Comparison of Various Analytical Techniques for the Determination of Essential Fatty Acids in Hardened Fats

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Abstract

In partially hydrogenated fats containing residual linoleic acid, linoleic acid isomers are found which have no essential fatty acid activity but contribute to the C-18 diene acid values; i.e., to the so-called linoleic acid values obtained by the usual physicochemical methods. Such hydrogenated fats and oil blends, particularly margarine oils, have now been analyzed by a biochemical procedure in the attempt to measure specifically the essential fatty acid content by a direct method. The latter procedure makes use of a lipoxidase enzyme specific for the *cis, cis,* methylene interrupted diene structure in polyunsaturated fatty acids having two or more double bonds. It is concluded that the biochemical method is equally as reliable as the combined use of the spectrophotometric and thiocyanometric procedures for estimating with precision the essential fatty acid content of hydrogenated fats containing residual dienes; the simplicity and speed of the biochemical method make it the procedure of choice.

Introduction

A GREATER AWARENESS of the importance of essential fatty acids in nutrition (1) makes it necessary to have available simple, accurate and precise nonbiological methods for the determination of essential fatty acids in hydrogenated fats as well as in nonhydrogenated oils. Inaccuracies result when the commonly used physicochemical methods are employed for the determination of the essential linoleic acid content of fats in the presence of the geometric and positional isomers of linoleic acid that are produced during hydrogenation (2).

When the physicochemical methods are applied to nonhydrogenated vegetable seed oils for the determination of naturally occurring *cis*-9, *cis*-12-linoleic acid, the values obtained are in good agreement with those found by biological assay. Mixtures of such oils with certain hydrogenated fats (those completely hydrogenated or those hydrogenated to the point of almost complete conversion of the polyunsaturated to the monounsaturated fatty acids) also give values which are in agreement with those obtained by bioassay (2). However, only a fraction of the linoleic acid found by the alkali-isomerization, spectrophotometric assay (the most reliable of any one of the physicochemical methods) of selectively hydrogenated margarine fats was found to be biologically active (2).

In the present report a comparison is made of various analytical methods for the determination of linoleic acid (more properly, of the diene fatty acids) in hydrogenated vegetable oils. With the advent of liquid oil margarines (3), the margarine fats, today, run the gamut from those which are high in the isomeric, nonessential, forms of linoleic acid, to those which contain practically all of the linoleic acid in its naturally occurring *cis*-9, *cis*-12-form, the only form which exhibits essential fatty acid activity (4,5). Thus, the present-day margarines provide excellent test systems for evaluating the usefulness of the various analytical techniques for measuring this essential nutrient.

Experimental

The physicochemical methods investigated in the current study were the alkali-isomerization, spectrophotometric procedure (6), the thiocyanometric procedure (7), and column chromatography for saturated fatty acids coupled with iodine value (8,9). In addition, an enzymic method (10,11) for the direct measurement of natural linoleic acid was also evaluated. The latter, a biochemical method, is based on the specificity of a lipoxidase enzyme for fatty acids containing *cis, cis* methylene interrupted double bonds as in natural linoleic, linolenic, and arachidonic acids. The conjugated diene hydroperoxide produced following atmospheric oxidation of the specific polyunsaturates absorbs light strongly at 234 mμ and the extent of this absorption is measured spectrophotometrically. This method does not measure the polyunsaturated fatty acids with conjugated double bonds, those with widely spaced double bonds, or with the double bonds in the *trans* configuration (10–12).

Briefly, the enzymic method of assay (10,11) consists in reacting a 3.0 ml aliquot of the potassium salts of polyunsaturated fatty acids (5–25 μg) with 0.1 ml of active lipoxidase solution (200 μg/ml). The blank contains 3.0 ml aliquot of the potassium salts of the same polyunsaturated fatty acids and 0.1 ml of boiled (inactivated) lipoxidase solution. All solutions are prepared in a 0.2 M potassium borate buffer, pH 9.0. The reaction is timed immediately after the addition of the active lipoxidase reagent to the sample. Both sample and blank are allowed to stand at room temperature for 25 min. The light absorption of the sample containing the active lipoxidase enzyme is read, in a spectrophotometer, at 234 mμ against the sample containing the heat inactivated enzyme as a blank.

The major problem with the method is in obtaining a lipoxidase enzyme that catalyzes rapidly and quantitatively the peroxidation of potassium salts of the triglyceride. To determine the activity and, hence, acceptability of the lipoxidase enzyme,[1] a sample of nonhydrogenated cottonseed oil (containing 0.5 mg of polyunsaturated fatty acids) is saponified (under an atmosphere of nitrogen, overnight, in the dark, and at room temperature) in a 100 ml volumetric flask, with 1 ml of 0.5 N alcoholic potassium hydroxide. After saponification, 20 ml of 1 M potassium borate buffer is added to the flask. This is followed by 1 ml of 0.5 N hydrochloric acid and then water to the 100 ml mark. Two 3.0 ml aliquots are taken for a sample and a blank and are treated as previously described with the exception that the light absorption of the sample is read in the spectro-

[1] Worthington Biochemical Corporation, Freehold, New Jersey or Nutritional Biochemicals Corporation, Cleveland, Ohio.

photometer at 1 min intervals until a plateau indicating maximum absorption is reached. For satisfactory activity, the maximum absorption should be reached within 5 min after initiation of the reaction.

The specific extinction coefficient for the product of the lipoxidase-catalyzed peroxidation of polyunsaturated fatty acids was obtained, using as substrates [2] saponified trilinolein, methyl linoleate, and a variety of vegetable seed oils. The average value of 82.6 so obtained was used throughout the present study in calculating the contents of the *cis*-polyunsaturated fatty acids in the test fats. This specific extinction coefficient is in fairly good agreement with the figure of 79.9 obtained by MacGee (11).

Results in Applying the Test Methods and Discussion

Eighteen brands of commercial margarines, made by ten different manufacturers, were purchased on the open market. According to the label declarations, about one-half of the margarines contained, as their major component, a liquid vegetable seed oil of domestic origin which was blended with one or more hydrogenated oils to form the oil phase of the margarine.

A comparison is made in Table I of the fatty acid composition of the oil blend in the margarines, as determined by the alkali-isomerization, spectrophotometric technique (6), by the thiocyanometric procedure (7) and by the iodine value balance method following the estimation of saturated fatty acids by column chromatography (8,9). The latter procedure has regularly given values comparable to those obtained by gas liquid partition chromatography in tests conducted on vegetable seed oils, liquid and hydrogenated and essentially free of linolenic acid.

It should be noted that, in most of the margarines listed in Table I in which liquid oil is listed first as a major component in the blend, the values for linoleic acid found by the three different physicochemical methods are in good agreement with each other. The values for saturated fatty acids in such fats obtained by the three procedures are also in fairly good agreement. It is concluded that good agreement among the values found by all three of the physico-chemical methods for linoleic acid is an indication that these margarine fats contain relatively little or none of the isomeric forms of linoleic acid. However, when isomers of linoleic acid are present, as in most margarines of the conventional type (those in Table I which list only partially hydrogenated fats as the margarine fats or as the predominating components of the blends) the agreement is poor among the values yielded by the different methods. The large differences among the values reported for linoleic acid in such margarines are attributed to the presence of geometric and double-bond positional isomers in the partially hydrogenated components and these isomers are included, in varying amounts, in the linoleic acid values yielded by the different physicochemical methods (2).

While each of these physicochemical methods fail in themselves to give accurate values for the essential fatty acid content in partially hydrogenated fats containing isomeric C-18 diene acids, a reliable estimation of essential fatty acid content can be made by the combined use of the alkali-isomerization, spectrophotometric and thiocyanometric procedures (2). This involves substracting from the spectrophotometrically found value for nonconjugated linoleic acid the difference between a) the *total* linoleic acid value obtained spectrophotometrically, and b) that obtained by the thiocyanometric procedure (2). Values so obtained are listed in the next to last column of Table I. This procedure has been applied only to the values obtained in analyzing products which obviously contained linoleic acid isomers. These fats exhibit progressively higher linoleic acid values as less specific methods of assay for *cis*-9, *cis*-12-linoleic acid are used, the thiocyanometric in place of the more specific spectrophotometric procedure, and the least specific column chromatographic in place of the thiocyanometric procedure. All of the margarines of the conventional type (those made predominantly with hydrogenated oils) and a few of the liquid oil margarines are in this category.

For those margarines in which the individual values for linoleic acid determined by all three physicochemical methods are in good agreement, i.e. within $\pm 5\%$ (relative) of the average, the values obtained by all three physicochemical methods have been averaged, corrected for conjugated fatty acids present, and reported as essential fatty acids by physicochemical assay in Table I. These products comprised, for the most part, the margarines with a liquid vegetable seed oil as the major component.

The need for a direct method for measuring, with precision and specificity, the essential fatty acid content of all types of fats and oils is apparent from the data in Table I. The results of studies (not herein reported) have shown that the enzymic method, like any one of the physicochemical methods, gives reliable values for natural linoleic acid in analyzing nonhydrogenated vegetable oils. Boatman and Hammond (13) have also reported agreement between spectrophotometric and enzymic methods in analyzing another type of nonhydrogenated fat (milk fat).

However, the more complicated test systems for assessing the value of the enzymic method are those involving margarine fats known to contain linoleic acid isomers. In the last column of Table I are listed the essential fatty acid contents of such margarine fats as obtained by the biochemical method. In every case this method gives the lowest value for essential fatty acid content in the tests conducted on margarine fats known to contain significant amounts of linoleic acid isomers (those fats that show a progressive increase in apparent linoleic acid content as progressively less specific methods for *cis*-9, *cis*-12-linoleic acid are used).

A comparison of the results in the last two columns of Table I, obtained in analyzing the margarine fats for essential fatty acids by the enzymic procedure with those calculated from the combined use of the spectrophotometric and thiocyanometric methods, is of interest. These two unrelated procedures give values in good agreement, the differences between the values being essentially within the experimental errors of the assay methods. The values by one method are as often above as they are below those yielded by the other procedure. The average absolute difference between values obtained by the combined physicochemical methods and those by the enzymic method is -0.20% (the enzymic method yielding slightly lower values). An evaluation of the differences between the values for the test fats (Student's "t" test for paired differences) has shown that this

[2] The purities of the substrates were established by gas liquid partition chromatographic and by alkali-isomerization spectrophotometric analyses.

TABLE I

Fatty Acid Values Obtained by Physicochemical and Lipoxidase Enzymic Assays of Commercial Margarines
(Values reported as per cent of triglycerides)

Code identification	Ingredient declaration	Iodine value (Wijs)	Fatty acids found by physicochemical assays							EFA content[b]	EFA by enzymic assay
			Assay method[a]	Conj. trienoic	Conj. dienoic	Linolenic	Linoleic	Oleic	Saturated		
CP-M	Liquid corn oil, partially hardened soybean and cottonseed oils	96.3	Spec.	0.1	0.00	0.3	29.0	47.2	19.0		
			Thio.	.....	.....	....	28.7	49.1	17.8	29.4*	28.2
			Col.	.....	.....	....	30.4	45.9	19.3		
CP-M	Liquid corn oil, partially hardened soybean and cottonseed oils	98.2	Spec.	0.04	0.8	0.4	27.4	51.3	15.7		
			Thio.	.....	.....	....	29.8	49.1	16.7	28.6*	27.7
			Col.	.....	.....	0.4	29.7	48.2	17.3		
CP-M	Liquid corn oil, partially hardened soybean and cottonseed oils	96.7	Spec.	0.03	0.6	0.2	28.2	49.0	17.6		
			Thio.	.....	.....	....	29.1	48.9	17.6	28.6*	28.2
			Col.	.....	.....	0.2	29.2	48.1	18.1		
CP-N	Liquid cottonseed oil (nonhydrogenated) partially hydrogenated soybean oil	89.9	Spec.	0.02	0.00	0.0	26.3	46.9	22.4		
			Thio.	.....	.....	....	27.3	44.9	23.2	26.9*	27.1
			Col.	.....	.....	....	27.2	45.2	23.2		
CP-N	Liquid cottonseed oil (nonhydrogenated) partially hydrogenated soybean oil	92.2	Spec.	0.03	0.00	0.0	27.7	46.6	21.3		
			Thio.	.....	.....	....	29.4	43.1	23.1	28.4*	27.3
			Col.	.....	.....	....	28.1	46.0	21.5		
MF-CO	Liquid corn oil, *partially hardened corn oil*	98.3	Spec.	0.02	0.00	0.6	26.0	55.3	13.7		
			Thio.	.....	.....	..	27.2	54.6	13.8	27.5*	26.3
			Col.	.....	.....	0.6	28.0	51.2	15.8		
MF-R	Partially hardened soybean and cottonseed oils	89.1	Spec.	0.01	0.00	0.8	14.3	67.8	12.7		
			Thio.	.....	.....	....	16.1	66.7	12.8	13.3Ø	12.3
			Col.	.....	.....	0.8	19.3	57.8	17.7		
SB-F-HCO	Hardened corn oil	85.0	Spec.	0.02	1.8	0.0	9.5	72.1	12.3		
			Thio.	.....	.....	....	13.8	67.0	14.8	7.0Ø	5.6
			Col.	.....	.....	....	16.3	61.9	17.4		
SB-F-HCO	Hardened corn oil	91.8	Spec.	0.14	4.9	0.2	8.1	74.9	7.4		
			Thio.	.....	.....	. .	16.7	68.4	10.5	4.7Ø	4.3
			Col.	.....	.....	0.2	22.0	57.2	16.2		
SB-F-CO	Liquid corn oil, partially hydrogenated corn oil	94.9	Spec.	0.04	0.00	0.4	22.4	59.1	13.7		
			Thio.	.....	.....	....	24.4	56.4	14.8	20.8Ø	21.7
			Col.	.....	.....	0.4	25.9	52.2	17.1		
SB-F-CO	Liquid corn oil, partially hydrogenated corn oil	101.2	Spec.	0.04	0.00	0.5	28.2	54.1	12.8		
			Thio.	.....	.....	....	30.7	50.7	14.2	26.2Ø	26.8
			Col.	.....	.....	0.5	31.2	48.2	15.7		
SB-BB	Partially hydrogenated vegetable oils (soya and cottonseed)	88.0	Spec.	0.02	1.3	0.6	13.1	67.0	13.6		
			Thio.	.....	.....	...	17.2	63.1	15.3	10.9Ø	12.5
			Col.	... ..	.....	0.6	17.9	60.0	17.1		
LB-I	Partially hardened soybean oil, liquid corn oil, partially hardened cottonseed oil, butter	93.4	Spec.	0.02	0.00	1.3	23.0	53.6	17.7		
			Thio.	.....	.....	...	25.6	52.2	17.8	25.4*	23.7
			Col.	.....	.....	1.3	25.1	49.4	19.8		
LB-GL	Partially hardened soybean and cottonseed oils	83.1	Spec.	0.01	0.00	0.8	10.7	68.5	15.6		
			Thio.	.....	.....	....	12.8	66.4	16.4	9.4Ø	9.3
			Col.	.....	.....	0.8	15.9	58.0	20.9		
K-D	Partially hardened corn oil, liquid corn oil	94.0	Spec.	0.03	1.6	0.4	18.4	63.0	12.2		
			Thio.	.....	.....		22.0	60.1	13.5	16.8Ø	16.3
			Col.	.....	.....	0.4	24.2	54.6	16.4		
K-P	Partially hardened soybean oil and cottonseed oil	83.9	Spec.	0.02	1.6	0.6	8.5	71.3	13.6		
			Thio.	.....	.....	....	11.3	70.6	13.7	7.9Ø	8.5
			Col.	.....	.....	0.6	15.0	61.4	18.6		
S-AW	Liquid cottonseed oil, partially hardened cottonseed oil	96.5	Spec.	0.08	0.00	0.1	37.4	31.5	26.5		
			Thio.	.....	.....	..	38.6	29.6	27.4	37.7*	37.3
			Col.	.....	.....	0.1	37.0	32.6	25.9		
S-AS	Partially hardened soybean liquid soybean and cottonseed oils	87.2	Spec.	0.01	0.00	1.0	14.0	65.9	14.7		
			Thio.	.....	.....	...	15.7	65.6	14.3	13.3Ø	13.8
			Col.	.....	.....	1.0	17.0	59.7	17.9		
AP-CO	Partially hardened corn oil	89.2	Spec.	0.02	1.2	0.2	10.6	74.9	8.7		
			Thio.	.....	.....	...	17.0	64.9	13.7	5.6Ø	6.6
			Col.	.....	.....	0.2	18.9	60.5	16.0		
AP-NT	Hardened soybean and cottonseed oils	84.0	Spec.	0.001	0.00	0.5	11.3	69.2	14.6		
			Thio.	.....	.....	....	13.1	67.0	15.5	10.0Ø	10.5
			Col.	.....	. ....	0.5	15.6	60.6	18.9		
PM-E	Corn oil (not hardened) coconut oil (hardened)	100.3	Spec.	0.04	0.00	0.8	41.6	25.3	27.9		
			Thio.	.....	.....	....	43.3	24.4	28.0	42.9*	41.7
			Col.	.....	.....	....	.....	.....	.....		
C-D	Liquid corn oil, partially hydrogenated corn, cottonseed and soya oils	96.1	Spec.	0.03	0.00	0.0	27.2	52.1	16.3		
			Thio.	.....	.....	....	28.6	49.2	17.8	25.8Ø	26.5
			Col.	.....	.....	....	29.9	46.7	19.0		
B-ND	Coconut, palm kernel and peanut oils	32.3	Spec.	0.02	0.00	0.0	9.0	17.8	68.8		
			Thio.	.....	.....	....	8.7	18.2	68.7	8.9*	9.2
			Col.	.	.....	..	...	.....	.....		

[a] Spec. = alkali-isomerization spectrophotometric method; Thio. = thiocyanogen procedure + iodine value; Col. = column chromatography + iodine value.

[b] EFA = Content of essential fatty acids; values with an "*" are the average figures obtained in good agreement by the three physicochemical methods, after correcting for the presence of conjugated fatty acids; values with an "Ø" are based upon the combined use of the spectrophotometric and thiocyanometric methods (2) since these products obviously contained linoleic acid isomers; see text.

average difference is not statistically significant. A correlation coefficient, $r = 0.99$, has been obtained showing a real linear relationship to exist between the values yielded by the two procedures.

Of interest is the fact that two of the margarines in the present study were the very same products employed by Alfin-Slater and Melnick in their study (2) of the essential fatty acids of various fats. These products had been held continuously in deep-freeze storage. For purpose of easy comparison, the results by all methods of assay are summarized in Table II. It is readily apparent that the enzymic method

TABLE II

Essential Fatty Acid Content of Two Margarine Fats

Assay procedure	Cis-9, cis-12-linoleic acid in Blended corn oil margarine [a]	Hydrogenated corn oil margarine [b]
	% of triglycerides	
Bioassay (2)	28.9	5.3
Combined physicochemical assays (2)	29.4	7.0
Enzymic oxidation-present findings	28.2	5.6
Values by conventional methods	Apparent linoleic acid Blended corn oil margarine [a]	Hydrogenated corn oil margarine [b]
	% of triglycerides	
Alkali-isomerization, spectrophotometric	29.0	9.5 [c]
Thiocyanogen + iodine value	28.7	13.8
Column chromatography + iodine value	30.4	16.3
Gas liquid partition chromatography (14–16)	29.6	17.4

[a] Made with liquid corn oil as the major ingredient, the hydrogenated moieties essentially free of linoleic acid and its isomers.
[b] Highly isomerized, selectively hydrogenated fat.
[c] Does not include the found 1.8% of linoleic acid with double bonds in conjugated position; such conjugated linoleic acid is separately measured in the spectrophotometric method.

gives values in good agreement with the bioassay findings and that this occurs regardless of whether linoleic acid isomers are present or absent.

Whereas the combined use of the spectrophotometric and thiocyanometric procedures also gives reliable values for the essential fatty acids in all types of fats, it lacks the simplicity of the enzymic method. The latter, as a *direct* measure of the *cis*-9, *cis*-12-linoleic acid, should appeal to most analysts. One analyst can complete in an 8-hr working day ten analyses, setting up ten saponifications on a given day and completing on the same day the enzymic oxidations, readings and calculations on the ten saponified samples from the previous day. The enzymic method should be the method of choice in analyzing all types of fats and oils for essential fatty acid content.

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