the SFO fed animals but not in the CNO or TLO ones. The results after administration of similar amounts of labeled cholesterol suggest that the ingestion of the *trans* isomers is not as effective as the *cis* isomers in lowering serum cholesterol levels. However, in agreement with our feeding experiment, Weigenberg and McMillan (11) found serum cholesterol levels to be similar when rabbits were fed either linoleic acid or an elaidinized linoleic acid which contained a mixture of *cis* and *trans* isomers.

Considerable interest has been aroused as to the disposition of the cholesterol when the blood levels are lowered by the ingestion of polyunsaturated fatty acids. In this study both the liver lipid level and total radioactivity was significantly increased by the *cis* isomer over that when either the *trans* isomer or CNO was fed. The cholesterol retained in the liver after the TLO feeding was intermediate in amount (131%) between that of CNO (set at 100%) and SFO (155%).

The above results suggest that the ingestion of TLO may be intermediate between that of CNO and SFO in its effect upon the production of maximum cholesterol levels in both serum and liver. Although not as effective in lowering cholesterol levels as the *cis* isomers, the *trans* isomers are apparently not any more objectionable as dietary constituents than saturated fats and less so than are fatty acids containing conjugated double bonds.

ACKNOWLEDGMENTS

Supported in part by grants from the Robert A. Welch Foundation, Houston, Texas and Division of Research and Development, Contract No. DA-49-193-MD-2499. The *trans, trans* linolein was prepared by the Hormel Institute, Austin, Minn., 95% pure as estimated by TLC. U.S. Department of Agriculture Research Service and H. P. Dupuy supplied the tung oil. George Nunn gave technical assistance.

REFERENCES

1. Hegsted, D. M., A. Gotsis and J. Stare, J. Nutr. *63*, 272 (1957).
2. Coots, R. H., J. Lipid Res. *5*, 473 (1964).
3. Aaes-Jorgensen, E., Proc. Nutr. Soc. *20*, 156 (1961).
4. Nutr. Reviews *22*, 247 (1964).
5. Barnes, R. H., E. Kwong, L. R. Mattick and J. K. Loosli, Proc. Exp. Biol. Med. *103*, 468 (1961).
6. Anderson, J. T., F. Grande and A. Keys, J. Nutr. *75*, 388 (1961).
7. Edwards, H. M., J. Nutr. *83*, 365 (1964).
8. Stern, I., and B. Shapiro, J. Clin. Path. *6*, 158 (1953).
9. Mamose, T., Y. Ueda, K. Vamamoto, T. Masumura and K. Ohta, Anal. Chem. *35*, 1751 (1963).
10. Burr, W. W., Jr., C. Dunkelberg, J. C. McPherson and H. C. Tidwell, J. Biol. Chem. *210*, 531 (1954).
11. Weigenberg, B. I., and G. C. McMillan, J. Nutr. *83*, 314 (1964).

[Received January 18, 1965—Accepted July 21, 1965]

A Rapid Method for Concentrating Highly Unsaturated Fatty Acid Methyl Esters in Marine Lipids as an Aid in Their Identification by GLC

PETER M. JANGAARD, Fisheries Research Board of Canada, Technological Research Laboratory, Halifax, Novia Scotia, Canada

Abstract

The selective solubility of unsaturated fatty acid methyl esters in nitromethane at temperatures down to −20C can be used to concentrate highly unsaturated methyl esters. With a typical sample of marine oils methyl esters having an iodine value of 110–190, a concentrate can be ready for GLC analysis in an hour or less and the nitromethane layer can be injected directly for analysis in GLC apparatus with ionization detectors. Examples of the use of the method in the identification of component fatty acids in herring oil are given.

Introduction

SEVERAL METHODS are available for the concentration or isolation of highly unsaturated fatty acid methyl esters in lipids. Most commonly used are the urea complex procedure (1,2,3,), low temperature solvent crystallization (4), silver nitrate column chromatography (5,6) and countercurrent distribution (7,8). In this laboratory, where the work is chiefly concerned with marine lipids of complex fatty acid composition, a rapid method for concentration of unsaturated fatty acid methyl esters was needed in conjunction with the log-plot-separation factor procedure (9,10,11) for the provisional identification of fatty acids.

The use of nitromethane as a solvent for pilot-plant scale countercurrent distribution of marine oils methyl esters was described in an earlier communication from this laboratory (12). In the course of investigating fractions obtained from the extraction column employed, the advantages of the present method were recognized.

Cannon et al. reported the use of a nitromethane-nitroethane, pentane-hexane system for the separation of methyl esters of fatty acids (13), and Schmid et al. have determined the critical solution temperature of several fatty acid methyl esters in nitromethane (14).

Experimental

Glass-stoppered centrifuge tubes with a capacity of 2.5 ml were used for these experiments. Nitromethane, practical grade, was freshly distilled, and the first 10% of the distillate discarded. The herring oil was a commercial oil from British Columbia and the methyl esters were prepared by methanolysis using 0.5% NaOH as catalyst. An insulated beaker containing ethanol-dry ice was used for cooling the samples in the centrifuge tubes. By adding small pieces of dry ice to the beaker, any temperature in the desired range could be reached quickly and held for several minutes.

Methyl esters (0.5 ml) were added to the centrifuge tube followed by nitromethane (1.5 ml). The

TABLE I

Component Fatty Acids of Herring Oil and of Methyl Ester Fractions Soluble in Nitromethane at various Temperatures

Fatty Acid	Double bond positions	Herring oil, wt %	% Comp. of M.E. mixture soluble at				Concentration factor CF = (conc. − 10C)/(Conc. + 40C)
			+40C[b]	+20C	0C	−10C	
14:0	....	7.0	7.2	7.0	7.6	7.2	1
16:0	....	17.8	17.9	14.9	11.2	6.0	0.33
16:1ω7	9[a]	8.9	9.6[c]	9.2	10.0	9.2	1
17:0	....	0.7					≈ 0.2
16:2ω4	9,12	0.7	0.7[c]	1.5	1.6	1.7	2.4
17:1	?	0.1		T	T	T	≈0.8
16:3ω4	6,9,12	0.8	2.7[c]	1.3	1.5	2.0	2.5
18:0	....	1.8		1.4	T	T	<0.1
18:1ω9	9[a]	20.8	20.8	19.2	14.3	13.6	0.65
16:4ω1	6,9,12,15	0.7	0.7	2.4	3.0	4.3	6.1
18:2ω6	9,12	0.7	0.6	0.9	0.9	1.1	1.8
18:3ω3	9,12,15	0.7	9.4[c]	1.2	1.3	1.4	2.0
20:1ω9	11[a]	8.8		5.4	3.5	3.0	0.34
18:4ω3	6,9,12,15	1.2	1.1	3.5	4.2	4.6	4.2
20:4ω6	5,8,11,14	0.4	11.4[c]	0.6	0.7	1.0	2.5
22:1ω11	11[a]	10.9		5.1	3.1	2.1	0.19
20:4ω3	8,11,14,17	0.3	0.3	0.6	0.7	0.7	2.3
20:5ω3	5,8,11,14,17	10.5	10.5	17.1	23.3	26.9	2.6
21:5ω2	7,10,13,16,19,(?)	0.4	0.9[c]	0.5	0.6	0.9	2.3
24:1	(?)	0.3		0.2	0.1	T	<0.1
22:5ω3	7,10,13,16,19	1.0	1.0	1.2	1.4	1.5	1.5
22:6ω3	4,7,10,13,16,19	5.0	4.8	8.1	11.5	12.8	2.6
Others		1.1					

[a] Other isomers may be present or predominate.
[b] Completely soluble at this temperature.
[c] No separation on this column under conditions employed.

tube was flushed with nitrogen, stoppered and warmed gently until the methyl esters dissolved completely. With marine oils this usually occurred at 35–40C. On cooling, the more saturated methyl esters separated and rapidly rose to the surface due to the large difference in specific gravity between nitromethane and the methyl esters.

To obtain maximum enrichment of the highly unsaturated fatty acid methyl esters, the samples were cooled to as low a temperature as possible without solidifying, preferably −10 to −20C. The lowest temperature that is possible to use is −29C, the freezing point of nitromethane. Some samples with a high percentage of saturated and long-chain monounsaturated fatty acids gelled if cooled rapidly to lower temperatures, and stepwise cooling (5C steps) was then employed. Increasing the amount of nitromethane was also helpful and, in some cases, it was advantageous to remove the top layer from each step as it was formed. A refrigerated centrifuge could be used to bring about a quicker separation of the two layers, but if not available, the sample was allowed to warm slowly in the alcohol bath until clearing started near the bottom. A syringe was then inserted to the bottom and a sample taken for GLC analysis. As a guide to the sample size, the herring oil ester bottom layer contained about 8% methyl esters b.wt. at room temperature and 3–4% at −15C. The syringes used were Hamilton No. 7001-N for the esters and Hamilton No. 701-N for the nitromethane solution.

The gas-liquid chromatograph employed was a Barber-Colman Model 10 equipped with a Model 5121 flame ionization detector and a Disc Co. Integrator. The columns used were of glass, 6 ft in length, 3 mm I.D., and packed respectively with 15.5% EGSS-Y on 100–120 Gas Chrom P and 10% EGSS-X on 100–120 mesh Gas Chrom P. (Applied Science Laboratories Inc., State College, Pa.). The operating conditions for the runs in Table I were: Column 200C, flash heater 275C, argon carrier gas at 10 psig. Tentative identifications of the peaks were made by the log-plot-separation factor procedure (15) and correction factors for the quantitative determination were applied (16). A sample was hydrogenated and analyzed similarly to check on the presence of odd-numbered acids, especially C_{17} and C_{21}.

When a larger amount of unsaturated esters were needed for study, the complete bottom layer was withdrawn and the nitromethane evaporated in a rotary evaporator.

Results and Discussion

In Table I the average results of several analyses of the component fatty acids of herring oil on two polyester columns are listed in the first column. In the following columns the composition of the mixture of methyl esters soluble in nitromethane at +40C, +20C, OC and −10C is tabulated as determined by GLC analysis on one polyester column (EGSS-X). Since the methyl esters completely dissolved at +40C, this analysis therefore represents the starting material. On this column, under the conditions employed, several fatty acids overlap, and the method described is especially useful for qualitatively determining if one or more acids are present in one peak.

Most notable of the results in Table I is the relatively large increase in the concentration of 16:4 and 18:4 acids at lower temperatures, as compared to the other fatty acids appearing in the same region. The method is therefore especially useful in demonstrating the presence of these acids. On highly polar DEGS and EGSS-X columns the 16:4 is usually masked by the 18:2 peak; in the example in Table I they separate cleanly since they are present in small and equal amounts. On the same columns 18:4 falls after 20:1, often as a shoulder on this peak. Some other pairs that are difficult to separate on these columns are 18:0 and 16:3 and also 17:1 and 16:2. Since 18:0 completely disappears at −10C, the first pair can easily be determined, and in the second pair, 16:2 is considerably enriched whereas 17:1 will not change much.

Of the longer-chain acids, the concentration of 22:1 drops from about 11% in the original herring oil esters to about 2% in the fraction soluble at −10C, and the presence of arachidonic acid, 20:4ω6, in the same region can be demonstrated. The concentration of 24:1 drops to near zero and can be separated from the acid tentatively identified as 21:5ω2 by log-plot and separation factor procedure.

On less polar EGSS-Y columns, 16:4 usually falls under stearic acid or just before oleic acid and can

easily be identified at −10C. 18:4 on these columns falls before 20:1, 16:3ω4 and 17:1 overlap and also 16:2ω4 and 17:0.

Myristic acid, 14:0, although completely saturated, is still quite soluble at −10C due to its short chain length, and conversely, 22:5 only shows slight enrichment in spite of five double bonds in the molecule. Isomers of 22:4 and 20:3 are usually present in marine oils in small amounts, but are not enriched by this method. The concentration of isomers of 20:2, when present, decreases slightly.

The method described has been used routinely in this laboratory for some time, although only methyl esters from marine lipids have been segregated. The method should, however, also be useful in the analysis of some animal and vegetable lipids with complex fatty acid compositions.

REFERENCES

1. Abu-Nasr, A. M., W. M. Potts and R. T. Holman, JAOCS *31*, 16 (1954).
2. Schlenk, H., Progress in the Chemistry of Fats and Other Lipids. Vol. II, Pergamon Press, London, 1953.
3. Ackman, R. G., R. D. Burgher and P. M. Jangaard, Can. J. Biochem. Physiol. *41*, 1627 (1963).
4. Brown, J. B., JAOCS *32*, 646 (1955).
5. DeVries, B., Chem. Ind., 1049 (1962).
6. DeVries, B., JAOCS *40*, 184 (1963).
7. Scholfield, C. R., *Ibid. 38*, 562 (1961).
8. Therriault, D. G., *Ibid. 40*, 395 (1963).
9. Ackman, R. G., *Ibid. 40*, 558 (1963).
10. Ackman, R. G., *Ibid. 40*, 564 (1963).
11. Ackman, R. G., and P. M. Jangaard, *Ibid. 40*, 744 (1963).
12. Vandenheuvel, F. A., and P. M. Jangaard, Can. Chem. Processing *41*, 40 (1957).
13. Cannon, J. A., K. T. Zilch and H. J. Dutton, Anal. Chem. *24*, 1530 (1952).
14. Schmid, H. H. O., H. K. Mangold and W. O. Lundberg, Microbiochem. J. 7, 287 (1963).
15. Ackman, R. G., and R. D. Burgher, JAOCS *42*, 38 (1965).
16. Ackman, R. G., and J. C. Sipos, *Ibid. 41*, 377 (1964).

[Received March 4, 1965—Accepted June 17, 1965]

Autoxidation of Cholesteryl Linoleate in Aqueous Colloidal Suspension[1]

L. N. NORCIA and W. F. JANUSZ,[2] Department of Biochemistry, Temple University Medical School, Philadelphia, Pennsylvania

Abstract

The autoxidation of the cholesteryl moiety of cholesteryl linoleate stabilized in aqueous colloidal suspension with sodium dodecyl sulfate has been studied at 85C. The overall rate of this oxidation is more rapid than that for unesterified cholesterol and oxidation also occurs to a greater extent for the linoleate ester. These results are in contrast to those for more saturated fatty acyl esters of cholesterol which show diminished susceptibility to attack by oxygen in such a system. Autoxidation of cholesteryl linoleate by an intramolecular free-radical mechanism is considered.

INFORMATION CONCERNING the autoxidation of cholesteryl esters is relatively meager whereas studies of autoxidation of unesterified cholesterol have been reported somewhat extensively. Bergstrom and Wintersteiner (1) reported that esterification of cholesterol by acetate, palmitate, or oleate greatly diminished the susceptibility of the cholesterol in aqueous colloidal suspension to attack by oxygen. Cook (2) compared the oxidation of cholesteryl acetate with unesterified cholesterol in xylene containing phthalocyanine. The literature of the autoxidation of cholesterol has been reviewed (3).

Since cholesterol occurs in the animal body in both the unesterified and esterified forms it was felt that the chemical reactivity of the esters, particularly those of the more unsaturated fatty acids, of cholesterol in aqueous suspension should be elucidated further. Cholesteryl linoleate was chosen for study. It was hypothesized that the high susceptibility of the linoleate 9,12 diene system to autoxidative attack might render the cholesteryl moiety of cholesteryl linoleate rather more susceptible to autoxidative attack than is the case for cholesteryl acetate, palmitate, and oleate (1). Experimental study revealed that cholesteryl linoleate in aqueous colloidal suspension has a markedly different susceptibility to autoxidative attack than the more saturated fatty acyl esters. It is the purpose of this communication to report this finding.

Experimental

The procedure of autoxidation was similar to that of Bergstrom and Wintersteiner (1). Modifications involved the amounts of cholesterol [cholesterol or cholesteryl esters (99% pure, Applied Science Laboratories, Inc., State College, Pa.) were used in equimolar amounts] and sodium dodecyl sulfate. pH was adjusted to 7.0 ± 0.2 with M/15 phosphate buffer. Autoxidation was carried out at 85C. Aliquots of the reaction mixture were acidified, extracted with diethyl ether, the ether extract water-washed, the solvent evaporated, and the extract analyzed for unoxidized cholesterol by the Sperry-Webb (4) procedure. Use of this analytical method for following the time course of autoxidation of cholesterol has been described (5). As a further check of the analytical procedure we have tried the method on standard solutions of 7-ketocholesterol, 7-β-hydroxycholesterol, and 3β, 5α, 6β-cholestanetriol. The first two compounds behaved

TABLE I

The Autoxidation of Cholesterol and Its Esters by Aeration in Aqueous Colloidal Suspension, pH near neutrality, 85C, 5 hr.

Compound	Percent of Cholesterol Autoxidized	
	Bergstrom-Wintersteiner (1,6)	Norcia-Janusz
Cholesterol	60	60
Cholesteryl acetate	16[a]	....
Cholesteryl palmitate	10	....
Cholesteryl oleate	<5	0
Cholesteryl linoleate	....	78

[a] Autoxidation for 4 hr.

[1] Presented at the AOCS Meeting in Houston, Texas, April, 1965.
[2] Work conducted during the tenure of a Summer Research Student Fellowship.