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Spectrophotometric Micromethod for Determining Polyunsaturated Fatty Acids

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There is considerable need for a micromethod of analysis of polyunsaturated fatty acids in fats or lipides, because often more than a few milligrams of sample may be difficult or impractical to obtain. A spectrophotometric method is described which requires 1 to 10 mg. of fat for determination of acids containing from two to five double bonds. Results of analyses by the micromethod were in agreement with those obtained by macromethods. The micromethod should find greatest application in studies of the changes in lipide composition of fluids and tissues of living animals and plants owing to the small quantity of sample required for analysis.

IMPROVEMENTS in spectrophotometric methods for determining polyunsaturated acids in fats and oils have been discussed in recent papers (*6, 7*). Although the methods currently in use require only 100 mg. of sample, there is a great deal of interest in a micromethod that would require only a few milligrams. Numerous inquiries concerning such an adaptation of the method have been received from persons engaged in biological and medical research, because often more than a few milligrams of lipide may be difficult or impractical to obtain—for example, in analysis of body fluids taken from living animals.

A search of the literature has furnished further evidence of keen interest in spectrophotometric micromethods. Considerable work in this direction has been published (*4, 9, 10, 11, 12*). Unfortunately, these investigators did not have pure, natural, unsaturated acids available as reference standards from which they could determine the correct spectrophotometric constants for their special conditions of alkali isomerization. This is particularly true for acids of greater unsaturation than linolenic acid. Consequently, these workers did not recommend their published constants, and advised others to determine their own independently.

In spite of the fact that no satisfactory micromethod resulted from this earlier work, the greater amount of conjugation produced by isomerizing in higher concentrations of potassium hydroxide in glycol indicated possibilities for a more sensitive method. For example, Holman and Burr (*9*) reported an extinction coefficient, $E^{1\%}_{1\text{cm.}} = 622$, for tetraene (3000 A.) from debromination–arachidonic acid isomerized in glycol solution containing 22 to 23 grams of potassium hydroxide per 100 ml. for 8 minutes at 178° C. This value is more than twice as great as the value 258, obtained under the conditions used by Beadle and Kraybill (*3*).

More recently, Herb and Riemenschneider (*7*), working with natural acids isolated by physical methods, reported optimum conditions for producing maximum triene, tetraene, and pentaene conjugation from corresponding nonconjugated acids. They heated 70 to 80 mg. of sample in 11 grams of 21% potassium hydroxide–glycol solution for 15 minutes at 180° C. The extinction coefficients determined were much greater than those obtained by standard methods (*1, 3, 6*) for all polyunsaturated acids except linoleic acid, in which case there was essentially no difference. These higher values lend greater sensitivity to the method.

The present investigation was undertaken to determine whether the optimum conditions of isomerization, with attendant greater sensitivity, could be adapted satisfactorily to the determination of unsaturated constituents in microsamples (1 to 10 mg.). A method was developed which gave results in agreement with those obtained by macromethods.

EXPERIMENTAL

The initial experiments consisted in isomerizing 10 mg. of fat in 11.0 grams of 21% potassium hydroxide–glycol for 15 minutes at 180° C. This procedure was not entirely satisfactory. It was difficult to obtain accurate spectral densities when determining minor components of the fat because of the large amount of reagent that had to be diluted to a relatively small volume of solution. This objection, of course, can be overcome by reducing the amount of reagent. Analysis indicated, however, that the results were more reproducible when the depth of the reagent was kept about the same as that obtained with 11 grams in the standard 1 × 10 inch reaction tube. Accordingly, the standard tube was modified so that the appropriate depth was obtained with 5 grams of reagent.

Equipment for Isomerization. The equipment for the isomerization was identical with that described for a standard macromethod (*1*) except for a modified reaction tube. The standard 1 × 10 inch reaction tube was cut off about 5.5 inches from the bottom, and a 5.5-inch length of test tube $^5/_8$ inch in diameter was sealed on. Weighing cups for introducing the sample to the reaction tube were made from standard heat-resistant glass tubing and were 8 mm. in diameter and 6 mm. in height.

Preparation of 21% Potassium Hydroxide–Glycol Reagent. The procedure for preparing the isomerizing reagent was similar to that described for the standard method (*1*). Ethylene glycol was heated to 190° C. for 10 minutes and allowed to cool to 150° C., and the calculated amount of potassium hydroxide was added to give a solution of 21.0% potassium hydroxide by weight (approximately 28 grams of 85% potassium hydroxide pellets to 100 grams of glycol). The alkali solution was again heated to 190° C. for 10 minutes and then cooled to room temperature. A blanket of nitrogen was kept over the reagent at all times. The

strength of the solution was checked by titrating a weighed aliquot with standard acid and adjusting to ±0.1 of the desired percentage by weight with ethylene glycol that had been dried by heating to 190° C. for 10 minutes. The clear, essentially colorless reagent was stored under nitrogen in a refrigerator at about 40° F.

Method. The reaction tube containing 5.0 grams of 21% potassium hydroxide–glycol reagent was blanketed with oxygen-free nitrogen and heated in the bath at 180° C. for 15 minutes. An accurately weighed 1- to 10-mg. sample of fat in a weighing cup was then added to the reaction tube by inclining the tube in the bath and allowing the cup to slide in gently. The tube was removed from the bath and shaken vigorously for 5 seconds and then replaced. Shaking was repeated at 30-second intervals until the contents appeared clear and homogeneous. Three such shakings were always employed; occasionally a fourth was necessary. A reaction tube containing reagent but no sample was treated similarly for use as a blank. The reaction was accurately timed with a stop watch from the moment the sample was added. After heating for a total of exactly 15 minutes, the tube was removed from the bath and cooled rapidly in cold water. The isomerized mixture was diluted to known volumes with absolute methanol until suitable spectral densities were reached. For components present in low concentration, the dilution with absolute methanol was usually made in a 25-ml. volumetric flask, and cells 2.5 or 5.0 cm. long were employed. Only calibrated volumetric flasks and pipets, and aliquots of not less than 10 ml., were used in making dilutions. Appropriate readings were made in a Model DU Beckman spectrophotometer.

Table I. Specific Extinction Coefficients of Pure Natural Polyunsaturated Acids Isomerized in 21% Potassium Hydroxide–Glycol and 11% Potassium Hydroxide–Glycerol Reagents

Acid	Wave Length, mμ	Specific Extinction Coefficients[a] 21% KOH–glycol[b]	11% KOH–glycerol[c]
Linoleic	233	91.6	93.9
Linolenic	233	47.5	58.6
	268	90.5	48.6
Arachidonic	233	39.7	55.0
	268	48.2	46.8
	315	60.6	20.3
Eicosapentaenoic	233	39.4	48.9
	268	41.2	33.3
	315	82.4	26.8
	346	87.5	15.0
Docosapentaenoic	233	43.5	50.0
	268	46.0	35.2
	315	56.9	23.8
	346	50.4	10.9
50% C_{20}–50% C_{22}	233	41.5	49.5
	268	43.6	34.3
	315	69.7	25.3
	346	69.0	13.0

[a] In methanol solutions.
[b] Isomerized for 15 minutes at 180° C. under nitrogen.
[c] Isomerized for 45 minutes at 180° C. under nitrogen.

A microanalytical balance is not necessarily a prerequisite for this method. When 10 to 30 mg. of sample are available it can be weighed, with an accuracy within 1%, on a good analytical balance. This weighed amount can be dissolved and mixed thoroughly with a weighed quantity of pure lauryl alcohol. The amount of lauryl alcohol added is such that a 10- to 30-mg. aliquot will contain 1 to 3 mg. of the lipide sample. This aliquot is placed in the small glass weighing cup, and the isomerization with 21% potassium hydroxide–glycol is carried out by the method described. Of course, only the actual weight of lipide in the aliquot is used in calculating the composition.

This alternative procedure has an additional advantage. For example, weighed aliquots of the lauryl alcohol solution can also be used to determine iodine and saponification values, thus making it possible to obtain rather complete analyses on 10 to 30 mg. of fat or lipide without requiring a microanalytical balance.

It is essential to determine the absence of interfering impurities in the lauryl alcohol. For example, when lauryl alcohol comparable in amount with that used in the spectrophotometric determination was subjected to the alkali isomerization procedure, no significant absorption resulted.

In the application of any spectrophotometric method to the analyses of biological materials, such as lipide extracted from body fluids, it is conceivable that occasionally impurities or conjugated compounds may be present which absorb in the ultraviolet spectral region. Depending on their concentration and intensity of absorption in the specific spectral regions used in the method, the impurities or conjugated compounds could influence the fatty acid analysis significantly. This initial absorption can be detected by spectral density measurements of the lipide materials before alkali isomerization and corrections can be made in the manner described in previous work (*6*). In some instances, it may be necessary to saponify the lipide material and remove the unsaponifiable matter. The spectral analyses are then made on the recovered fatty acids.

RESULTS AND DISCUSSION

The specific extinction coefficients to be used with these isomerizing conditions and the values for the 11% potassium hydroxide–glycerol method were compared in a recent publication (*7*). They are reproduced in Table I. The extinction coefficients for all polyunsaturated acids except linoleic acid, at their principal maxima, are much greater when determined by the 21% potassium hydroxide–glycol isomerization than when determined by the 11% potassium hydroxide–glycerol method. Linolenic acid, at 268 mμ, has a coefficient of 90.5 as compared with 48.6; arachidonic acid, at 315 mμ, 60.6 as compared with 20.3. The greatest difference was observed for eicosapentaenoic acid, which has a principal maximum at 346 mμ. In this instance, the comparison is 87.5 to 15.0. These differences indicate a two- to six-fold increase in sensitivity for determining the respective components. Similar comparison could be made between the 21% potassium hydroxide–glycol and 6.5% potassium hydroxide–glycol isomerization methods, since the coefficients are approximately the same for the latter method and for the 11% potassium hydroxide–glycerol method.

The specific extinction coefficient of a fat or oil at a selected wave length is equal to the sum of the specific extinction coefficients of the components, each multiplied by its proportion in the mixture. Therefore, simultaneous equations may be set up for the system and solved for each component. The data in Table I were used in deriving the following equations for the simultaneous determination of linoleic, linolenic, arachidonic, and pentaenoic acids in fats and oils when (*a*) the pentaenoic acid present is known to be a C_{20} acid, (*b*) the pentaenoic acid is known to be a C_{22} acid, and (*c*) the chain length of the pentaenoic acid is unknown; the assumption is made that it is a mixture of 50% C_{20} and 50% C_{22} acids, as discussed in a previous paper (*7*).

(*a*) Samples containing C_{20} pentaenoic acid:

$$\text{Linoleic acid, } \% = 1.092\, k_{233} - 0.573\, k_{268} - 0.259\, k_{315} + 0.023\, k_{346}$$

$$\text{Linolenic acid, } \% = 1.105\, k_{268} - 0.879\, k_{315} + 0.307\, k_{346}$$

Table II. Analyses of Several Vegetable Oils by Micromethod and Macromethod A

(All results reported as percentage of acid in sample)

Sample	Method[a]	Sample Weight, Mg.	Linoleic, %	Linolenic, %
Cottonseed oil	Macro *A*	100	52.4	...
	Micro	10	52.8	...
Soybean oil 1	Macro *A*	100	52.6	8.3
	Micro	10	52.6	8.1
Soybean oil 2	Macro *A*	100	52.3	8.3
	Micro	10	51.6	8.0
Linseed oil	Macro *A*	100	16.4	50.6
	Micro	10	15.9	51.0

[a] Micro; 21% KOH–glycol; 15 minutes' isomerization at 180° C. under N_2; constants reported in Table I. Macro A; 6.5% KOH–glycol; 25 minutes' isomerization at 180° C. under N_2; natural acid constants (*6*).

Table III. Analyses of Samples Containing Pentaenoic Acid by Micromethod and Macromethods

(All results reported as percentage of acid in sample)

Sample	Method[a]	Sample Weight, Mg.	Linoleic, %	Linolenic, %	Arachidonic, %	Pentaenoic, %
Lard 2	Macro B	100	11.3	0.91	0.28	...
	Micro	10	11.0	0.83	0.30	0.09
Methyl esters from lard 1, fraction 7[b]	Macro B	100	25.2	7.9	4.8	1.8
	Micro	10	26.3	6.5	4.9	1.3
Methyl esters from lard 1, fraction 8[b]	Macro B	100	21.8	11.3	8.7	5.3
	Micro	1–1.5	23.7	9.3	8.5	4.0
Fatty acids from lard 2, fraction 10[c]	Macro B	100	12.3	36.2	14.1	8.4
	Micro	10	12.3	36.7	15.8	7.5
Fish oil	Macro A	100	7.5	−18.5	47.0	...
	Micro[d]	10	3.1	4.2	5.1	20.5

[a] Micro: 21% KOH–glycol; 15 minutes isomerization at 180° C. under N_2; constants reported in Table I. Macro A: 6.5% KOH–glycol; 25 minutes isomerization at 180° C. under N_2; natural acid constants (*6*). Macro B: 11% KOH–glycerol; 45 minutes isomerization at 180° C. under N_2; constants reported in Table I.
[b] Concentrate of polyunsaturates obtained by low temperature crystallization and high vacuum distillation.
[c] Fraction obtained by low temperature crystallization and adsorption separation on silicic acid (*8*); unsaponifiable material removed.
[d] Also had a specific extinction coefficient of 4.30 at 374 mμ.

Arachidonic acid, % = $1.650\ k_{315} - 1.554\ k_{346}$

C_{20} pentaenoic acid, % = $1.143\ k_{346}$

(*b*) Samples containing C_{22} pentaenoic acid:

Linoleic acid, % = $1.092\ k_{233} - 0.573\ k_{268} - 0.259\ k_{315} - 0.126\ k_{346}$

Linolenic acid, % = $1.105\ k_{268} - 0.879\ k_{315} - 0.016\ k_{346}$

Arachidonic acid, % = $1.650\ k_{315} - 1.863\ k_{346}$

C_{22} pentaenoic acid, % = $1.984\ k_{346}$

(*c*) Samples containing pentaenoic acid of unknown chain length (calculated as 50% C_{20}, 50% C_{22} pentaenoic acids):

Linoleic acid, % = $1.092\ k_{233} - 0.573\ k_{268} - 0.259\ k_{315} - 0.033\ k_{346}$

Linolenic acid, % = $1.105\ k_{268} - 0.879\ k_{315} + 0.190\ k_{346}$

Arachidonic acid, % = $1.650\ k_{315} - 1.667\ k_{346}$

Pentaenoic acid % = $1.449\ k_{346}$

The equations under (*c*) were employed in all analyses of fats and oils by the micromethod reported in this paper.

Analyses of Fats and Oils. A number of samples were analyzed by the micromethod described in this paper, and the results were compared with those obtained by either macromethod *A*—isomerization in 6.5% potassium hydroxide–glycol reagent at 180° C. under nitrogen for 25 minutes, using the constants for natural acids (*6*)—or macromethod B—isomerization in 11% potassium hydroxide–glycerol reagent at 180° C. for 45 minutes under nitrogen, using the constants for natural acids given in Table I.

Table II shows results of analyses of several common vegetable oils by the micromethod and macromethod A. The results by the two methods are in good agreement. These oils, however, are relatively simple in composition, and they do not contain acids of greater unsaturation than that of linolenic acid.

In the analyses of more complex fats and oils, which contain tetraenoic and pentaenoic acids as well as the more common linoleic and linolenic acids, the choice of a method becomes much more important. It is essential to select a method that provides spectrophotometric constants for all polyunsaturated acids in the sample. Constants for pentaenoic acids are available for the micromethod and macromethod B, but not for macromethod A. Table III shows results of analyses of several complex samples by the micromethod and macromethod B. The results by these two methods are in good agreement.

Results of analyses of fish oil by macromethod A and the micromethod are included to stress the importance of employing methods that provide all the necessary constants. It is evident that the former method gives impossible values. The values by the micromethod cannot be accepted as an accurate representation of composition either because this sample had a significant absorption maximum at 374 mμ (k = 4.3). This absorption indicates the probable presence of a hexaenoic acid, for which no constants have yet been determined. Hence, none of the present methods will give accurate values for the composition of this type of oil.

Table IV. Reproducibility of Micromethod

(Single determinations reported as percentage of acid in sample)

Sample	Sample Weight, Mg.	Linoleic, %	Linolenic, %	Arachidonic, %	Pentaenoic, %
Linseed oil	10	15.7	51.5	...	...
		16.2	50.8	...	...
		15.7	50.8	...	...
	2[a]	16.0	51.8	...	...
		16.0	52.0	...	...
		15.8	51.9	...	...
Methyl esters[b] from lard 1, fraction 8	10	23.6	9.04	8.79	4.58
	1–1.5	23.9	9.35	8.50	4.02
		23.6	9.37	8.41	3.89
		23.6	9.22	8.61	4.16
	5[a]	24.7	9.15	8.59	5.08
		24.7	9.14	8.64	5.02
	2[a]	24.0	8.97	8.96	4.80
		24.0	8.87	9.37	4.89
Fish oil	10	3.02	4.21	5.13	20.3
		3.11	4.09	5.13	20.7
		3.12	4.19	5.01	20.6
	2[a]	2.66	3.93	5.32	20.3
		2.62	3.87	5.35	20.2
		2.77	4.68	5.71	20.7

[a] Lauryl alcohol diluent procedure used in weighing of sample.
[b] Concentrate of polyunsaturates obtained by low temperature crystallization and high vacuum distillation.

To show the reproducibility of the micromethod, results of single determinations of the polyunsaturated content of several samples are listed in Table IV. In some instances the sample was weighed directly on a microanalytical balance; in others it was weighed on a macroanalytical balance as an aliquot from a lauryl alcohol solution. In general, based on experience with both the standard macromethods and the micromethod, it is the authors' opinion that the reproducibility of the latter method is fully comparable with the reproducibility of the macromethods. A reasonable appraisal of the reproducibility of the spectrophotometric macromethods can be gained from previous publications (*2, 5, 6*).

The method without modification has given equally good results on sample weights up to 40 mg.

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