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ALL-*CIS*-3,6,9,12,15-OCTADECAPENTAENOIC ACID: A PROBLEM OF RESOLUTION IN THE GC ANALYSIS OF MARINE FATTY ACIDS

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Key Word Index—Octadecapentaenoic acid; gas-liquid chromatography; phytoplankter fatty acids; marine lipids; hydrazine reduction.

Abstract—The GC properties of 18:5*n*3 (all-*cis*-3,6,9,12,15-octadecapentaenoic acid) of algal origin are described for analysis on the popular polyglycol liquid phase Carbowax-20M 'bonded' in flexible fused silica open-tubular columns. It is shown with oyster lipids that this fatty acid could be confused with early eluting 20:1 isomers such as the 20:1*n*11, common in marine animal oils and lipids, and coincides exactly with 20:1*n*15 of plant origin. Partial hydrazine reduction is demonstrated as a useful technique for confirming the structure of 18:5*n*3.

INTRODUCTION

The existence of a C_{18} fatty acid with five ethylenic bonds in marine lipids was unsuspected until the last decade despite considerable earlier efforts on the identification of marine fatty acids [1]. The tentative structural identification of an octadecapentaenoic acid in the cultured unicellular alga *Prorocentrum minimum* was a rather complex exercise [2] in partial hydrazine reduction and open-tubular GC. A more thorough demonstration [3] of the structure confirmed the initial view of an all *cis*-3,6,9,12,15,-octadecapentaenoic acid (18:5*n*3).

Concentration of marine oils is currently of much interest because of the rapid development of activity of marine polyunsaturated fatty acids, especially eicosapentaenoic acid (20:5*n*3), as inhibitors of human cardiovascular problems [4, 5]. The structural similarity of 18:5*n*3 and 20:5*n*3 will lead to enrichment of 18:5*n*3 when fatty acids or esters are concentrated by most processes, especially urea complexing, but it is not commonly listed in such concentrates (e.g. [6, 7]).

It is important that all possible polyunsaturated fatty acids in fish or shellfish lipids, and in fish oils or in concentrates of fish oils, be identified when they are fed in nutritional or biochemical studies. Primarily, this means by GC, but confirmation by other means is highly desirable. GC combined with mass-spectrometry (GC/MS) is an attractive alternative technique, but fatty acids and their simple alkyl esters suffer rearrangement of the double bonds under electron impact ionization conditions, and give spectra with extensive fragmentation which cannot be interpreted to locate the orignal double bond. To overcome this problem several derivatives of fatty acids were proposed in the literature [8], which are excellent for double bond location in single fatty acids of enhanced purity. However, those derivatives are not helpful for mixtures of fatty acids, especially if the fatty acid of interest is present as a minor component in the mixture, as in the case of 18:5*n*3. The reason for this is that these derivatives are too polar to allow satisfactory separation by GC.

GC on capillary columns is obviously the ideal technique for identification of 18:5*n*3. But unfortunately, no reference 18:5*n*3 is available from supply houses and GC also presents several problems which are addressed in this report.

Newer polar liquid phases have replaced the BDS (butanediol succinate polyester) for which 18:5*n*3 retention data had been tabulated earlier [2]. In addition, it has been proposed that 'bonded' Carbowax 20-M, wall-coated in flexible fused silica columns, be made the standard liquid phase and column for the identification of marine fatty acids [9–11]. We have, therefore, compared the retention behaviour of 18:5*n*3 on this low-polarity liquid phase in the form of bonded and cross-linked coatings in columns from two suppliers. Publications of the GC elution behaviour of 18:5*n*3 relative to other C_{18} fatty acids [2] are still valid, since such elution patterns are consistent on polar liquid phases [12]. The important overlap of 18:5*n*3 with C_{20} fatty acids on SILAR-5CP observed a decade ago [13] is still applicable since the phase continues in use in WCOT open-tubular GC. The original Apiezon retention elution order of Ackman *et al.* [2], also illustrated for the whole C_{18} group of fatty acids by Mayzaud *et al.* [13], can be assumed to be followed by the now very stable 'bonded' wall-coated flexible fused silica columns with methyl silicones as the liquid phase.

RESULTS AND DISCUSSION

The marine microalga *Isochrysis galbana* is widely used in aquaculture due to its good food value and its

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tolerance for a wide range of nutrient concentrations and salinity conditions [14].

The 3,6,9,12,15-octadecapentaenoic acid was detected in all the samples of *I. galbana*, typically at *ca* 3% of the total fatty acids. Equivalent chain lengths (ECL) were calculated for most of the fatty acids detected in *I. galbana* for two different chromatographic columns: DB-Wax and SUPELCOWAX-10 (Table 1).

Figure 1a is a chromatogram of the total lipid fatty acid methyl esters (FAME), showing the characteristic elution order for the proposed 18:5*n*3 and other identified compounds. After isolation by silver nitrate thin layer chromatography ($AgNO_3$–TLC) of the basic pentaenoic/hexaenoic band (which also contains some 6,9,12,15-octadecatetraenoic acid (18:4*n*3), the fatty acids were both partially reduced using hydrazine [15], and totally reduced by catalytic hydrogenation on PtO_2. Figure 1b shows a chromatogram corresponding to the original pentaenoic/hexaenoic band and Fig. 1c shows the FAME pattern of the same material after the hydrazine reduction. Hydrazine is a mild, homogenous reducing agent for ethylenic bonds and does not affect the geometry and position of the remaining bonds [2, 15]. Therefore, partial reduction of polyunsaturated fatty acids with hydrazine is a useful chemical technique for the analysis of unsaturated fatty acids. During the hydrazine reduction, a series of less unsaturated fatty acids were generated not only from 18:5*n*3 but also from the 18:4*n*3 present in the sample. The chromatogram also shows 22:5*n*6 and 22:6*n*3 and their reduction products. When comparison of the 18:5 products is made with the 18:4*n*3 area products, an extra peak is observed in the presumed tetraenoic acid region, with an ECL value of 19.95 (using SUPELCOWAX-10). This extra peak should be another 18:4 (different from 18:4*n*3) generated from the 18:5 fatty acid during its partial reduction. It is presumed to be *cis*-3,9,12,15-octadecatetraenoic acid since a *cis*-3 ethylenic bond confers a long retention time (R_t), as does the 9,12,15 'group'. Most other isomers can be excluded by calculation of R_t [2].

Table 1. Comparison of equivalent chain length values determined for total fatty acid methyl esters of *I. galbana* using two different columns based on Carbowax-20M

Fatty acid	DB-Wax*	SUPELCOWAX-10†
12:0	12.00	12.00
14:0	14.00	14.00
15:0	15.00	15.00
16:0	16.00	16.00
16:1*n*9	16.20	16.26
16:1*n*7	16.26	16.32
16:1*n*5	16.43	16.47
16:2*n*6	16.69	16.71
16:2*n*4	16.89	16.92
16:3*n*4	17.23	17.24
16:4*n*3	17.64	17.57
17:0	17.00	17.00
18:0	18.00	18.00
18:1*n*9	18.22	18.25
18:1*n*7	18.29	18.30
18:1*n*5	18.45	18.35
18:2*n*7	18.65	18.39
18:2*n*6	18.68	18.66
18:3*n*6	19.02	19.01
18:3*n*3	19.33	19.31
18:4*n*3	19.64	19.54
20:0	20.00	20.00
18:5*n*3	20.14	20.15
20:2*n*6	20.54	20.67
20:3*n*6	20.92	20.94
20:4*n*6	21.13	21.13
20:4*n*3	21.65	21.57
20:5*n*3	21.95	21.75
22:0	22.00	22.00
22:5*n*6	23.32	23.37
22:5*n*3	23.90	23.73
22:6*n*3	24.10	24.01

* Durabond-wax fused silica capillary column 35 m × 0.25 mm, 210°.

† SUPELCOWAX-10 fused silica capillary column 28 m × 0.25 mm, 170°.

A specific feature of hydrazine reduction, which may be used to confirm the 18:5*n*3 structure, is that before the partial reduction, (see Fig. 1b) the 18:5*n*3/18:4*n*3 ratio was >1, whereas after reduction (Fig. 1c, above) the ratio became <1. This can be explained by, (i) some additional 18:4*n*3 has been generated during the reduction and/or (ii) the peak of interest (i.e. 18:5*n*3) has a higher reduction rate than that of 18:4*n*3, in part due to the presence of an additional double bond [15]. Both interpretations support a 18:5 structure.

Additionally, and confirming this hypothesis, it was demonstrated that during the hydrazine reduction of unsaturated fatty acids of marine origin [15], *cis*-ethylenic bonds at the Δ^3 position (specifically that ethylenic bond which marks the difference between 18:5*n*3 and 18:4*n*3) are very rapidly reduced.

Figure 1d shows the complete hydrogenation of the $AgNO_3$–TLC band for 18:5*n*3 and 20:5*n*3. The generation of a single peak with a R_t corresponding to that of 18:0 is also suggestive that the 1b peak designated as 18:5*n*3 is indeed a C_{18} fatty acid. This relationship is semi-quantitatively valid, since the area ratio C_{22}/C_{18} in Fig. 1b is similar to the ratio 22:0/18:0 in Fig. 1d (1.50 and 1.79 respectively). The positions of saturated standards for reference are indicated in Fig. 2 and were obtained from a subsequent co-injection of authentic materials.

There is no apparent reason for the presence of 22:5*n*6 in a lipid mixture of fatty acids dominated by the *n*3 series. However, there has been speculation that fatty acids are sometimes elongated by four carbon atoms [16, 17], so a conversion of 18:2*n*6 to 22:5*n*6 could be a parallel to the more obvious 18:3*n*3 to 22:6*n*3 conversion. There are four novel peaks in Fig. 1c between 22:5*n*6 and 22:5*n*3. These could be (not necessarily in order) the four 22:5 fatty acids lacking one bond from the methylene- interrupted systems, viz: 4, –, 10, 13, 16, 19; 4, 7, –, 13, 16, 19; 4, 7, 10, –, 16, 19; and 4, 7, 10, 13, –, 19.

It is important to note that the R_t of 18:5*n*3 on capillary columns, and under chromatographic conditions similar to those used in the present study, leads to it eluting after the 20:0. This is a very complex area of the FAME chromatography of marine lipids as not only do several 20:1 isomers occur, but a group of non-methylene-interrupted dienes are also often present [18]. The

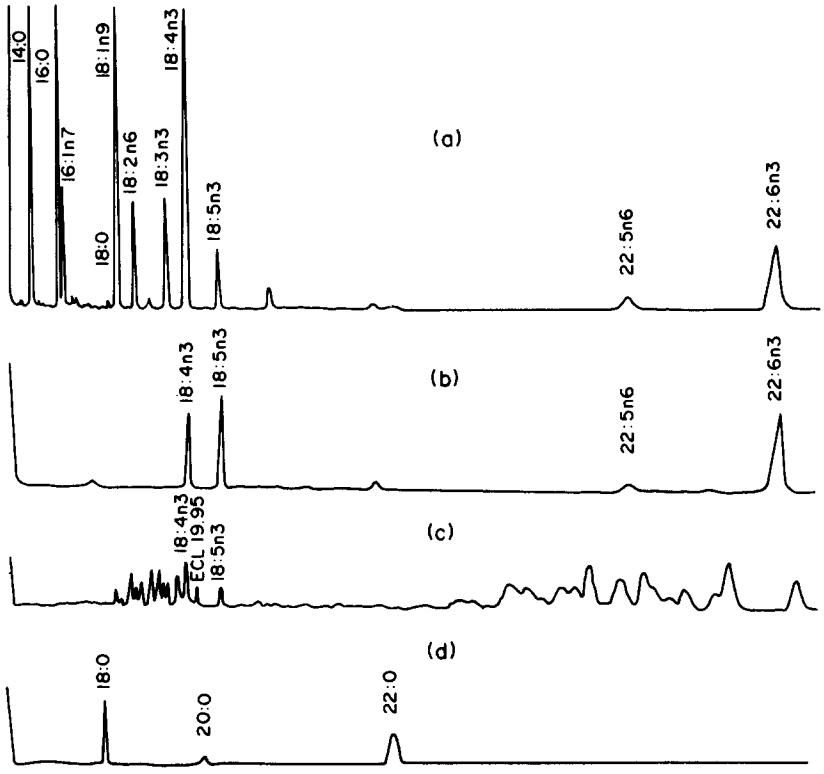

Fig. 1. Chromatogram of *Isochrysis galbana* on a SUPELCOWAX-10 column, 170° and 20 psig He. (a) Section of a total lipid FAME chromatogram, (b) FAME extracted from the pentaene- and hexaene-rich band after $AgNO_3$-TLC, (c) and (d) are the respective reduction products of the FAME shown in (b); (c) partial hydrazine reduction products, (d) complete hydrogenation over H_2/PtO_2.

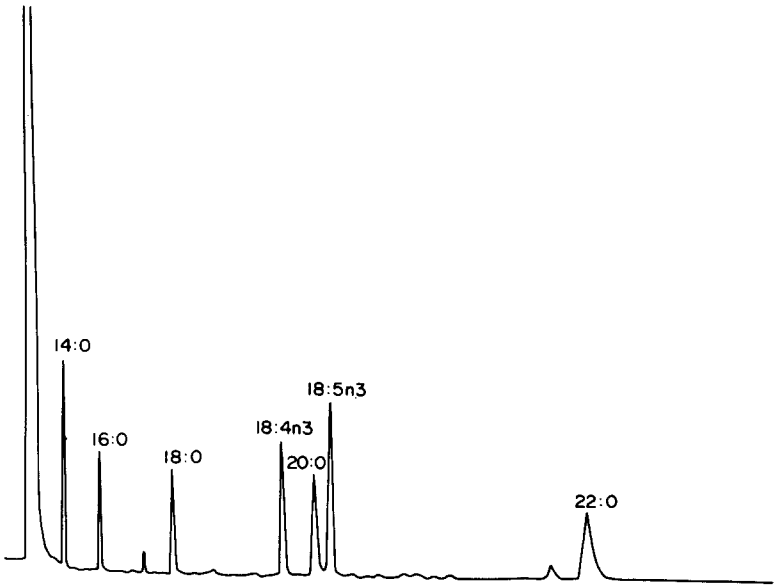

Fig. 2. Section of a Chromatogram obtained by co-injection of 18:4*n*3, 18:5*n*3 (from the pentaenoic acid-rich band of a $AgNO_3$-TLC plate) and some saturated standards, showing their relative position. GC conditions as Fig. 1.

resolution of 18:5*n*3 and 20:1 obviously would be much poorer by packed column GC than by capillary GC.

To illustrate the overlap problem in capillary GC, a sample containing 18:5*n*3 isolated from *I. galbana* was co-injected with the fatty acids of an adult European oyster *Ostrea edulis*. This organism has virtually no

18:5n3, but has moderate amounts of 20:1, the major isomers being n11, n9 and n7. Figure 3 is a section of the total FAME chromatogram obtained by the co-injection of FAMEs from both *I. galbana* and *O. edulis*. This chart clearly shows that 18:5n3 partially overlaps with 20:1n11.

Analyses of fish oil under our conditions have always shown 20:1n11 (ECL 20.16 on SUPELCOWAX-10) as a shoulder on the front of the 20:1n9 (ECL 20.20) component. An experienced eye can often detect the 'doubling' of the Gaussian curve for 20:1n11 if 18:5n3 is present. This resolution problem persists even at temperatures as low as 140°.

In some marine invertebrates 20:1n15 is observed as a species specific indicator fatty acid [19] and small amounts may be found in depot fats of carnivores [20]. When the total FAME of *I. galbana* were co-injected with a sample of FAME from the seed oil of *Limnanthes* sp., a plant with high content of 20:1n15 [21], it was observed that 18:5n3 and 20:1n15 eluted together producing a single sharp peak in the absence of 20:1n11. In an analysis [13] of marine invertebrate lipids on the cyanosilicone SILAR-5CP it appears that the same problem is presented by this chemically different type of liquid phase. SILAR-5CP in WCOT columns has *ca* the same polarity as Carbowax-20M [9]. An alternative low-polarity cyanosilicone liquid phase, Supelco SP-2300, in an efficient WCOT glass column, showed [19] two minor and only partially resolved peaks between 20:1n15 and 20:1n9, and possibly these are, respectively, 18:5n3 and 20:1n11. Therefore, it is possible to erroneously identify 18:5n3 as a single compound, perhaps as an isomer of 20:1, depending on the extent of chain length overlap as governed by polarity of liquid phase [12].

Due to the poor resolution of 18:5n3 from the 20:1 isomers even on a capillary column, the presence of small amounts of this fatty acid in marine lipids could have been overlooked in some samples, and therefore 18:5n3 may have a wider distribution than reported in the literature. If a significant peak is detected in the 18:5n3, 20:1n15, or 20:1n11 positions, it is necessary to explore the presence or absence of 18:5n3 by other supporting physical and chemical techniques, especially $AgNO_3$-TLC and catalytic hydrogenation.

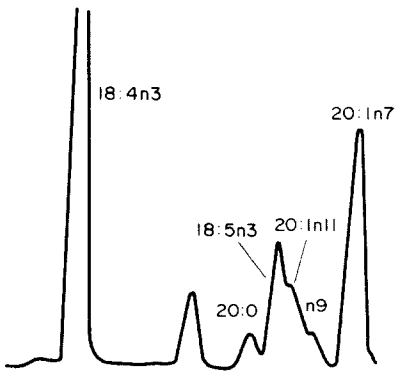

Fig. 3. Section of a chromatogram obtained by co-injection of total FAME from *I. galbana* and from *O. edulis*, showing the position of 18:5n3 with respect to the 20:1 isomers. GC conditions as Fig. 1.

Higher polarity open-tubular GC columns do not necessarily solve the identification problems. The increased overlap of chain lengths [22] will simply move the 18:5n3 into an elution zone already rich in small C_{20} components of the 20:1, 20:2 non-methylene-interrupted types, or 20:2n6 and 20:3n9 [23, 24]. Bonded Carbowax is already widely accepted as a stable and reliable liquid phase with only minor variations in polarity when originating from different suppliers [9, 25], and is thus especially recommended for interlaboratory comparisons of R_t data.

EXPERIMENTAL

Materials. I. galbana (Parke) clone T-iso (Haptophyceae) and *O. edulis* were obtained from the Aquaculture Laboratory of the Biology Department, Dalhousie University (courtesy of Dr. G. Newkirk). Algal cultures were developed under the routine conditions for feeding oyster larvae described in ref [26], using the f/2 nutrient mix of ref. [27]. Generally the isolation procedure for fatty acids and their esterification followed procedures used earlier [2, 3, 28].

Fatty acid standards were purchased from Serdary Research Laboratories, London, Ontario. Organic solvents were A.C.S. reagent grade (Anachemia), redistd in glass before use. Acids and other reagents were A.C.S. grade from Fisher Scientific Company (Canada).

General. Glassware was carefully cleaned before use by soaking overnight in a concd KOH–EtOH soln or by washing with detergent. It was then rinsed several times with H_2O, dist H_2O and Me_2CO before oven drying. All procedures except hydrazine reduction were carried out under N_2.

GC analysis of FAME. Analytical GC was carried out on a FID instrument using a flexible fused silica capillary column (30 m × 0.25 mm id) with SUPELCOWAX-10 as the liquid phase (Supelco, Inc. Bellefonte, PA). This liquid phase is essentially bonded Carbowax-20M, a phase for which much R_t data have already been tabulated [9, 11]. Additional FID-GC was carried out in a flexible fused silica capillary column 30 m × 0.25 mm id with DB-Wax (Durabond wax, also Carbowax-20M, bonded and crosslinked, J & W Scientific Inc., U.S.A) as the liquid phase.

R_ts and percent areas were recorded using a computing integrator. Relative areas were converted to percent wt of FAME by correcting the FID response using the computer program described in ref. [29].

Identification of 18:5n3. Since there is no absolute method for identifying fatty acids by GLC, the following series of confirmatory identification procedures were applied to all the samples used in this study.

(i) $AgNO_3$-*TLC followed by GC of the pentaenoic fraction.* FAME were sepd according to the number of double bonds present. Plates were prepd by dipping an Adsorbosil-5 (silica gel) 'Prekote' plate, (Applied Science Laboratories) in a 10% soln of $AgNO_3$ in MeCN for 30 min and drying at 110° for 1 hr. Samples were then applied using a plate streaker (Applied Science Laboratories). The developing solvent was benzene; FAME resolved into bands were visualized under UV. The silica was scraped from the plate, extracted with $CHCl_3$–hexane (1:1) and the recovered esters subjected to GC. The combined information from GC and $AgNO_3$-TLC was used to identify the fatty acids.

(ii) *Comparison of ECL values of fatty acids on different capillary GC columns.* Essentially this means comparisons with retention data published for the liquid phases BDS (the polyester butanediol succinate) and SILAR-5CP, a cyanosilicone [2, 11].

(iii) *Catalytic hydrogenation of the sample over* PtO_2. In order to verify the chain length of the polyunsaturated fatty acid in question, portions of all FAME samples (1–2 mg) were dissolved in EtOH (96%) and hydrogenated for 30 min with H_2 over PtO_2 (Adam's catalyst, 1 mg). The resulting saturated forms were subjected to GC.

(iv) *Hydrazine reduction of ethylenic bonds.* About 1 mg of free fatty acid was taken to dryness in a flat-bottomed flask, then 25 ml of 96% EtOH and 1 ml of hydrazine were added. If Me esters are to be reduced, a small amount of HOAc should be added to provide protons to accelerate the reaction in the absence of the carboxyl group [15]. Reduction was allowed to proceed at 35° under O_2 with constant stirring. After 1–2.5 hr the reaction was stopped by acidifying with 3 N HCl and adding 25 ml of H_2O. All fatty acids were recovered with hexane, esterified and then subjected to GC analysis.

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REFERENCES

1. Ackman, R. G. (1964) *J. Fish. Res. Bd Canada*, **21**, 247.
2. Ackman, R. G., Manzer, A. and Joseph, J. D. (1974) *Chromatographia*, **7**, 107.
3. Joseph, J. D. (1975) *Lipids*, **10**, 395.
4. Bang, H. O. and Dyerberg, J. (1983) *Adv. Lipids Res.* **1**, 22.
5. Kinsella, J. E. (1986) *Food Technol.* **40**, 89.
6. Haagsma, N., van Gent, C. M., Luten, J. B., Jong, R. W. and van Doorn, E. (1982) *J. Am. Oil Chem. Soc.* **59**, 117.
7. Bruckner, G. G., German B, Lokesh, B. and Kinsella, J. E. (1984) *Thromb. Res.* **34**, 479.
8. Christie, W. W. (1982) *Lipid Analysis*, 2nd Edn. Pergamon Press, Oxford.
9. Ackman, R. G. (1986) in *Analysis of Fats and Oils* (Hamilton, R. J. and Rossell, J. B., eds), pp. 137–207. Elsevier, New York.
10. Firestone, D. (1986) *J. Assoc. Off. Anal. Chem.* **69**, 246.
11. Ackman, R. G. (1986) in *CRC Handbook of Chromatography. Lipids, I*, (Mangold, H. K., ed.), pp. 95–151. CRC Press, Boca Raton, Florida.
12. Ackman, R. G. (1967) *Lipids* **2**, 502.
13. Mayzaud, P., Eaton, C. A. and Ackman, R. G. (1976) *Lipids* **11**, 858.
14. Epifanio, C. E. (1979) *Aquaculture* **16**, 187.
15. Ratnayake, W. M. N. (1980) Ph.D. Thesis, Dalhousie University, Nova Scotia, Canada.
16. Laakso, I., Hiltunen, R., Seppanen, T. and von Schantz, M. (1983) *Acta Pharm. Fenn.* **92**, 127.
17. Mukherjee, K. D. (1983) *Plant Physiol.* **73**, 929.
18. Ackman, R. G. and Hooper, S. N. (1973) *Comp. Biochem. Physiol.* **46B**, 153.
19. Takagi, T. Kaneniwa, M., Itabashi, Y. and Ackman, R. G., (1986) *Lipids* **21**, 558.
20. Ackman, R. G., Eaton, C. A. and Linke, B. A. (1975) *Fish Bull.* **73**, 838.
21. Smith, C. R. (1979) in *Fatty acids* (Pryde, E. H., ed.), pp. 29. Am. Oil Chem. Soc. Champaign.
22. Ackman, R. G. (1967) *Lipids* **2**, 502.
23. Ackman, R. G. and Hooper, S. N. (1973) *Comp. Biochem. Physiol.* **46B**, 153.
24. Takagi, T., Eaton, C. A. and Ackman, R. G. (1980) *Can. J. Fish. Aquat. Sci.* **37**, 195.
25. Kramer, J. K. G., Fouchard, R. C. and Jenkins, K. J. (1985) *J. Chrom. Sci.* **23**, 54.
26. Enright, C. T. (1983) Ph.D. Thesis. Dalhousie University, Nova Scotia, Canada.
27. Guillard, R. L. and Ryther, J. H. (1962) *Can. J. Microbiol.* **8**, 229.
28. Ackman, R. G. (1981) in *New Sources of Fats and Oils* (Pryde, E. H., Princen, L. H. and Mukherjee, K. D., eds), pp. 185–220 Am. Oil Chem. Soc., Champaign.
29. Ackman, R. G. and Eaton, C. A. (1979) *Fette Seifen Anstrichm.* **80**, 21.