15. Keppler, J. G., S. Sparreboom, J. B. A. Stroink, J. D. von Mikusch, JAOCS *36*, 308 (1959).
16. Knegtel, J. T., C. Boelhouwer, M. Tels, H. I. Waterman, *Ibid. 34*, 336 (1957).
17. Kolb, D. K., Thesis, The Ohio State University (1953).
18. Mathews, J. H., J. Am. Chem. Soc., *48*, 571 (1926).
19. Mumford, S. A., and J. W. C. Phillips, J. Chem. Soc., *75*, (1950).
20. Naves, Y. R., Helv. Chim. Acta, *32*, 2306 (1949).
21. Nevin, C. S., JAOCS *33*, 95 (1956).
22. Ralston, A. W., "Fatty Acids and Their Derivatives," John Wiley and Sons, New York, 1948, p. 509.
23. Richards, T. W., and H. M. Chadwell, J. Am. Chem. Soc. *47*, 2287 (1925).
24. Ruhoff, J. R., and E. E. Reid, *Ibid. 55*, 3827 (1933).
25. Schjänberg, E., Z. Phys. Chem. *172A*, 197 (1935).
26. Sie, S. T., and H. I. Waterman, De Ingenieur *72*, Ch.71, (1960).
27. Smittenberg, J., and D. Mulder, Rec. Trav. Chim. *67*, 813 (1948).
28. Sreenivasan, B., J. B. Brown, E. P. Jones, V. L. Davison, and Janina Nowakowska, JAOCS *39*, 255 (1962).
29. Swern, D., and W. E. Parker, *Ibid. 30*, 5 (1953).
30. Tatevskii, V. M., V. A. Benderskii, and S. S. Yarovoi, "Rules and Methods for Calculating the Physico-chemical Properties of Paraffinic Hydrocarbons," translated from Russian, Pergamon Press, 1961.
31. Tels, M., A. J. Kruidenier, C. Boelhouwer, and H. I. Waterman, JAOCS *35*, 163 (1958).
32. Timmermans, J., and Mme. Hennaut-Roland, J. Chim. Phys. *52*, 223 (1955).
33. Timmermans, J., and Mme. Hennaut-Roland, *Ibid. 56*, 984 (1959).
34. Toyama, Y., J. Chem. Ind. Japan, *25*, 1503 (1922).
35. Vandenheuvel, F. A., and P. Yates, Can. J. Res. *28B*, 556 (1950).
36. Vogel, A. I., J. Chem. Soc. 133 (1946), 607 (1948), 634 (1948).
37. Wheeler, D. H., and R. W. Riemenschneider, Oil and Soap *16*, 207 (1939).
38. Wilds, A. L., and A. L. Meader., J. Org. Chem. *13*, 763 (1948).
39. Young, S., Sci. Proc. Roy. Dublin Soc., N. S. *12*, 374 (1909–1910).

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Essential Fatty Acid Contents of Various Fats: Interpretations of Values by Physico-Chemical Tests

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Abstract

Biological assays of oil and fat products, free from isomers of the naturally-occurring *cis*-9, *cis*-12 linoleic acid, have been shown to provide estimates of essential fatty acid content which agree well with values obtained by spectrophotometric analysis. However, when partially hydrogenated fats, such as those used in margarines, are bio-assayed the estimates obtained are only about 60% of those derived by spectrophotometric tests.

In a blended corn oil margarine, good agreement was obtained for linoleic acid content by using biological assay or spectrophotometry, thiocyanometric procedure, column chromatography for saturates plus iodine value, and gas liquid partition (GLP) chromatography. This margarine fat contained about 29% of the essential form of linoleic acid, and had a ratio to saturated fatty acids of 1.6:1.

The hydrogenated corn oil margarine is unlike conventional margarines in providing high amounts of the isomeric forms of linoleic acid which lack essential fatty acid activity. For this reason, poor agreement was obtained between biological assay results and those by physicochemical measurements of linoleic acid content. Such fat contains only about 6% of the essential form of linoleic acid, with a ratio to saturated fatty acids of ca. 0.3:1.

From this study it is now possible to characterize, even without bio-assay data, the fatty acid composition of a highly isomerized fat, such as is found in hydrogenated corn oil margarine. The characterization groups the fatty acids into saturates and total linoleic acids, with the latter including estimates of the positional isomers of linoleic acid with widely spaced double bonds, *trans* forms of linoleic acid with methylene-interrupted double bonds, linoleic acids with the double bonds in conjugated position, and *cis*-9, *cis*-12 linoleic acid. The combined use of the spectrophotometric and thiocyanometric procedures makes it possible to estimate the essential fatty acid content of hydrogenated fats containing residual dienes.

Introduction

THE REQUIREMENT for essential fatty acids (EFA) by many animals is well documented (1–7). Not only are they required for proper growth, reproduction, lactation, longevity, and tissue structure, but also for regulation of plasma and liver cholesterol levels and liver lipid levels (7). However, the human requirement is less well understood. It has been shown that essential fatty acid deficiency in the human infant causes a dry and scaly skin which responds to essential fatty acid therapy (8); linoleic acid is also required for optimum utilization of the total calories ingested (8).

Values for the human requirement for essential fatty acids (viz., *cis*-9, *cis*-12-linoleic acid) have been reported to range from 1–4% of the caloric intake (8–10). Holman (9), in proposing his value of 2% of caloric intake as the minimal requirement, points out that "a judicious selection of foods should provide many times 2% linoleate calories, but diets containing high proportions of hard fats and sugars could lead to relative EFA deficiency." He also emphasizes that "for some physiological functions the requirement for EFA may be greater than 2% of calories. For example, if hypocholesterolemia is a desirable condition, the amount of highly unsaturated fatty acids required to maintain it appears to be several fold that amount." The fact that other polyunsaturated fatty acids, in addition to the essential fatty acids, exert hypocholesterolemic effects (10,11) should not lead one to regard the value of dietary linoleic acid as limited only to those functions for which it is essential. The hypocholesterolemic activity of the essential fatty acids indicates another important function, even though non-specific, of these nutrients in human nutrition.

Three essential fatty acids are now recognized: linoleic (*cis*-9, *cis*-12-octadecadienoic acid), linolenic (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid), and arachidonic (*cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatetraenoic acid). Arachidonic acid has been reported ca. three times as effective as linoleic in promoting growth (12), and linoleic acid is more effective than linolenic in relieving EFA deficiency symptoms (13). Since linoleic acid can form arachidonic acid in the animal body (14,15), investigators have concentrated atten-

TABLE I

Essential Fatty Acid Content of Oil and Fat Products Containing No Fatty Acid Isomers of Hydrogenation; Comparison with Results by Spectrophotometric Assay

Product		Spectrophotometric Assay [a]			Bioassay	
No.	Description	Saturated fatty acids	Oleic acid	Linoleic acid	*cis*-9, *cis*-12-linoleic acid	% of Spec. assay
		% triglycerides			% triglycerides	
1	Cottonsee oil, liquid whole	24.7	21.3	49.5	48.0	97
2	Cottonseed oil, (winterized)	21.3	20.5	53.8	51.7	96
3	Soybean oil, liquid whole	12.9	29.0	53.8 [b]	59.1	110
4	Corn oil, liquid whole	12.3	28.3	54.2	45.7	84
5	82.5 parts of liquid cottonseed oil interesterified with 17.5 parts of completely hydrogenated cottonseed oil (21)	39.2	13.6	42.8	44.0	103
6	Same as 5 above + edible antioxidants (0.022%)	39.2	13.6	42.8	41.5	97
7	Same as 6 above + completely hydrogenated coconut oil, 85 parts + 15 parts	47.5	11.7	36.4	34.5	95

[a] By the method of Brice et al. (22). By IR absorption analysis (23), products contained about 2% apparent *trans*-acids.
[b] Includes 7.3% linolenic acid.

tion on the determination, metabolism, and supply of this fatty acid. Only the *cis*-9, *cis*-12-form of linoleic acid possesses EFA activity; *cis-trans* and *trans-trans* isomers of linoleic acid lack EFA activity when fed to weanling rats at 50–100 mg/day (16,17).

Biological Assay for EFA Activity

Certain polyenoic fatty acids can be determined by spectrophotometric, chromatographic, and other techniques, and by animal bio-assay technique. Although bio-assays are time consuming, they have the advantage of differentiating between the essential form of linoleic acid and its inactive isomers. When a product containing linoleic acid isomers is assayed by physicochemical methods, the value reported as linoleic acid is erroneously high for the esential form of linoleic acid.

We have developed a bio-assay method for linoleic acid based on growth response (18,19). The weight gain of rats, depleted of essential fatty acids, was found proportional to the logarithm of the linoleate supplement given during the repletion period. The procedure involves depleting male rats of EFA by feeding a fat-free diet after weaning. Depletion is achieved when weight either declines or reaches a plateau over a 3-week period, usually after 12–14 weeks. Then the animals are divided into groups and linoleate supplements of established purity, or supplements of test substances, are added to the fat-free diet. Bio-assays are carried out over an 8-week period and weights are recorded weekly. At the end of this period, the log dose of the known pure linoleate supplements, expressed as linoleic acid, is plotted against the 8-week weight gain. From the straight line thus obtained, the linoleic acid content of the "unknown" substances is determined from the weight gain of the animals.

Bio-Assay vs. Spectrophotometric Findings

Samples with little or no linoleic isomers show reasonable agreements (20) between bio-assay and spectrophotometric values (Table I). Thus, fats containing only natural forms of fatty acids or mixtures of these and completely hydrogenated fats give, on bio-assay, an average of 97% of that obtained spectrophotometrically.

In samples containing partially hydrogenated oils, considerable amounts of *trans* isomers of the polyunsaturated fatty acids are present. These are partially measured by spectrophotometry but do not show up in bio-assay (20). Thus, only 44–80% of the total linoleic acid measured spectrophotometrically is shown to be of the EFA type according to bio-assay (Table II).

The *trans* fatty acids in partially hydrogenated margarine fats consist of *trans*-oleic and *trans*-linoleic acids. Although the *trans* oleic acids have been shown (24) to have no effect on the development of EFA deficiency in rats on a fat-free diet, or on the response following addition to the diet of *cis*-9, *cis*-12-linoleate, little has been reported on the effect of *trans*-linoleic acids. The spectrophotometric method measures all the *cis*-9, *cis*-12-linoleic acid present but, under the conditions of the test, only to a limited degree the

TABLE II

Essential Fatty Acid Content of Hydrogenated Margarine Fats; Comparison with Results by Spectrophotometric Assay

Product [a]		Spectrophotometric Assay [b]			Bioassay	
No.	Description	Saturated fatty acids	Oleic acid	Linoleic acid	*cis*-9, *cis*-12-linoleic acid	% of Spec. estimate
		% triglycerides			% triglycerides	
8	Soybean and cottonseed oil hydrogenated directly to margarine constants and blended	19.5	71.2	4.8	2.4	50
9	As No. 8, but another lot	18.9	71.1	5.5	2.4	44
10	As No. 8 and 9, but another lot	20.0	71.1	4.4	3.5	80
11	Same as No. 8 + liquid whole cottonseed oil, 98.5 parts + 1.5 parts	20.3	70.3	4.9	2.4	49
12	As No. 8–10, but another lot	19.5	71.1	4.9	3.7	76
13	Plastic margarine fat made from soybean and cottonseed oils hydrogenated excessively and blended with oils hydrogenated to a compensating lesser degree	17.7	68.0	9.7	6.9	71
14	As No. 13 but another product	18.5	66.9	10.2	6.1	60

[a] Margarine fats No. 8–10 and 12 were products directly hardened to margarine constants under selective condition of hydrogenation. In margarine fats No. 13 and 14, one portion of the oil blend was selectively hydrogenated to a degree in excess of that characteristic of whole margarine fats, and the other portion was selectively hydrogenated to a compensating lesser degree.
[b] By the method of Brice et al. (22). According to IR absorption analyses (23), the above products contained about 33% *trans*-fatty acids (expressed as elaidic acid).

TABLE III
Essential Fatty Acid Content of Two Types of Margarine Fats by Bioassay

Supplement to fat-free diet	No. of rats	Amount ingested	Avg. wt gain 8-week period	Calculated linoleic acid intake	*cis-9, cis-12*-linoleic acid content of fat
		mg/rat/day	*g*	*mg/day*	%
None—control	17	0	10	0	.....
Linoleic acid [a]—control	13	20	106	20	100
Linoleic acid [a]—control	10	50	136	50	100
Blended-corn-oil margarine [b]	13	93	114	26	28.9
Hydrogenated corn-oil margarine [b]	12	270	61	14	5.3

[a] Administered as pure methyl linoleate; quantity ingested expressed as linoleic acid.
[b] Separated margarine oil administered.

TABLE IV
Performance of EFA-Deficient Rats Following Repletion of Fat-Free Diet with Blended Corn-Oil Margarine or Hydrogenated Corn-Oil Margarine at Same Low Level

Criterion	Blended corn-oil margarine group (BCOM)	Hydrogenated corn-oil margarine group (HCOM)
At end of depletion period on fat-free diet		
Number of Animals	13	13
Avg body wt, g	219	215
After 8 wk repletion of same diet [a]		
Margarine fat fed/rat/day/mg	37	37
No. of animals surviving	13	11
Avg. wt of survivors, g	305	253
Avg. wt gain of survivors, g	86	38
After 26 wk of repletion of same diet		
Margarine fat fed/rat/day/mg	37	37
No. of animals surviving	10	7
Avg. wt of survivors, g	324	263
Avg. wt gain of survivors, g	105	48
After 43 wk of repletion of same diet		
Margarine fat fed/rat/day/mg	37	37
No. rats surviving	7	5
Avg. wt of survivors, g	286	216
Avg. wt gain of survivors, g	67	1

[a] In calculating *cis*-9, *cis*-12-linoleic acid content of the test fats from these data, the fat in BCOM contained 29.7% EFA-linoleic acid; the HCOM contained 6.5% linoleic acid of this type.

trans-isomers of linoleic acid (25,26). These *trans*-isomers lack EFA activity and, to the extent that they are present and included in the spectrophotometric measurement, the spectrophotometric value is an erroneously high estimate of EFA content. Part of the findings in Tables I and II have been published (19, 20).

Studies of Two New Types of Margarine Fats

In the more detailed experiments reported here, the EFA contents (as linoleic acid) of the fats in two widely differing types of margarine fats have been determined, using the bio-assay procedure described above. Both margarines were purchased on the open market in Los Angeles. The first, hereafter called blended corn oil margarine fat (BCOMF) is a liquid oil margarine (27) made with non-hydrogenated corn oil as its major ingredient; other components of the blend are a lightly hydrogenated cottonseed oil and a partially hydrogenated soybean oil. The corn oil in this product provides about 90% of the total linoleic acid; the remaining 10% is contributed by lightly hydrogenated cottonseed oil. According to the method of making this product (27) very little linoleic acid isomers would be expected. Iodine value (I.V.) of margarine fat of this type was 96.3.

In the second product, hereafter called hydrogenated corn oil margarine (HCOM), each oil component was hydrogenated to some degree. Hydrogenation coupled with a high degree of isomerization, is employed in making this fat, to provide a product having a high retention of octadecadienoic acids, but with most of the residual dienes being of the isomeric forms of linoleic acid. I.V. was 85.2.

Results of the bio-assay show in Table III; the BCOMF contained 28.9% *cis*-9, *cis*-12-linoleic acid and the hydrogenated product contained 5.3%. Dosage levels used were based upon results of spectrophotometric analyses for nonconjugated linoleic acid content, where the BCOM showed 29.0% linoleic acid, while the HCOM showed 9.5%; therefore, three times as much of the latter was fed. The intent in both cases was to obtain a growth response between that obtained by feeding two known levels of linoleic acid: 20 and 50 mg of pure methyl linoleate per animal/day. This was attained with the BCOM but not with the HCOM.

To determine the relative nutritional values of the two margarines the fats were fed at the same suboptimal level of 37 mg fat/animal/day. Growth responses of the EFA-deficient rats and survival records, when the fat-free diets were supplemented with these margarine fats, show in Table IV. Since reple-

TABLE V
Comparison of Assay Methods for Determining EFA Content of Two Margarine Fats

Assay Method [a]	Blended corn-oil margarine fat (BCOM)			Hydrogenated corn-oil margarine fat (HCOM)		
	Saturated fatty acids	Oleic acid	Linoleic acid	Saturated fatty acids	Oleic acid	Linoleic acid
	% of triglycerides			% of triglycerides		
Spectrophotometry (22)	19.0	47.2	29.0	12.3	72.1	9.5 [b]
Thiocyanogen + iodine value (28)	17.8	49.1	28.7	14.8	67.0	13.8
Column Chromatography for [c] saturates + iodine value (29) [d]	19.3	45.9	30.4	17.4	61.9	16.3
GLP Chromatography (30–32) [e]	18.5	45.0	29.6	17.5	58.2	17.4
Bioassay (18–20)	.....	.....	28.9	.....	.....	5.3

[a] By IR absorption values (33), the BCOM contained 25% *trans* acids; the HCOM contained 42% *trans* acids, expressed as elaidic acid.
[b] This value does not include 1.8% linoleic acid with double bonds in conjugated position; such isomers of linoleic acid are readily measured spectrophotometrically. These known isomers *obviously should not be included in a linoleic acid* value intended to reflect essential fatty acid activity, as they lack this special nutritional value.
[c] Gravimetric method depends on separation of saturated fatty acid methyl esters from mercuric-acetate derivatives of unsaturated fatty acid esters, latter held on the alumina column, with correction made by I.V. for small amount of methyl oleate in filtrate and washings.
[d] $\text{I.V. Balance} = \frac{(95.6 - \%S - \%L)}{100} \times 89.9 + \frac{\%L}{100} \times 181.1$

where S = % saturated fatty acids in the triglycerides
L = % linoleic acid in the triglycerides

$$\text{Hence, } \%L = \frac{100 \times \text{Iodine Value} - 89.9\ (95.6 - \%S)}{91.2}$$

and % Oleic acid = 95.6 − %S − %L
[e] Beckman Model GC-2 Gas Chromatograph equipped with 11 ft × ¼ in. stainless steel column; stationary phase 20% succinic acid-diethylene glycol polyester on 60–80 mesh acid-washed Chromosorb W (Wilkins Instrument and Research, Inc.); temp 200–210C; helium as carrier gas at flow rate to permit elution of fatty acids in 40 min; test solution 5 μl of 20% solution of methyl esters of sample in acetone.

TABLE VI

Fatty Acid Composition of Fat in Hydrogenated Corn Oil Margarines

Fatty Acids: Kind	% Triglycerides in margarine oils: I [a]	II [b]	Explanatory Notes
Saturated	17.5	16.4	(A) Avg of values obtained by direct test, column chromatography and GLP chromatography
Total Linoleic	16.9	21.7	(B) Avg obtained by combination of column chromatography and I.V. and by GLP chromatography
Positional isomers of linoleic with widely spaced double bonds	3.1	5.0	(C) Value by (B) minus that based on thiocyanometric procedure
Trans form of linoleic with methylene-interrupted double bonds	5.0	7.4	(D) Difference between total linoleic acid by thiocyanometric and spectrophotometric procedures multiplied by 2 [c]
Conjugated linoleic	1.8	4.9	(E) Obtained spectrophotometrically
Cis-9, *cis*-12-linoleic	7.0	4.4	(F) Spectrophotometric value for nonconjugated linoleic acids minus difference between the total linoleic acid values obtained by thiocyanometric and spectrophotometric procedures
	5.3		(G) By bioassay

[a] Margarine oil (I) bioassayed for essential fatty acid content; I.V. 85.2; 42% total *trans*-fatty acids, expressed as elaidic acid.
[b] Margarine oil (II) found to increase serum cholesterol level in humans (39); I.V. 91.8; 50% total *trans*-fatty acids, expressed as elaidic acid.
[c] Under standardized conditions of the spectrophotometric method, the specific absorption values for the *trans* forms of linoleic acid vary from 22% (*trans, trans*) to 66% (*cis, trans*) of that for *cis, cis*-linoleic acid (26). For the purpose of calculation, it was assumed that the spectrophotometric method includes in the linoleic acid estimate about 50% of the *trans* isomers present. Whereas the precision of this figure may be questioned, it does not introduce a significant error in making the required correction of the spectrophotometric estimate. The corrected value agrees well with the figure derived from bioassay, as shown above. Further confirmation is obtained by comparing bioassay results from margarine fats 8–12 of Table II with the spectrophotometric values before and after correction for the *trans* isomers included in the spectrophotometric estimates. The products, for which thiocyanogen values were available, contained ca. 6.8% linoleic acid by the thiocyanogen procedure and ca. 4.9% spectrophotometrically. The corrected spectrophotometric estimate then indicates ca. 3.0% *cis*-9, *cis*-12-linoleic acid in these products.

tion studies in Tables III and IV were begun about the same time, growth responses in the second study (Table IV) after 8 weeks of repletion may be considered replicate bio-assays estimating the EFA content as *cis*-9, *cis*-12 linoleic acid. The growth response curve in feeding 0, 20, and 50 mg linoleic acid (Table III) has provided the bio-assay reference curve where the three points are on a straight line, i.e., when the log doses of the known pure linoleate supplements are plotted against the 8-week weight gains. The findings were 29.7% linoleic acid in the BCOMF and 6.5% in the HCOMF, which agree with previous results (Table III).

Table IV shows that the weight gain of the animals fed the BCOMF was strikingly superior to that of the HCOMF. Also, the superior survival is worthy of emphasis. Animals receiving the hydrogenated fat had a poorer appearance, such as coarse hair, skin lesions, and matted fur.

Values by the physico-chemical methods and by bio-assay (Table III) show in Table V. Linoleic acid values by bio-assay and by the four physico-chemical tests are in good agreement for the BCOM. Findings indicate unequivocally that this product contains no significant amount of linoleic acid isomers. Liquid non-hydrogenated vegetable oils, such as cottonseed and corn oils, have also given good agreement for linoleic acid content by bio-assay and by the four physico-chemical tests. Liquid soybean oil, containing linolenic acid, offers some problems when assayed according to the thiocyanometric procedure (28) unless a direct and reliable test for saturated fatty acid content is first made. Thus, any of the five unrelated assay methods may be used to obtain the EFA nutritional value of products such as the BCOM.

For the fat in the HCOM, a different situation prevails. The physico-chemical methods, using GLP chromatography (30–32) and direct column chromatography for separating the saturated fatty acids (29), with calculations of linoleic and oleic acid contents based on the I.V. of the total unsaturated fatty acids, include all forms of octadecadienoic acid in the linoleic acid estimate. The difference between the total linoleic acid value, and that derived from the thiocyanometric measurements (28), is a measure of linoleic acid isomers with double bonds so far removed from each other that each double bond reacts with the thiocyanogen reagent like the one double bond in oleic acid. Such linoleic acid isomers are thus not included in the linoleic acid values by the thiocyanometric method and, to the extent that they are not included, the value for saturated fatty acids is erroneously low. The difference in linoleic acid found by the thiocyanometric and spectrophotometric procedures is a measure of *trans*-linoleic acids which are not included in the spectrophotometric estimate (25, 26). In the thiocyanometric method, the *trans* forms are measured to the same degree as *cis, cis*-linoleic acid (34,35); this led to the conclusion (36) that the thiocyanometric procedure gives results (37) higher than do biological tests. To the extent that *trans*-linoleic acids are not measured by the spectrophotometric method, the value for saturated fatty acids is further reduced below the true value. These low saturated fatty acid values by the spectrophotometric procedure are further reduced by the failure of this method to include in the linoleic acid estimate those isomers of linoleic with double bonds so far removed from each other that they cannot be brought into conjugated position by the alkali isomerization procedure. Therefore, it is apparent that of the non-biological methods reported to date, the spectrophotometric procedure is still the best single method for measuring the nutritional value of hydrogenated fats relatively high in linoleic acid isomers. This procedure permits measurement of isomers with double bonds in conjugated position, but these are readily differentiated from *cis, cis*-linoleic acid; positional isomers with widely spaced double bonds are missed by this method; and the *trans* isomers of linoleic acid with methylene interrupted double bonds are included in the spectrophotometric estimate only to a limited degree.

TABLE VII

Comparison of Results of Assay Methods for Determining Fatty Acid Composition of a More Highly Isomerized Fat in HCOM

Assay Method	More Highly Isomerized Margarine Fat [a]: Saturated fatty acids	Oleic acid	Linoleic acid
	% of triglycerides		
Spectrophotometry (22)	7.4	74.9	8.1 [b]
Thiocyanogen + I.V. (28)	10.5	68.4	16.7
Column chromatography for saturates + I.V. (29)	16.2	57.0	22.4
GLP chromatography (30–32)	16.7	50.6	21.0

[a] Sample contained ca. 50% *trans* acids, expressed as elaidic acid in the triglycerides, according to IR values (33).
[b] This value excludes the 4.9% linoleic acid with double bonds in conjugated position present in the fat before alkali isomerization.

TABLE VIII
Physico-Chemical Assays of Commercial Samples of Oils and Fats and Interpretations

Sample	Identity [a]	Assay Method [b]	Fatty Acids Found						Interpretations	
			Saturated	Oleic	Linoleic	Linolenic	Conjug. dienoic	Conjug. trienoic	EFA linoleic	Ratio of EFA linoleic: Sat.
			% trigl.	% trigl.	% trigl.	% trigl.	% trigl.	% trigl.	% trigl.	
Cottonseed oil	Non-hydrog. winterized	Spec.	21.4	20.4	53.3	0.2	0.0	0.2		
		Thio.	21.7	21.0	52.9	....	....	....	52.7	2.5
		Col.	20.4	23.4	51.8	....	....	....		
Corn oil	Non-hydrog.	Spec.	12.4	27.2	55.2	0.7	0.0	0.1		
		Thio.	12.2	28.5	54.9	....	....	....	54.7	4.6
		Col.	11.2	30.5	53.9	....	....	....		
Margarine oil (another lot of blended corn oil product)	Predominantly non-hydrog. CO + hydrog. SBO and hydrog. CSO	Spec.	17.4	49.0	28.9	0.3	0.0	0.1		
		Thio.	19.1	47.9	28.6	....	....	....	28.6	1.6
		Col.	18.8	48.4	28.4	....	....	....		
Margarine oil	Blend—hydrog. CSO and hydrog. SBO	Spec.	17.5	63.2	12.5	0.4	1.9	0.0		
		Thio.	19.7	59.7	16.2	....	....	....	11.1	0.5
		Col.	20.6	57.8	17.2	....	....	....		
Margarine oil	Hydrog. SBO + about 15% non-hydrog. CSO	Spec.	13.8	68.6	10.6	0.5	2.1	0.0		
		Thio.	16.2	63.3	16.1	....	....	....	7.7	0.4
		Col.	19.8	56.1	19.7	....	....	....		
Margarine oil	Blend—hydrog. SBO and hydrog. CSO + a few % butter oil	Spec.	14.0	72.9	7.5	0.3	0.0	1.0		
		Thio.	14.7	71.6	9.3	....	....	....	7.0	0.4
		Col.	18.8	63.4	13.4	....	....	....		
Margarine oil (another lot of the hydrog. corn oil product)	Hydrog. CO	Spec.	7.3	75.4	11.9	0.0	1.0	0.0		
		Thio.	13.5	64.5	17.6	....	....	....	7.2	0.4
		Col.	18.2	55.1	22.3	....	....	....		

[a] CO = corn oil; SBO = soybean oil; CSO = cottonseed oil.
[b] Spec. = spectrophotometric; thio. = thiocyanogen + iodine value; col. = column chromatographic + I.V.

For estimating the saturated fatty acid content of hydrogenated fats, particularly those high in linoleic isomer content, the preferred method is the direct test of column chromatography or GLP chromatography.

From the results of this study, it is now possible to characterize in detail the fatty acid composition of a highly isomerized margarine fat, such as that in the HCOM. The biological assay for estimating *cis*-9, *cis*-12 linoleic acid content is a costly and time-consuming test of ca. 20 weeks and hence will never be used as a routine test. However, fairly good characterization of the fatty acid composition of highly isomerized margarine fats can be achieved by using four of the physico-chemical procedures. This method is applicable to most margarine fats, those made with domestic vegetable oils and containing no residual linolenic acid. When the latter is present, for each per cent of linolenic acid the value for saturated fatty acids by the thiocyanometric procedure is correspondingly low.

Table VI gives a characterization of the fatty acids in the HCOM (I) which had been bio-assayed. This table also gives the characterization of another production lot of this same margarine (II), an even more highly isomerized fat, but with the same solids content index (38) values. The latter fat has recently been shown to increase the serum cholesterol levels of humans (39) when compared with a fat of the same fatty acid pattern but free from isomers.[1] Table VII summarizes the results of the analyses of this fat by the four physico-chemical tests. The ratio of isomeric forms to natural linoleic acid was 4:1. This high ratio may explain why this product increased the serum cholesterol level in contrast to the finding by others (40) that linoleic acid isomers up to 8% of the fats fed do not elevate the cholesterol level. In the latter study (40) the ratio of isomeric forms to natural linoleic acid in the hydrogenated fats was relatively small, varying from a low of 0.1:1 to a high of 0.4:1. Hence, results noted by Anderson, et al. (39) could readily have been missed in the McOsker, et al. study (40) where less critical test systems were investigated.

Since the fat in the BCOM contains almost no isomers of linoleic acid, the characterization of the fatty acids present may be taken as the average of the values obtained by all five assay methods listed in Table V. Thus, this fat contains 29.3% *cis*-9, *cis*-12 linoleic acid, 46.8% oleic acid (25%, absolute, being *trans* oleic acid), and 18.7% saturated fatty acids in the triglycerides; essential to saturated fatty acids is 1.6:1. In the fats of the HCOM, essential to saturated fatty acids is 0.3:1.

Physico-Chemical Assays and Nutrition Value

From this study it is possible to obtain a good estimate of the essential fatty acid content of hydrogenated fats containing residual dienes. By subtracting from the spectrophotometrically found value for non-conjugated linoleic acid the difference between a) the *total* linoleic acid value obtained spectrophotometrically, and b) that obtained by the thiocyanometric procedure, a figure in reasonable agreement with the biological assay estimate is obtained. Such application of the physico-chemical methods is shown in Table VIII which gives the assays on a number of commercial oils and fats. In the first three samples, each practically free of linoleic acid isomers, the values for linoleic acid and those for the saturated fatty acids by all three assay methods are averaged for nutritional interpretations. For the latter four samples, containing linoleic acid isomers, the total of conjugated fatty acids and apparent linolenic acid found by spectrophotometric analyses is added to the figure for non-conjugated linoleic acid, and the sum subtracted from the linoleic acid value obtained by the thiocyanometric procedure. This difference is then subtracted from the spectrophotometric figure for non-conjugated linoleic acid content to provide the value for EFA-linoleic acid. The latter is divided by the figure for saturated fatty acids obtained by col-

[1] Anderson, et al. (39) in comparing results obtained with those of their prediction equation, unfortunately attributed the serum cholesterol raising effect of this margarine fat to the *trans* monoenes present. No such departure of results from those of the prediction equation was noted in their three earlier tests with hydrogenated vegetable oils, containing the same concentrations of *trans* monoenes but very little linoleic acid isomers; they found serum cholesterol results in these tests were either in agreement with the values of the prediction equation or were actually less than expected.

umn chromatography (and/or GLP chromatography) to give the ratio of EFA-linoleic acid to saturated fatty acids.

REFERENCES

1. White, E. A., J. R. Foy, and L. R. Cerecedo, Proc. Soc. Exp. Biol. Med., *54*, 301 (1943).
2. Reid, M. E., *Ibid.*, *86*, 708 (1954).
3. Dam, H., and F. Christensen, Acta Pathol. Microbiol. Skand., *30*, 236 (1952).
4. Hansen, A. E., and H. F. Wiese, Texas Reports Biol. Med., *9*, 49 (1951).
5. Lambert, M. R., N. L. Jacobsen, R. S. Allen, and J. H. Zaletel, J. Nutr., *52*, 259 (1954).
6. Hill, E. G., E. L. Warmanen, H. Hayes, and R. T. Holman, Proc. Soc. Exp. Biol. Med., *95*, 274 (1957).
7. Aaes-Jorgensen, E., Physiol. Rev., *41*, 1 (1961).
8. Wiese, H. F., A. E. Hansen, and D. J. D. Adam, J. Nutr., *65*, 345 (1958).
9. Holman, R. T., J. Am. Med. Assoc., *178*, 930 (1961).
10. Council on Foods and Nutrition of the American Medical Association, *Ibid.*, *181*, 411 (1962).
11. American Heart Association, Planning Fat-Controlled Meals for 1200 and 1800 Calories, (1962).
12. Turpeinen, O., J. Nutr., *15*, 351 (1938).
13. Hume, E. M., L. C. A. Nunn, I. Smedley-MacLean, and H. H. Smith, Biochem. J., *32*, 2162 (1938).
14. Steinberg, G., W. H. Slaton, Jr., D. R. Howton, and J. F. Mead, J. Biol. Chem., *220*, 257 (1956).
15. Mead, J. F., Am. J. Clin. Nutr., *8*, 55 (1960).
16. Mattson, F. H., J. Nutr., *71*, 366 (1960).
17. Nutr. Rev., *19*, 62 (1961).
18. Greenberg, S. M., C. E. Calbert, E. E. Savage, and H. J. Deuel, Jr., J. Nutr., *41*, 473 (1950).
19. Deuel, H. J., Jr., S. M. Greenberg, L. Anisfeld, and D. Melnick, *Ibid.*, *45*, 535 (1951).
20. Melnick, D., and H. J. Deuel, Jr., JAOCS, *31*, 63 (1954).
21. Melnick, D., and C. M. Gooding, U.S. Patent 2,921,855 (1960).
22. Brice, B. A., M. L. Swain, S. F. Herb, P. L. Nichols, Jr., and R. W. Riemenschneider, JAOCS, *29*, 279 (1952).
23. Swern, D., H. B. Knight, O. D. Shreve, and M. R. Heether, *Ibid.*, *27*, 17 (1950).
24. Alfin-Slater, R. B., L. Aftergood, L. Bingemann, G. D. Kryder, and H. J. Deuel, Jr., Proc. Soc. Exp. Biol. Med., *95*, 521 (1957).
25. Nichols, P. L., Jr., R. W. Riemenschneider, and S. F. Herb, JAOCS, *27*, 329 (1950).
26. Jackson, J. R., R. F. Paschke, W. Tolberg, H. H. Boyd, and D. H. Wheeler, *Ibid.*, *29*, 229 (1952).
27. Melnick, D., and F. H. Luckmann, U.S. Patent 2,955,039 (1960).
28. Official Methods of the AOCS, 1957–1959, Cd 2-38.
29. AOCS, "Direct Determination of Saturated Fatty Acids, Collaborative Study," 1960; Kuemmel, D. F., Anal. Chem., *34*, 1003 (1962).
30. Craig, B. M., and N. L. Murty, JAOCS, *36*, 549 (1959).
31. Lipsky, S. R., R. A. Landowne, and J. E. Lovelock, Anal. Chem., *31*, 852 (1959).
32. Keulemans, A. I. M., "Gas Chromatography," Reinhold Publishing Corp., New York, N.Y., 1957, pp. 33–34.
33. Report of Spectroscopy Committee, JAOCS, *36*, 629 (1959); now ACOS Tentative Method, Cd 14-61.
34. Kass, J. P., and G. O. Burr, J. Am. Chem. Soc., *61*, 1062 (1939).
35. Kass, J. P., W. O. Lundberg, and G. O. Burr, Oil and Soap, *17*, 50 (1940).
36. Committee on Fats of the Food and Nutrition Board, National Research Council, A Report on Margarine, June 15 (1943).
37. Andrews, J. T. R., and A. S. Richardson, Oil and Soap, *20*, 90 (1943).
38. Fulton, N. D., E. S. Lutton, R. L. Willie, JAOCS, *31*, 98 (1954).
39. Anderson, J. T., F. Grande, and A. Keys, J. Nutr., *75*, 388 (1961).
40. McOsker, D. E., F. H. Mattson, H. B. Sweringen, and A. M. Kligman, J. Am. Med. Assoc., *180*, 380 (1962).

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Isomerization of Mono Ethenoid Acids During Hydrogenation[1]

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Abstract

Methyl petroselinate, methyl oleate, and methyl erucate were hydrogenated under conditions used in industry for selective hydrogenation. The resulting products were separated into saturated esters and *trans*- and *cis*-unsaturated esters on a silver nitrate impregnated silicic acid column. The positional isomers in the total hydrogenated samples and the *cis* and *trans*-fractions were determined by oxidation with permanganate-periodate and GLC analysis of the resulting dicarboxylic fragments. Positional isomers were found in both *trans* and *cis*-fractions with equal shifting of the bond toward and away from the carboxyl group, regardless of whether the bond was originally in the 6,9, or 13 position. The ratio of *trans* to *cis*-form in the positional isomers in all cases was higher than the reported equilibrium proportions of 2:1.

Introduction

ISOMERIZATION of mono ethenoid acids during partial hydrogenation has been studied by several investigators. From partially hydrogenated ethyl oleate, Moore (9) isolated both positional and geometric isomers, while Hilditch and Vidyarthi (7), working with methyl oleate, obtained *trans*-isomers with bonds in the 8 and 10 positions. Boelhouwer et al. (4) and Knegtel et al. (8) showed the formation of large amounts of positional isomers during the hydrogenation of unsaturated fatty acids. Allen and Kiess (3) demonstrated that bond shifting toward and away from the carboxyl group was equal during hydrogenation of oleic acid. By separating the *trans* from the *cis*-acids by acetone crystallization and determining the positional isomers present in the purified *trans*-fractions, these authors concluded that the positional isomers were composed of an equilibrium mixture of *cis* and *trans*-isomers. Feuge and Cousins (6) studied the influence of temperature, rate of hydrogen dispersion, type of catalyst, and catalyst concentration on the bond shifts during hydrogenation of methyl oleate. The amount of *trans*-isomers formed was not proportional to either the degree of hydrogenation or the extent of migration of the double bonds. Effects of operating variable during partial hydrogenation of methyl oleate were also studied by Albright and Wisniak (1). They found that the ratio of *trans* to *cis*-isomers approached 2, and that the rates of hydrogenation for *cis, trans* and positional isomers were the same. This latter point was also noted in a recent paper by Allen (2) on the hydrogenation of methyl *cis* 6-, *cis* 9-, and *cis* 12-octadecenoates. Scholfield et al. (11), studying the partial hydrogenation of methyl linolenate, isolated the monoenes formed and fractionated these into the *cis* and *trans*-isomers by countercurrent distribution between methanolic silver nitrate and petroleum ether. In the *cis*-fraction, the major part of the double bonds remained in the original positions, while in the *trans*-fraction the bonds were widely scattered. In the monoenes with double bonds in other than the original positions 13-25% were *cis*.

The present paper describes the application of a silver nitrate impregnated silicic acid column, as described by de Vries (5), to the quantitative separation of saturated esters and *trans* and *cis*-isomers formed during the partial hydrogenation of methyl petroselinate, methyl oleate, and methyl erucate under selective conditions. Positional isomers in the hydrogenated esters and their *cis* and *trans*-fractions

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