proportions necessary in the first solvent system depend on the nature of the sample; 99:1:0.1 was found to be optimal for model lipid classes. Where there are large tailing peaks from free fatty acids in the sample, slightly more formic acid is necessary to ensure that a near baseline return is obtained at the point at which the scan should be halted prior to the second development. The remaining neutral lipids may then be separated in a more polar solvent system containing between 75 and 92% hexane by volume. In both developments, constant humidity and temperature are important for reproducible separations.

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REFERENCES

1 R.G. Ackman, *Methods Enzymol.*, 72 (1982) 205.
2 J.K.G. Kramer, R.C. Fouchard and E.R. Farnworth, *J. Chromatogr.*, 198 (1980) 279.
3 W.W. Christie and M.L. Hunter, *J. Chromatogr.*, 171 (1979) 517.
4 R.G. Ackman, *Chem. Ind.*, Oct. 17 (1981) 715.
5 H.R. Harvey and J.S. Patton, *Anal. Biochem.*, 116 (1981) 312.
6 P. van Tornout, R. Vercaemst, H. Caster, M.J. Lievens, W. de Keersgieter, F. Soetewey and M. Rosseneu, *J. Chromatogr.*, 164 (1979) 222.
7 C.C. Parrish and R.G. Ackman, *Lipids*, in press.
8 J.K. Kaitaranta and N. Nicolaides, *J. Chromatogr.*, 205 (1981) 339.