Thin-Layer Chromatography–Pyrolysis-Gas Chromatography–Mass Spectrometry: a Multidimensional Approach to Marine Lipid Class and Molecular Species Analysis

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Abstract

A new multidimensional chromatographic method is described in which material separated into lipid-class bands on silica-coated quartz thin-layer chromatography (TLC) rods (Chromarods) is desorbed using a pyrolysis unit interface and introduced directly into a gas chromatograph–mass spectrometer for molecular species analysis. Steryl esters, wax esters, hydrocarbons, ketones, and fatty-acid methyl esters (FAMEs) are thermally desorbed without pretreatment. In order to desorb free sterols, monoacylglycerols (MAGs), aliphatic alcohols, and free fatty acids, the esters are converted to trimethylsilyl derivatives on the rod. Triacylglycerols and phospholipids are converted to FAMEs by thermochemolysis with tetramethylammonium hydroxide. The method's utility is demonstrated with lipids from seawater particulate matter by first confirming the identity of lipid bands with the appropriate standards. The wax ester–steryl ester TLC band contained no more than 8% steryl esters. Wax esters of up to C_{42} are detected. In six individual acyl lipid classes, C_{14}–C_{22} fatty acids are detected with C_{16} acids predominant in all but wax esters. C_{16}–C_{22} MAGs are identified in the complex acetone-mobile polar lipid band. The method successfully extends the scope of Iatroscan TLC–flame-ionization detection on Chromarods, which is a widely used technique for lipid-class analysis. Modification of the pyrolysis probe to handle intact TLC rods is a future objective.

Introduction

In the past fifteen years, Iatroscan (Iatron Laboratories, Tokyo, Japan) thin-layer chromatography (TLC)–flame-ionization detection (FID) has become a widely accepted technique for lipid-class analysis. The analytical system consists of 15-cm quartz rods (Chromarods, Iatron Labs) that are 0.9 mm in diameter and coated with a 75-µm thick layer of porous silica-gel particles (5-µm diameter) sintered into a supporting glass frit (1). The sample is spotted at one end of the rod and developed in the same way as in plate TLC. The rod is then passed through an FID in an Iatroscan instrument, quantitating each separated band by combustion. Partial scanning of the rods and the further development of uncombusted material allow multidimensional separation and detection that is impossible with conventional plate TLC (Figure 1).

When applied to lipid classes in marine-particulate matter or sediments, Iatroscan TLC–FID can distinguish energy storage classes, pollutants, or indicators of organic-matter degradation (2,3) as well as the contributions of specific taxa of organisms to the environmental lipid pool (4,5). Other applications have included using total triacylglycerol (TAG)–sterol ratios to gauge the condition of fish, crustacean, and bivalve larvae (6), thus determining the quality of halibut eggs by quantitating storage lipid content (7) and estimating the total sterols in various seafood (8).

The principal drawback of TLC is its inability to resolve individual closely related molecular species in complex samples. The molecular species analysis of a particular lipid-class—usually done by gas chromatography (GC) or high-performance liquid chromatography (HPLC)—often requires prefractionation, saponification, derivatization, or other treatments (9). A method allowing for the further analysis of individual compounds within a Chromarod band would greatly increase the amount of information available from a Chromarod lipid-class separation and permit confirmation of peak purity.

Sterols present particular difficulties in the TLC–FID method as it is currently practiced. They occur in the marine environment in several different forms of varying polarities. In a typical three-development lipid-class separation on Chromarods used in our laboratory (10), sterol species appeared in several bands (Figure 1). Free sterols were resolved after the second development, and sterol esters eluted with wax esters in the first development.

Various methods have been reported to couple rapid, inexpensive, low-resolution TLC separation directly to the further separation or accurate quantitation by GC or specific identification by mass spectrometry (MS). Direct transfer from TLC to MS has

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been accomplished by Xe^+ bombardment (11) or laser desorption (12–14). Lyle and Tehrani (15,16) introduced polymers and water-soluble vitamins from cut TLC plates into a GC by pyrolysis. In another study, Nishikawa et al. (17) combined plate TLC and pyrolysis GC–MS for the analysis of methylbenactzylum bromide in human urine. In all these studies, the GC step was used primarily for accurate quantitation (already possible in TLC–FID) rather than further separation. Furthermore, these studies relied on TLC as the principal separation step for molecular species instead of taking advantage of its broad profiling capabilities for complex naturally occurring mixtures. Also, the methods have been developed for plate TLC rather than Chromarod TLC.

This study extends the scope of Chromarod TLC by using an analytical pyrolyzer to thermally desorb the lipid-class bands directly into a GC–MS, thus providing further information on the molecular species within each separated class and clarifying the purity and composition of the TLC bands. Sterol-containing bands in Chromarod separations were emphasized in conjunction with our ongoing investigations of marine sterol biogeochemistry. Lipids were either desorbed intact, pyrolyzed in order to give diagnostic breakdown products, or converted in situ to characteristic methyl or trimethylsilyl (TMS) derivatives.

Experimental

Chemicals and glassware

All solvents used for rinsing, diluting, and developing were of trace analysis grade (Optima, Fisher Scientific, Nepean, Canada) except for diethyl ether (ACS-grade, BDH, Toronto, Canada) and acetone (Spectrograde, Caledon, Toronto, Canada). The water that was used in the developments was distilled and deionized (Nanopure II, Barnstead) and triple-extracted with chloroform. Most of the lipid standards were purchased from Sigma (St. Louis, MO), and additional sterols were obtained from Steraloids (Wilton, NH).

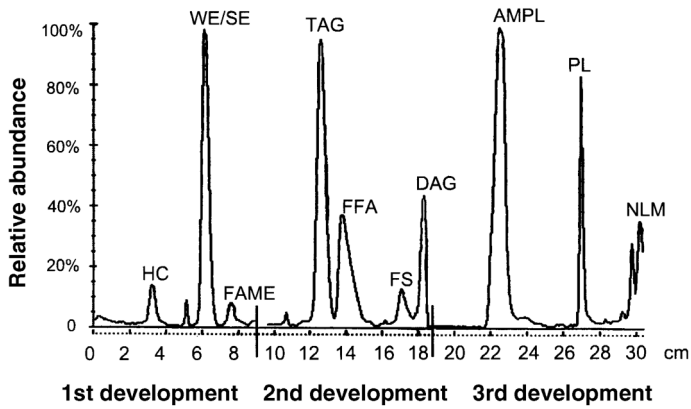

Figure 1. Typical Iatroscan TLC-FID chromatogram of the hydrogenated TLE of settling particles (Station 7, 75-m depth) showing three developments and scans from right to left. Peak identification is based on the elution of representative standard compounds for each lipid class: hydrocarbons, HC; wax esters, WE; steryl esters, SE; fatty-acid methyl esters, FAME; triacylglycerols, TAG; free fatty acids, FFA; free sterols, FS; diacylglycerols, DAG; acetone-mobile polar lipids, AMPL; phospholipids, PL; and nonlipid material, NLM.

Marine samples

As part of a study of marine lipids in Newfoundland coastal waters, settling particles were sampled using sediment traps moored for 28 days at 75- and 100-m depths at an offshore site (Station 7, 48°08.2' N, 53°22.0' W, 363-m water depth) in Trinity Bay, Newfoundland (18). The material was cold-extracted using the Folch method (19) modified for small samples (20). The lipids were recovered in chloroform, and the total lipid extract (TLE) was stored under N_2 at –20°C until analyzed.

Hydrogenation of TLE

Aliquots (1.5 mL) of the TLEs of the sediment-trap samples were hydrogenated with approximately 5 mg of a platinum (IV) oxide catalyst (Sigma) in 4-mL vials. A gentle stream of hydrogen was bubbled through the extracts via stainless steel needles piercing loosely fitted Teflon-faced septa for 20 min, which was followed by vigorous stirring (6 h). This method is equivalent to hydrogenation on a commercial atmospheric-pressure apparatus (21). After catalyst removal, the extracts were evaporated to dryness under N_2 and rediluted to 0.3 mL.

Chromarod development and scanning

Solutions were spotted onto Chromarods below the origin using a 25-µL blunt-tipped #702 syringe with a repeating dispenser or a 10-µL #701 syringe (Hamilton Co., Reno, NV). Prior to development, racks of Chromarods were dipped in acetone until the solvent front reached the origin—a process repeated twice in order to focus spotted material. A three-development three-scan procedure (Figure 1) reported by Parrish (10) was used. The first developments in hexane–diethyl ether–formic acid (99:1:0.05) separated hydrocarbons, wax and steryl (acyl) esters, methyl esters, and ketones. The second developments in hexane–diethyl ether–formic acid (80:20:0.1) separated TAGs, free fatty acids, aliphatic alcohols, free sterols, and diacylglycerols. The final developments were in 100% acetone followed by chloroform–methanol–water (5:4:1), which eluted the acetone-mobile polar lipids (AMPLs), pigments, glycoglycerolipids, monoacylglycerols (MAGs), and phospholipids.

Desorption–GC–MS

A Pyroprobe 120 platinum coil pyrolyzer and GC interface (Chemical Data Systems, Oxford, PA) (Figure 2) was used to desorb lipids from the Chromarod coating and introduce them onto a GC column. The interface was mounted on a HP 5890 II GC and 5971A mass-selective detector (Hewlett-Packard, Palo Alto, CA) fitted with a 25-m × 0.25-mm-i.d. 100% polydimethylsiloxane column (CP-Sil 5CB, 0.12-µm film) (Chrompack, The Netherlands). The MS was operated in the electron-impact mode (70 eV). After chemical treatment (as will be described), sections of the Chromarod (1–2 cm) were placed in a quartz tube in the platinum wire coil of the pyrolysis probe (Figure 2). After the introduction of the probe into the heated interface (250°C), the resealing of the system, and the restoration of the carrier gas (He) pressure, the probe was flash-heated for 10 s at

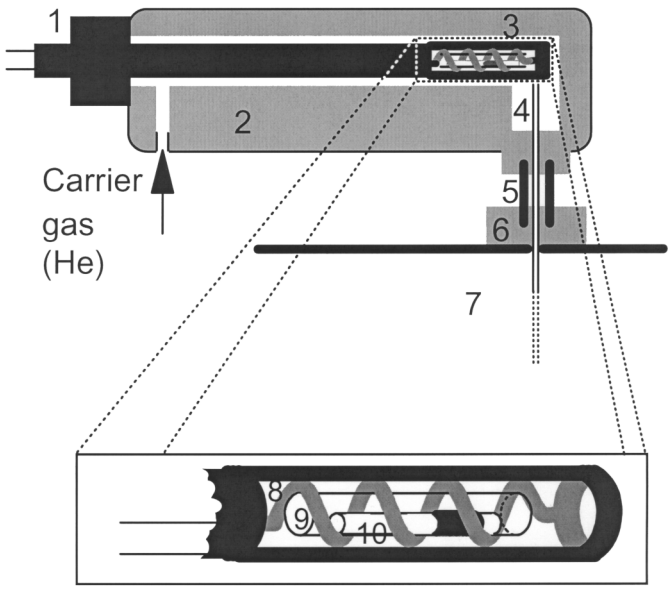

Figure 2. Pyroprobe and interface used to desorb and pyrolyze lipids off silica Chromarod coating: pyroprobe with Pt coil, 1; heated interface, 2; probe tip, 3; capillary GC column threaded into interface, 4; steel transfer tube, 5; modified injector-port nut, 6; GC oven, 7; Pt heating coil, 8; quartz tube, 9; and Chromarod section with lipid band, 10.

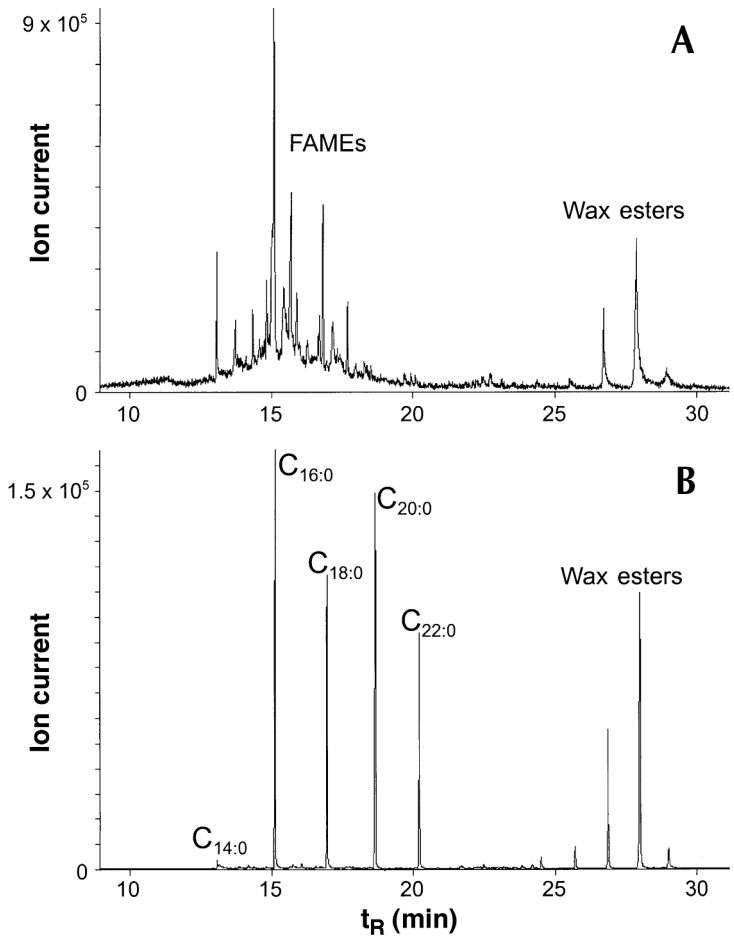

Figure 3. Effect of hydrogenation on the TLE of settling particles (Station 7, 75-m depth). TICs from thermochemolysis with methanolic TMAH for (A) original TLE (42 µg total lipid spotted) and (B) hydrogenated TLE (14 µg total lipid).

300°C for hydrocarbons, free fatty acids, and aliphatic alcohol analysis; 400°C for free sterols, fatty-acid methyl esters (FAMEs), and ketones; 450°C for TAGs and wax and steryl esters; or 500°C for MAGs and phospholipids. The GC injector (heated at 275°C) was used in the split mode. The total carrier flow from the pyrolysis unit was 30 mL/min. An initial column flow of 5 mL/min (1 min) was used to sweep desorbed material rapidly onto the column, after which the flow was dropped to 1 mL/min. Two temperature programs were used. Program A (which was used for steryl esters, wax esters, and free sterols) began at 50°C for 2 min, was raised to 200°C at 30°C/min, raised again to 300°C at 5°C/min, and then held for 5 min. Program B was used for MAGs, aliphatic alcohols, hydrocarbons, ketones, fatty-acid TMS esters, and FAMEs as well as for any bands from marine samples in which compounds with a large range of molecular weights were anticipated. It began at 50°C for 2 min, was raised to 300°C at 10°C/min, and then was held for 5 min.

Initial experiments involved the desorption of standards (1–10 µL) spotted onto 1–2-cm sections of Chromarods and focused into a narrow band as described previously. The rod section was positioned in the coil so that the band was at the end closest to the column head (Figure 2).

In order to isolate individual lipid bands for desorption after development, the Chromarods were etched with a knife and broken by holding them tightly with forceps. The bands were located on the basis of FID scans of adjacent rods spotted with the same sample. For cases in which the band that was required for desorption eluted in the second or third development, material from prior developments was removed from the rod by combustion in the Iatroscan.

Trimethylsilylation

For certain lipid classes containing free hydroxyl or carboxyl groups, TMS ethers or esters were formed by applying 2–3 µL *N,O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Supelco, Bellefonte, PA) to the material on the Chromarod surface. The rod section was then heated in a foil-covered 2-mL vial (60°C, 10–15 min) before desorption and pyrolysis in the Pyroprobe coil.

Thermochemolysis

In order to convert involatile acyl lipids directly to the corresponding FAMEs by thermochemolysis, 5 µL of 5% methanolic tetramethylammonium hydroxide (TMAH, pentahydrate, Sigma) was applied to the bands on Chromarod sections before pyrolysis at 450°C (TAGs) or 500°C (phospholipids).

Results and Discussion

Our interest in settling marine particles stems from their role in transporting biogenic carbon and pollutants from productive surface waters to marine sediments, thereby connecting pelagic and benthic food webs (22) and thus having important implications for commercial fisheries. Furthermore, this transport of atmospheric carbon into sediments influences global climate (23).

Hydrogenation

The hydrogenation of TLEs is valuable in TLC–FID lipid analysis because it improves peak shapes, increases response, decreases peak splitting that may arise because of different degrees of unsaturation within a lipid class, and improves the stability of lipid extracts in regards to oxidation (24). Hydrogenation gives a clearer picture of the acyl carbon-number distribution of settling particulate lipids (as can be seen in Figure 3B), which shows the total ion chromatogram (TIC) of the rod-spotted hydrogenation treated by TMAH thermochemolysis as compared with the original TLE sample in Figure 3A. Tailing of the wax ester peaks is reduced, presumably because of the existence of fewer molecular species within each peak. This approach is particularly useful for cases in which many species are expected in a certain lipid class band with each individually being below the detection limit. Hydrogenation combines these species into fewer peaks while still allowing useful carbon-number information to be obtained. Yang et al. (21) also used this approach to improve resolution in the direct GC analysis of marine neutral lipids. The persistence of some wax esters also illustrates that they are not fully decomposed by TMAH thermochemolysis.

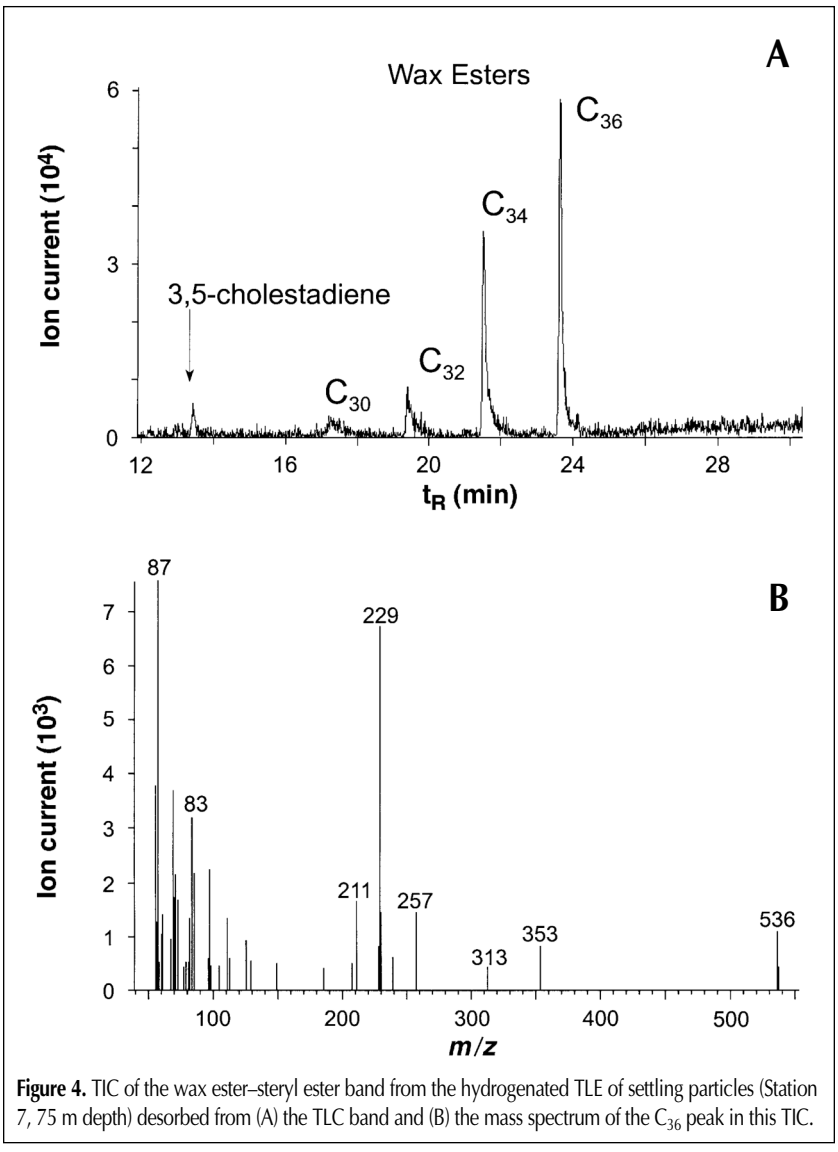

Figure 4. TIC of the wax ester–steryl ester band from the hydrogenated TLE of settling particles (Station 7, 75 m depth) desorbed from (A) the TLC band and (B) the mass spectrum of the C_{36} peak in this TIC.

Steryl and wax esters

Two cholesteryl esters (cholesteryl hexadecanoate and cholesteryl eicosanoate) both yielded a pyrolysis product (cholesta-3,5-diene) as a result of deacylation. Thus, for steryl esters, a product diagnostic of the sterol moiety can be obtained. The intact steryl esters were not observed, nor were any products attributable to the acyl moieties. Intact steryl esters can be separated at high temperatures with GC columns (25) or by carbon number on short (5 m) columns (21).

Wax-ester standards—16:0/16:0 (0.5 µg), 18:0/ 16:0 (0.5 µg), and 18:0/18:0 (1 µg) in which x:y/x:y indicate the number of carbons (x) and double bonds (y) in the alkyl and acyl portions, respectively—were desorbed from the Chromarod surface intact.

The wax ester–steryl ester band of hydrogenated particulate lipids (75 m) revealed that the contribution from steryl esters was small (approximately 8% of the total band based on the percentage peak area of TIC) and a direct result only of C_{27} sterols (cholestadiene peak) (Figure 4A). This was consistent with a marine source for steryl esters (26). The wax-ester carbon-number distribution increased from C_{30} to C_{36}, above which no other wax esters were observed. This distribution differed not only from the more normal one reported by Wakeham and Frew (25), but also from the standard wax esters analyzed (detected by desorption of spotted and unseparated material, 14-g TLE) in which wax esters with molecular masses up to 564 amu (C_{38}) were detected. At a higher sample load (42-µg TLE) on an undeveloped rod section, traces of the wax esters up to 620 amu (C_{42}) were detected, although spotting this much material reduces TLC resolution. When Chromarods are developed, each individual wax ester may not be uniformly distributed throughout the band, and if the full band is not included in the section cut from the rod, this would bias the distribution of wax esters seen by desorption. The scanning of an intact rod by pyrolysis would alleviate this problem.

The mass spectra in each GC peak contained prominent $[RCO_2H_2]^+$ ions of several chain lengths (in which R was derived from the wax-ester acyl moiety), demonstrating that each carbon number (Figure 4B) contained several alkyl/acyl combinations (25). Assuming equal MS

ionization efficiencies for all of these, the C_{36} wax ester ($M^+ = 536$) (Figure 4B) was mainly C_{22}/C_{14} (66%) ($[RCO_2H_2]^+ = 229$) with some contribution from C_{24}/C_{12} (16%) ($[RCO_2H_2]^+ = 211$) and C_{20}/C_{16} (14%) ($[RCO_2H_2]^+ = 257$). The C_{34} peak contained in decreasing order of abundance of C_{20}/C_{14} (75%), C_{16}/C_{18} (16%), and C_{18}/C_{16} (6%). C_{16}/C_{16} (57%) and C_{14}/C_{18} (37%) predominated among the C_{32} esters. These were in good agreement with the ratios for each peak in the undeveloped extract.

Free sterols

Treatment of the sterol standard cholestanol with BSTFA after spotting and before desorption gave a clear peak for sterol TMS ethers. C_{27} sterols were the most abundant sterols in the trimethylsilylated sterol band obtained from a hydrogenated TLE of settling marine particles (100-m depth) with smaller amounts of C_{28}, C_{29}, and C_{26} sterols present (Figure 5A). The TMS ether mass spectra (Figure 5B) revealed that hydrogenation had not reduced the $\varnothing^5$ double bond in the steroid nucleus, as was also found by Yang et al. (21) using this procedure. Therefore, the small amounts of cholestanol, 24-methylcholestanol, and 24-ethylcholestanol in the chromatogram were present in the original unhydrogenated sample (an observation confirmed by traditional saponification/derivatization GC analysis).

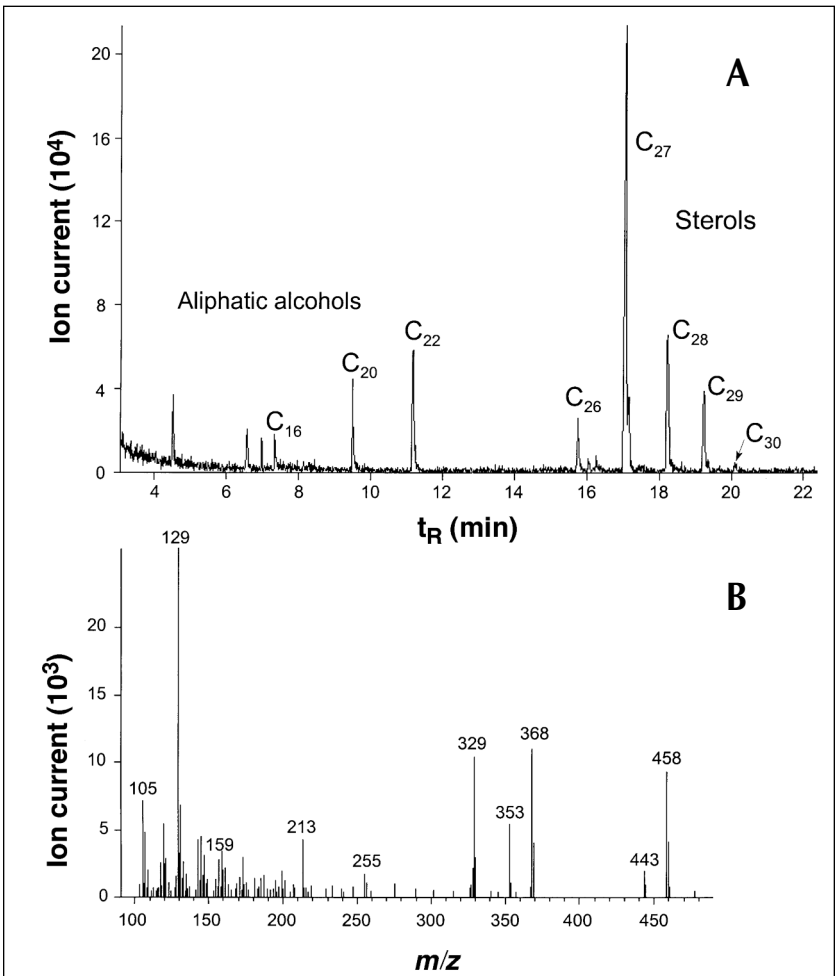

Figure 5. TIC of the free sterol band from the hydrogenated TLE of settling particles (Station 7, 100-m depth) desorbed from a Chromarod as TMS ethers (A) and the mass spectrum of the C_{27} peak in this TIC (B).

TAGs

The desorption of TAG standards illustrated the value of thermochemolysis with TMAH. The thermal desorption of untreated TAGs was unsuccessful up to 500°C. Moreover, tristearin (trioctadecanoylglycerol) did not elute from the GC column that was employed at a temperature compatible with the stationary phase (325°C) when injected. Methanolic TMAH treatment of the TAG standards tricaprin (tridecanoylglycerol), tristearin, and triarachidin (trieicosanoylglycerol) on a Chromarod section yielded capric (decanoic), stearic (octadecanoic), and arachidic (eicosanoic) acid methyl esters with almost complete conversion.

The C_{16} FAME was the most common in the TAG band from the 75-m settling particles thermochemolyzed with TMAH, which had C_{18} and C_{20} FAMEs nearly as abundant and smaller amounts of C_{14} and C_{22} FAMEs present. The degree of unsaturation for the fatty acids was difficult to determine using TMAH as the hydrolysis/methylating reagent. Studies have shown that the strong basic conditions result in the partial isomerization of polysaturated fatty acids (26).

Hydrocarbons

Although standard *n*-nonadecane was readily desorbed after the first TLC development, very few hydrocarbons could be clearly detected and identified in the Chromarod-separated settling particulate lipids from 75- or 100-m depths. In the 100-m chromatogram, phytane (2,6,10,14-tetramethylhexadecane), a degradation product of algal pigments, was present along with an earlier hydrocarbon peak at *m/z* 268 in the mass spectrum—possibly pristane (2,6,10,14-tetramethylpentadecane), which is a further breakdown product of phytane.

Free fatty acids

Hexadecanoic and octadecanoic acids could not be desorbed directly from Chromarod sections by temperatures up to 400°C. This was likely because of the strong absorption of the carboxyl groups onto the polar silica. Conversion to their TMS esters using BSTFA allowed both acids to be desorbed in good yields. In the free fatty-acid band from the TLE of settling particles at the 75-m depth, C_{16} acids predominated (66% of total-assuming equal MS detection efficiencies for all acids) with smaller amounts of C_{18} (14%) and C_{20} (8%) acids and minor amounts of C_{14} (3%), C_{15} (4%), and C_{17} (4%) acids also present. The two peaks having mass spectra consistent with a C_{15} acid TMS ester could have been because of *iso* and *anteiso* branched C_{15} fatty acids, both of which are bacterial markers (27).

Ketones, aliphatic alcohols, and methyl esters

Hexadecan-3-one and *n*-hexadecanol standards were desorbed after the first and second TLC developments, respectively. The ketone was desorbed without further treatment, and the alcohol had to be converted to its TMS ether with BSTFA

before desorption analysis. Neither of these classes were studied further. Some aliphatic alcohols (C_{16}, C_{20}, and C_{22}) were detected as TMS ethers in the free sterol TLC band (Figure 5), reflecting the difficulty of accurate band location and thus the need to develop a system that accommodates intact Chromarods.

FAMEs occur naturally in the marine environment (2). Iatroscan TLC–FID detected these as a minor lipid class in 75-m settling particles (Figure 1). After the development of unhydrogenated lipids from 75-m settling particles, desorption of the FAMEs revealed C_{14}, C_{16}, C_{18}, and C_{20} species with comparable quantities of saturated and mono-unsaturated C_{16} FAMEs. Also, the C_{18} and C_{20} esters were largely mono-unsaturated. It is possible that any polyunsaturated species present underwent thermal degradation (26).

AMPL

The complex AMPL peak was found in the Iatroscan TLC–FID groups glycoglycerolipids, pigments, and MAGs. 1-Monopalmitin (1-monopalmitoyl-*rac*-glycerol), a standard for the AMPL peak in TLC-FID, was successfully desorbed as its di-TMS ether. Analysis of the AMPL band of hydrogenated lipids from settling particles at 100 m after BSTFA treatment gave a range of 1-MAG di-TMS ethers with acyl groups ranging from C_{16} to C_{22} (Figure 6). No TMS products as a result of 2-MAGs were observed.

To date, the AMPL band in TLC–FID analyses of lipids from settling marine particles has been used to gauge primary productivity because it contains glycoglycerolipids and photosynthetic pigments attributable to phytoplankton (2). However, the presence of MAGs suggests that all lipids in the AMPL band cannot always be unambiguously attributed to algae. MAGs may be formed by the breakdown of TAGs, diacylglycerols, or other acyl lipids and may therefore indicate bacterial activity or other degradations of organic matter.

In addition to MAGs, two compounds in the AMPL band (peaks A and B of Figure 6) have sterol characteristics (*m/z* 215, 257, and 255), which raises the intriguing possibility of sterol-containing species in this band. Steryl chlorin esters, which are products of zooplankton herbivory (28), may be very similar to pigments in their elution behavior on Chromarods and may give a sterol moiety on thermal cleavage.

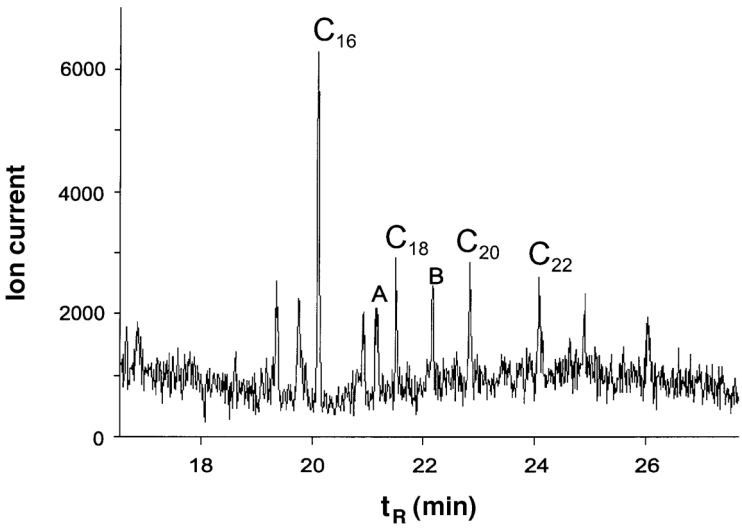

Figure 6. TIC for the AMPL band from settling particles (100-m depth) after BSTFA treatment showing C_{16}–C_{22} di-TMS ethers of 1-MAGs. Peaks A and B are unidentified steroids.

Phospholipids

Phospholipids could not be desorbed without further treatment. This was likely because of their very polar head groups. Thermochemolysis with TMAH yielded FAMEs from the phospholipid acyl groups, with the treatment of dihexadecanoylphospatidylcholine giving an abundant C_{16} FAME peak. To date, only phosphatidylcholine and phosphatidylethanolamine standards have been investigated. The phospholipid band of hydrogenated settling particulate lipids (100 m) that were desorbed in this way showed a maximum at C_{16} in its carbon-number distribution with roughly equal amounts of C_{14}, C_{18}, C_{20}, and C_{22} present at approximately one-third this level. Very low levels of C_{14}, C_{16}, and C_{18} FAMEs were detected in the phospholipid TLC band at 75 m.

Conclusion

Ten neutral and polar lipid classes were desorbed from silica-coated Chromarods for direct GC–MS analysis. Different classes were either desorbed without further treatment (allowing for the detection of intact molecules or diagnostically useful cleavage products) or converted to either TMS derivatives on the rod using BSTFA or FAMEs using TMAH. This allowed for the quantitative or semiquantitative analysis of separated lipid bands and was particularly useful with those containing more than one lipid class (wax and steryl ester, AMPL), as has been demonstrated with lipids from seawater particulate matter. Furthermore, the fatty-acid chain lengths that were present in six individual acyl lipid classes were revealed, which is information unobtainable by transmethylation of the whole TLE. Future improvements will include modifying the Pyroprobe interface to accommodate an intact rod and desorb each band by sequential heating in different places along its length, thus preserving the rod for repeated use, which is one of the major inherent advantages of Chromarod TLC (10). The use of a shorter GC column with high-temperature capability will be adopted in order to more rapidly scan the intact rods. A wider range of derivatization reagents will also be studied to identify those that produce readily desorbed and identifiable derivatives without isomerization and those allowing the desorption of currently recalcitrant lipid classes such as diacylglycerols.

Acknowledgments

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References

1. R.G. Ackman, C.A. McLeod, and A.K. Banerjee. An overview of analyses by Chromarod-Iatroscan TLC–FID. *J. Planar Chromatog.* **3:** 450–62 (1990).
2. C.C. Parrish. Dissolved and particulate marine lipid classes: a review. *Mar. Chem.* **23:** 17–40 (1988).
3. J.K. Volkman and P.D. Nichols. Applications of thin layer chromatography–flame ionization detection to the analysis of lipids and pollutants in marine and environmental samples. *J. Planar Chromatogr.* **4:** 19–26 (1991).
4. J.K. Volkman, D.A. Everitt, and D.I. Allen. Some analyses of lipid classes in marine organisms, sediments and seawater using thin-layer chromatography–flame ionization detection. *J. Chromatogr.* **356:** 147–62 (1986).
5. M. Goutx, C. Gerin, and J.C. Bertrand. An application of Iatroscan thin-layer chromatography with flame ionization detection—Lipid classes of microorganisms as biomarkers in the marine environment. *Org. Geochem.* **16:** 1231–37 (1990).
6. A.J. Fraser. Triacylglycerol content as a condition index for fish, bivalves, and crustacean larvae. *Can. J. Fish. Aquat. Sci.* **46:** 1868–73 (1989).
7. E.S. Daniel, C.C. Parrish, D.C. Somerton, and J.A. Brown. Lipids in eggs from first-time and repeat spawning Atlantic halibut, *Hippoglossus hippoglossus* (L.). *Aquaculture Fish. Management* **24:** 187–91 (1993).
8. C.G. Walton, W.M.N. Ratnayake, and R.G. Ackman. Total sterols in seafoods: Iatroscan TLC/FID versus Kovacs GLC/FID method. *J. Food Sci.* **54:** 793–95 (1989).
9. R. Wood. *Analysis of Fats, Oils and Lipoproteins.* E.G. Perkins, Ed. American Oil Chemists Society, Champaign, IL, 1991, pp. 236–69.
10. C.C. Parrish. Separation of aquatic lipid classes by Chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection. *Can. J. Fish. Aquat. Sci.* **44:** 722–31 (1987).
11. Y. Kushi and S. Handa. Direct analysis of lipids on thin layer plates by matrix-assisted secondary ion mass spectrometry. *J. Biochem.* **98:** 265–68 (1985).
12. A.I Gusev, O.J. Vasseur, A. Proctor, A.G. Sharkey, and D.M. Hercules. Imaging of thin-layer chromatograms using matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **67:** 4565–70 (1995).
13. L. Ramaley, M.-A. Vaughan, and W.D. Jamieson. Characteristics of a thin-layer chromatogram scanner-mass spectrometer system. *Anal. Chem.* **57:** 353–58 (1984).
14. T. Fanibanda, J. Mills, and J. Gormally. Thin layer chromatography–mass spectrometry using infrared laser desorption. *Int. J. Mass Spectrom. Ion Processes* **140:** 127–32 (1994).
15. S.J. Lyle and M.S. Tehrani. Pyrolysis-gas chromatography of separated zones on thin-layer chromatograms. I. Apparatus and method. *J. Chromatogr.* **236:** 25–30 (1982).
16. S.J. Lyle and M.S. Tehrani. Pyrolysis-gas chromatography of separated zones on thin-layer chromatograms. II. Application to the determination of some water-soluble vitamins. *J. Chromatogr.* **236:** 31–38 (1982).
17. M. Nishikawa, M. Tatsuno, S. Suzuki, and H. Tsuchihashi. Analysis of methylbenactyzium bromide in human urine by thin-layer chromatography and pyrolysis gas chromatography. *Forensic Sci. Int.* **29:** 197–203 (1991).
18. C.C. Parrish. Lipid biogeochemistry of plankton, settling matter and sediments in Trinity Bay, Newfoundland. I. Lipid classes. *Org. Geochem.* **29:** 1531–45 (1998).
19. J. Folch, M. Lees, and G.H. Sloane-Stanley. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509 (1957).
20. C.C. Parrish. *Lipids in Freshwater Ecosystems.* M.T. Arts and B.C. Wainman, Eds. Springer-Verlag, New York, NY, 1999, pp. 4–20.
21. Z. Yang, C.C. Parrish, and R.J. Helleur. Automated gas chromatographic method for neutral lipid carbon number profiles in marine samples. *J. Chromatogr. Sci.* **34:** 556–68 (1996).
22. J.C. Colombo, N. Silverberg, and J.N. Gearing. Biogeochemistry of organic matter in the Laurentian Trough. I. Composition and vertical fluxes of rapidly settling particles. *Mar. Chem.* **51:** 277–88 (1996).
23. J.L. Sarmiento and E.T. Sundquist. Revised budget for the oceanic uptake of anthropogenic carbon dioxide. *Nature* **356:** 589–93 (1992).
24. N.C. Shantha. Thin-layer chromatography–flame ionization detection Iatroscan system. *J. Chromatogr.* **624:** 21–35 (1992).
25. S.G. Wakeham and N.W. Frew. Glass capillary gas chromatography–mass spectrometry of wax esters, steryl esters and triacylglycerols. *Lipids* **17:** 831–43 (1982).
26. D. Jun-Kai, J. Wei, Z. Tian-Zhi, S. Ming, Y. Xiao-Guang, and F. Chui-Chang. The effect of isomerization and degradation of polyunsaturated fatty acids from oils by different volume proportions of tetramethylammonium hydroxide in thermally assisted hydrolysis and methylation. *J. Anal. Appl. Pyrol.* **42:** 1–8 (1997).
27. P. Scribe, J. Fillaux, J. Laureillard, V. Denant, and A. Saliot. Fatty acids as biomarkers of planktonic inputs in the stratified estuary of the Krka River, Adriatic Sea: relationship with pigments. *Mar. Chem.* **32:** 299–312 (1991).
28. P.J. Harradine, P.G. Harris, R.N. Head, R.P. Harris, and J.R. Maxwell. Steryl chlorin esters are formed by zooplankton herbivory. *Geochim. Cosmochim. Acta.* **60:** 2265–70 (1996).

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