Though these curves indicate a minimum of about one hour for a practical holding-time, it seems quite probable from other available information (1, 3) that still shorter chilling periods can be used when the miscella is agitated during the chilling period. In the present experiments the miscella was chilled from room temperature by setting the centrifuge bottle in a bath at the desired temperature and was not stirred. It has been shown (1, 3) that crystallization proceeds much more rapidly when the solutions are even gently agitated every 10 minutes. It can thus be expected that with the relatively violent agitation in industrial chilling units the curves corresponding to those in Figure 1 would flatten out much sooner and that therefore a chilling period considerably below one hour would be feasible under those conditions.

Cold Tests. Cold tests were obtained by a modification (3) of the A.O.C.S. Official Method Cc 11-42 Cold Test. Winterized oils were obtained in all the above experiments in which a holding-time of one hour or longer was used. There was very good agreement between the cold tests obtained and those predicted for an oil of this particular iodine value (see Ref. 3, Fig. 10). If 5.6% of solid is removed from an oil having an iodine value of 110, like that of the present oil, the winterized oil would be expected to fail the cold test between 6 and 20 hours. The actual failure found when this amount of solid was removed from a 30% oil concentration at —12°C. with a 1-hour holding-time, was between 8 and 15 hours. A 2-hour holding-time at the same concentration and temperature resulted in the removal of 6.8% solid and a cold-test failure somewhere between 24 and 40 hours. The predicted cold test would have been just below 20 hours. All samples that had more than 7.0% solid removed during the winterization experiments passed more than a 40-hour cold test.

Settling Qualities of the Solid Separating. No special settling tests in graduated conical centrifuge tubes were performed. Measurements of the settling were however made as before (1) on each sample after centrifuging in the 250-ml. centrifuge bottles. The results indicated that the reduction in holding-time did not have an appreciable effect upon the settling characteristics of the solid.

Summary

The purpose of this investigation was to determine whether and under what conditions it would be practicable to use holding-times of less than three hours in the solvent winterization of cottonseed oil in 85-15 acetone-hexane mixture. Since the output of the chilling unit varies inversely as the holding-time required, it is advantageous to use the shortest feasible holding-time. Systematic winterization data were obtained on a laboratory scale for different oil-solvent ratios and temperatures using the technique previously described.

The results indicate that, under the static chilling conditions used in these experiments, the holding-time can be reduced from three hours to 1 hour by using either a higher oil concentration, a lower chilling temperature, or both. However, on the basis of previously reported data it is known that with agitation during chilling, such as would be encountered in commercial continuous chilling units, the rate of crystal formation would be further increased. Under practical conditions therefore the adjustment of temperature and concentrations should make it feasible to use holding-times even shorter than one hour with a corresponding increase in the capacity of the chilling units.

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Influence of Alkali Concentration and Other Factors on the Conjugation of Natural Polyunsaturated Acids as Determined by Ultraviolet Absorption Measurements[1]

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ULTRAVIOLET spectrophotometric analysis is the most important method employed today for determination of the polyunsaturated constituents of fats and oils. Since publication in 1943 of a spectrophotometric method for determination of linoleic and linolenic acids in fats and oils (12), the method has been subjected to numerous changes or to suggested changes by various investigators. The method has been extended to include arachidonic acid (2) and modified to increase the transparency of the medium (14, 4). Efforts to improve the sensitivity and accuracy of the method by including certain correction factors (4, 5, 17) or by variations of the conditions of isomerization (1, 3, 9, 10) have been described. Recently this laboratory has published new and more accurate constants for use in the spectrophotometric analyses of the more common natural fats and oils (6). These new constants were determined on acids isolated by physical means in their natural geometrical configuration whereas the earlier constants were determined on chemically prepared bromination-debromination acids. The latter acids contain substantial proportions of geometrical isomers other than the natural all-cis type (11). Geometrical isomers vary in their rates of conjugation during alkali treatment, resulting in significant dif-

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ferences in their observed specific extinction coefficients (7, 13).

The effect of alkali concentration and time of isomerization was studied by Holman and Burr (10). They found that maximum yields of diene from linoleic acid are produced over a wide range of alkali concentrations, but to produce maximum triene, tetraene, and pentaene from their respective unconjugated acids a strong alkali concentration is needed. Application of their optimum conditions more than doubled the specific extinction coefficient of arachidonic acid, as compared with that obtained by the isomerization conditions of Beadle and Kraybill (2). Owing to the presence of pentaene as an impurity in their arachidonic acid and low yields and poor reproducibility of conjugated isomers from linoleic and linolenic acids, Holman and Burr did not recommend any definite set of spectrophotometric constants for use with their isomerization conditions. Their polyunsaturated acids were prepared by a bromination-debromination method.

Since unconjugated diene, triene, tetraene, and pentaene acids in their natural configuration were available to us from previous work (7, 8, 16), it was of interest to extend the study of the effect of alkali concentration and time of heating on the spectral properties of these acids. Attention was also given to the selection of isomerizing conditions that could be used as a basis for a more sensitive spectrophotometric method of analysis, particularly for tetraene and pentaene acids. Analyses of a series of fats in which the selected conditions were employed were compared with analyses by standard methods.

Experimental

Natural methyl linoleate, methyl linolenate, methyl arachidonate, methyl eicosapentaenoate, and docosapentaenoate prepared by physical methods (7, 8, 16) had iodine values of 172.2, 259.1, 313.7, 393.3, and 368.0, respectively, as determined by the half hour Wijs method.

Preparation of Alkali-Isomerizing Reagents. The procedure for the preparation of KOH-glycol reagents was essentially that described in the A.O.C.S. Tentative Method Cd7-48 (15). Ethylene glycol was heated to 190°C. for 10 minutes and allowed to cool to 150°C., and the calculated amount of alkali was added to give solutions of 6.6, 11.0, 18.0, 21.0, and 27.0% KOH by weight. The alkali solutions were again heated to 190° for 10 minutes and then cooled to room temperature. A blanket of nitrogen was kept over the reagents at all times. The strength of each solution was checked by titrating a weighed aliquot with standard acid, and then adjusted to ±0.1 of the desired weight percentage with ethylene glycol that had been dried by heating to 190° for 10 minutes.

Method for Isomerization. The isomerizing equipment, i.e., test tubes, nitrogen-distributing head, and bath, was identical with that described in the A.O.C.S. method. A 1- x 10-inch test tube containing 11 grams of KOH-glycol reagent was placed in the bath (maintained at 180°C.), blanketed with nitrogen, and heated for 15 minutes. An accurately weighed 70-80-mg. sample contained in a 1-ml. glass cup was added to the reagent, and the tube was removed from the bath and shaken vigorously for 5 seconds and then replaced. This shaking operation was repeated at 30-second intervals until the contents appeared clear and homogeneous. A test 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 of addition of the sample. After being heated for the desired length of time, 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 optical densities were reached. Appropriate readings were made in a Model DU Beckman spectrophotometer.

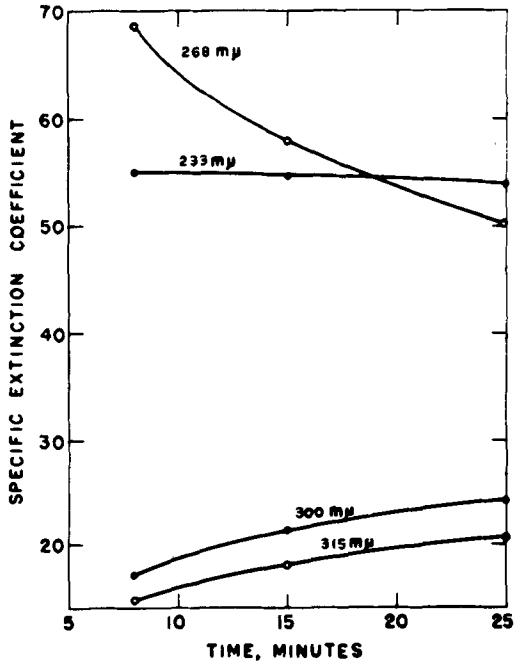

FIG. 1. Specific extinction coefficients of methyl arachidonate vs. time of isomerization at 180°C. in 6.6% KOH glycol.

Results and Discussion

Effect of KOH Concentration and Time of Heating on Degree of Conjugation of Methyl Arachidonate. Preliminary experiments on a somewhat impure sample of methyl arachidonate (I.V. 306) were carried out under the conditions suggested by Holman and Burr (10), that is, 5 ml. of reagent (23 g. KOH/100 ml. ethylene glycol solution), 15 to 25 mg. of sample, 8 minutes of reaction time but isomerization at temperature of 180°C. instead of 178°. Under these conditions it was difficult to get reproducible results.

After some experimentation in varying the amount of reagent and weight of sample, it was found that satisfactory reproducibility could be obtained with 11.0 g. of reagent, 70 to 80 mg. of sample, 8 minutes' reaction time, and a temperature of 180°C. It was noted that the specific extinction coefficient at 300 mμ under these conditions was just a little more than half that obtained by Holman and Burr. Time curves at different KOH concentrations, other factors remaining constant, were then determined in an effort to approach their maximum value.

The plots of the specific extinction coefficients[3] of methyl arachidonate (I.V. 306) against time with different KOH concentrations are shown in Figures 1 to 5. As the KOH concentration is increased, the maxi-

[3] Specific extinction coefficient = D/bc where D = spectral density of the solution (compared with solvent), b = length of cell, and c = concentration in grams/liter.

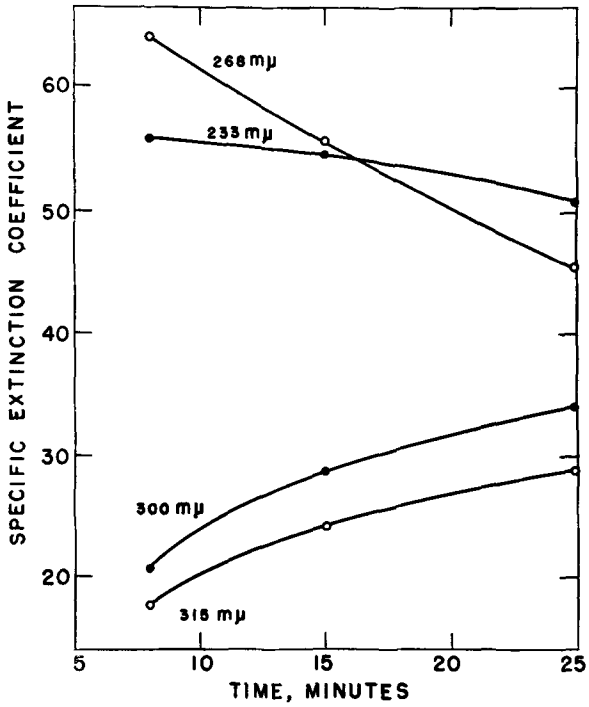

FIG. 2. Specific extinction coefficients of methyl arachidonate vs. time of isomerization at 180°C. in 11% KOH glycol.

mum conjugated diene formation decreases. The lower KOH concentrations cause a tendency to plateau near a maximum conjugated diene formation whereas the higher KOH concentrations cause a maximum diene conjugation in less than 8 minutes and then show a decrease. With the 27% KOH concentration however the plateau again appears although at a lower level. The maximum triene conjugation from methyl arachidonate is in the same relative order; the greater the KOH concentration the lower the maximum triene conjugation. There is also a rather definite decline in triene with an increase in time. Conjugated tetraene formation however is just the reverse; the greater the KOH concentration the greater the formation of conjugated tetraene until the maximum is reached with 21% KOH concentration. With the 27% KOH concentration, maximum conjugated tetraene falls

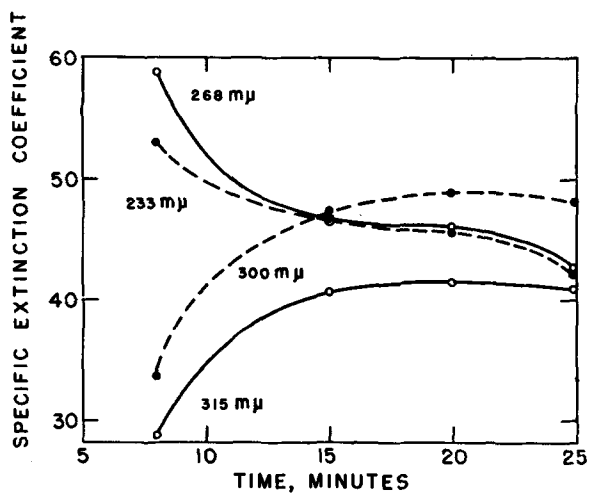

FIG. 3. Specific extinction coefficients of methyl arachidonate vs. time of isomerization at 180°C. in 18% KOH glycol.

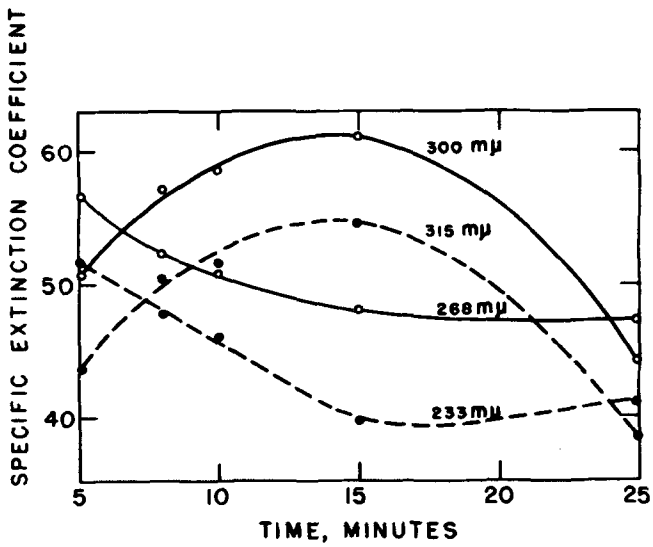

FIG. 4. Specific extinction coefficients of methyl arachidonate vs. time of isomerization at 180°C. in 21% KOH glycol.

sharply to a low level. During isomerization at this KOH concentration considerable difficulty was experienced in keeping the sample in solution which may account for the erratic values.

From these results it appears that a concentration of 21% KOH and 15 minutes' reaction time gives maximum tetraene conjugation. Despite the fact that this sample of methyl arachidonate was somewhat impure, these conditions of isomerization gave a specific extinction coefficient at 300 mμ that is in good agreement with the maximum value found by Holman and Burr (10).

In Figure 6 the specific extinction coefficients of methyl arachidonate are plotted against percentage of KOH for a reaction time of 15 minutes. The sharp peak for tetraene production with relatively small changes in alkali concentration emphasizes the care that must be taken in preparing the reagent.

Effect of KOH Concentration on Pure Polyunsaturated esters. In the preceding experiments to deter-

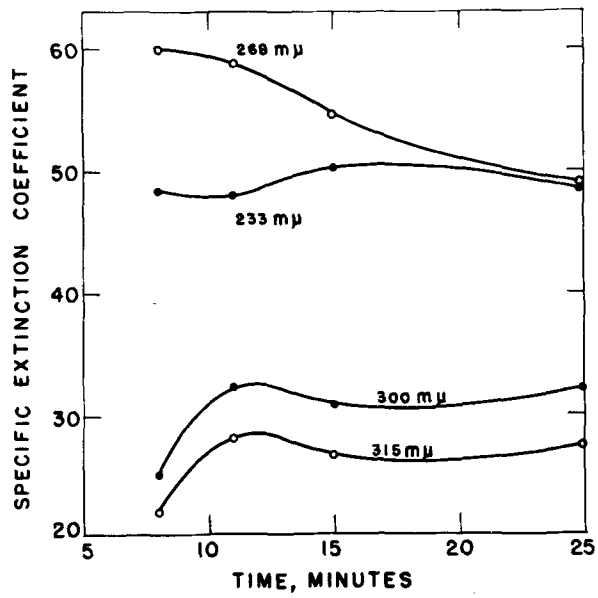

FIG. 5. Specific extinction coefficients of methyl arachidonate vs. time of isomerization at 180°C. in 27% KOH glycol.

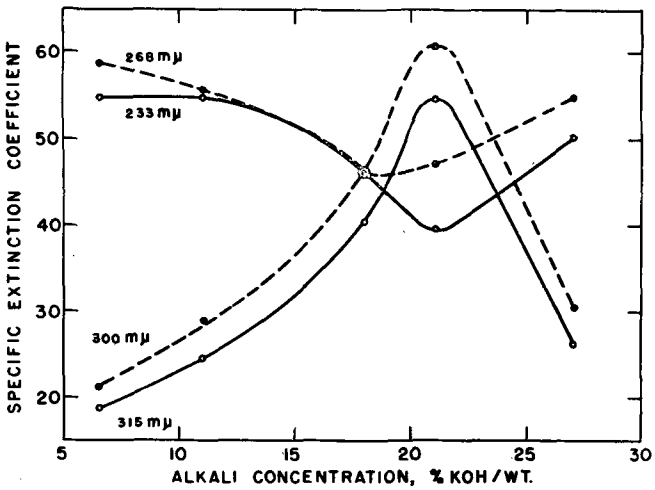

FIG. 6. Specific extinction coefficients of methyl arachidonate vs. KOH concentration when isomerized at 180°C. for 15 minutes.

mine optimum conditions of isomerization a slightly impure sample of methyl arachidonate was used. In the following work methyl arachidonate of greater purity and other polyunsaturated esters were employed. A comparison was made of the absorption curves produced when the methyl esters of the pure naturally occurring acids were isomerized for 15 minutes at 180°C. with 21% KOH glycol reagent and when isomerized for 45 minutes at 180°C. with 11% KOH glycerol (6). All dilutions were made with absolute methanol. These curves are shown in Figures 7 to 11.

The methyl linoleate curves are almost identical for the two methods of isomerization. For the other esters, methyl linolenate, methyl arachidonate, methyl eicosapentaenoate, and methyl docosapentaenoate, it is evident that the increase in alkali concentration favors the formation of a mixture of conjugated isomers in which the isomers having the greatest possible number of conjugated double bonds predominate.

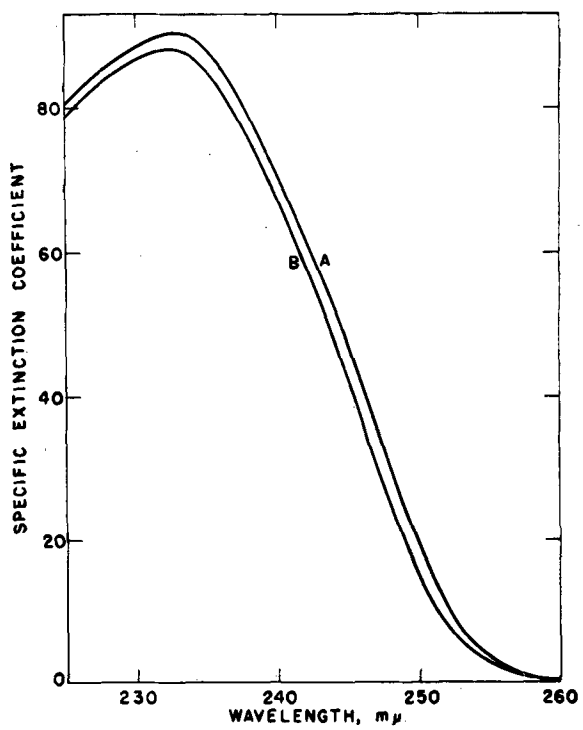

FIG. 7. Absorption spectra of methyl linoleate: (A) isomerized at 180°C. for 45 minutes in 11% KOH glycerol under nitrogen; (B) isomerized at 180°C. for 15 minutes in 21% KOH glycol under nitrogen.

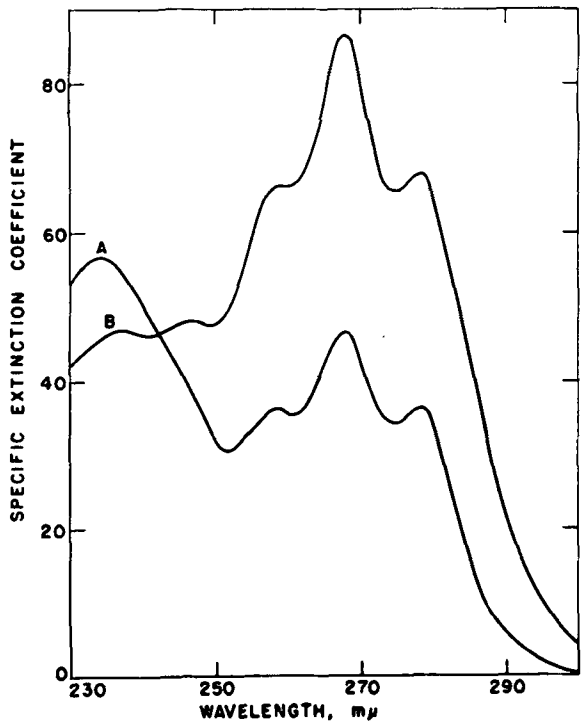

FIG. 8. Absorption spectra of methyl linolenate: (A) isomerized at 180°C. for 45 minutes in 11% KOH glycerol under nitrogen; (B) isomerized at 180°C. for 15 minutes in 21% KOH glycol under nitrogen.

Greater differences than expected were observed in the spectral properties of the two pentaenes when conjugated with 21% KOH. These differences could possibly be ascribed to a) an unknown impurity in the preparations, b) conditions of alkali isomerization not optimum for producing maximum conjugation in each compound, c) different positions of the double bonds, or d) different geometrical configurations of the C_{20} and C_{22} pentaene acids. Unfortunately the amount of material available precluded a more detailed study of this phenomenon.

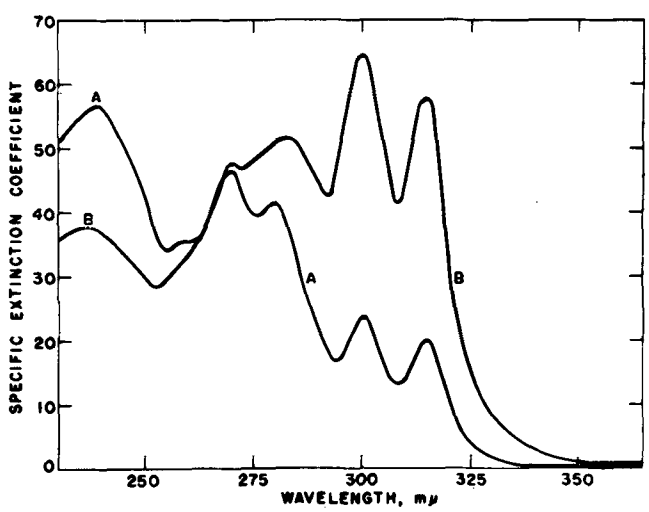

FIG. 9. Absorption spectra of methyl arachidonate: (A) isomerized at 180°C. for 45 minutes in 11% KOH glycerol under nitrogen; (B) isomerized at 180°C. for 15 minutes in 21% KOH glycol under nitrogen.

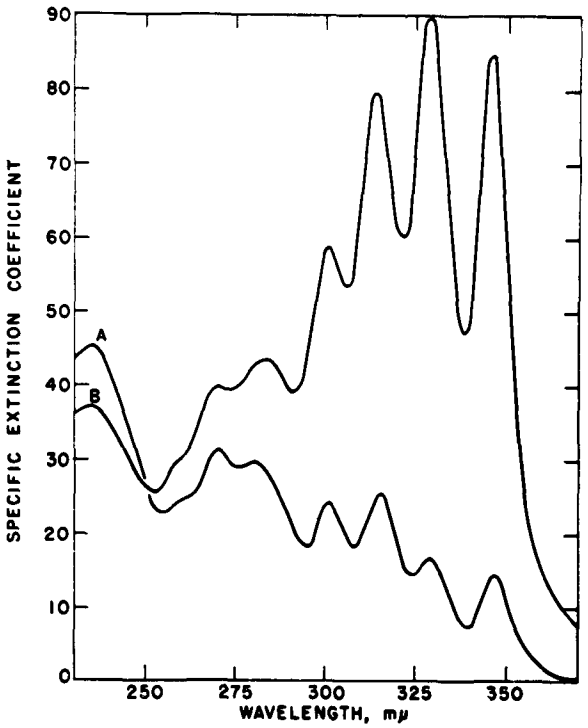

FIG. 10. Absorption spectra of methyl eicosapentaenoate: (A) isomerized at 180°C. for 45 minutes in 11% KOH glycerol under nitrogen; (B) isomerized at 180°C. for 15 minutes in 21% KOH glycol under nitrogen.

The absorption curves shown in Figure 11 for isomerized methyl docosapentaenoate are somewhat different from corresponding curves shown in a previous paper (8, Figure 2). The present curves represent average values of a greater number of determinations taken on several preparations of comparable unsaturation.

The specific extinction coefficients of the naturally occurring acid esters when isomerized for 15 minutes with 21% KOH glycol reagent are given in Table I.

TABLE I

Specific Extinction Coefficients of Pure Natural Polyunsaturated Esters (Adjusted to Acid Basis) Isomerized in 11% KOH Glycerol and 21% KOH Glycol

Acid	Wavelength	Specific extinction coefficients[1]	
		21% KOH glycol[2]	11% KOH glycerol[3]
	mμ		
Linoleic	233	91.6	93.9
Linolenic	233	47.5	58.6
	268	90.5	48.6
Arachidonic	233	39.7[4]	55.0
	268	48.2[4]	46.8
	315	60.6[4]	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

[1] In methanol solutions.
[2] Isomerized for 15 minutes at 180°C. under nitrogen.
[3] Isomerized for 45 minutes at 180°C. under nitrogen.
[4] Corrected values—see text.

Methyl arachidonate available for this study had an iodine value of 313.7, which is slightly lower than a previously isolated sample (7). Therefore a small correction was made by plotting the specific coefficients against the iodine values of a number of samples ranging from I.V. 300 to I.V. 314 and extrapolating the coefficients to the theoretical iodine value of 318.8. For comparison with values obtained by the 21% KOH glycol method, coefficients are included for these esters when isomerized for 45 minutes with 11% KOH glycerol (6, 8). The data in Table I show that the sensitivity of the method has been increased considerably for all acids except linoleic, for which the sensitivity remains unchanged.

The spectrophotometric method of analysis will not differentiate between acids that have the same number of double bonds but different chain length, e.g., between C_{20} and C_{22} pentaenes. Hence, when the two pentaene acids are present in a fat and their chain lengths are unknown, it would be necessary to have an independent determination of one for an accurate analysis. No satisfactory method however is available for this purpose. Therefore in the spectrophotometric analyses reported in this paper the assumption was made that the pentaene acids were present in equal quantities. This assumption, although not necessarily true in all fats, is in agreement with the approximate proportions found previously in beef suprarenal lipids (8). Constants for equal proportions of C_{20} and C_{22} pentaene acids are also included in Table I.

Analyses of Fats and Oils. Analyses of some common oils and concentrates of polyunsaturated components from these oils are given in Table II. The analyses were determined on a 70- to 80-mg. sample isomerized in 21% KOH glycol reagent for 15 minutes at a temperature of 180°C. For comparison the samples were also determined by either the modified A.O.C.S. Tentative Method Cd7-48 (15) or the method employing 11% KOH glycerol and 45 minutes reaction time (6). In general, the results are in good agreement with the standard methods.

Owing to lack of absolute values for the fatty acid composition of the samples, no claim is made that the 21% KOH glycol method is more precise than standard methods. It may be superior however because of the increased sensitivity in most analytical regions,

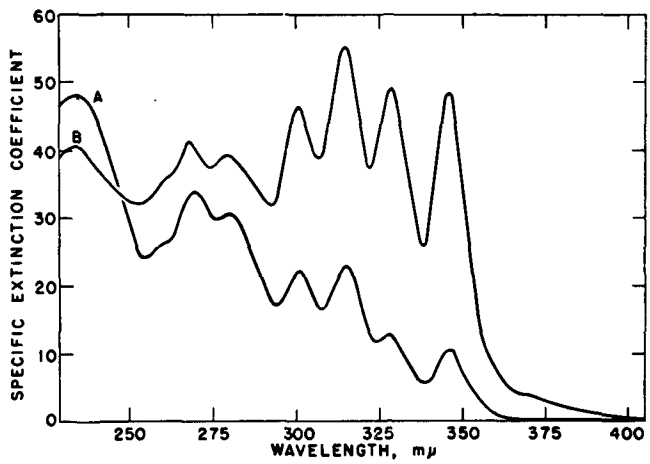

FIG. 11. Absorption spectra of methyl docosapentaenoate: (A) isomerized at 180°C. for 45 minutes in 11% KOH glycerol under nitrogen; (B) isomerized at 180°C. for 15 minutes in 21% KOH glycol under nitrogen.

and for the same reason it should be more adaptable to micro quantities of sample. Work on this adaptation is under way.

An interesting observation was made on the composition of lard. The two concentrates of polyunsaturated constituents of lard methyl esters (Table II) exhibited a definite peak in the pentaene region. The position of the peak and the magnitude of the absorption strongly indicate the presence of pentaene acids in the original lard. This is believed to be the first report that lard contains acids of greater unsaturation than arachidonic.

TABLE II

Spectrophotometric Analyses of Various Samples of Fats and Oils Isomerized by Standard Methods and by the 21 % KOH Glycol Method

Sample	Component acid	Method[1]	
		21% KOH glycol	Standard[2]
		%	%
Cottonseed oil 1	Linoleic	51.6	51.5
Cottonseed oil 2	Linoleic	49.1	49.4
Soybean oil 1	Linoleic	50.8	52.6
	Linolenic	7.7	8.3
Soybean oil 2	Linoleic	50.9	52.2
	Linolenic	7.7	8.3
Methyl esters[3] from cottonseed oil	Linoleic	73.3	71.9
Methyl esters[3] from soybean oil	Linoleic	62.8	62.6
	Linolenic	9.0	9.5
Methyl esters[3] from linseed oil	Linoleic	18.1	16.6
	Linolenic	49.0	49.9
Methyl esters[4] from lard 1 fraction 7	Linoleic	23.8	25.2
	Linolenic	6.8	7.9
	Arachidonic	4.4	4.8
	Pentaenoic (50% C_{20}-50% C_{22})	1.0	1.8[6]
Methyl esters[4] from lard 1 fraction 8	Linoleic	21.2	21.8
	Linolenic	10.6	11.3
	Arachidonic	7.4	8.7
	Pentaenoic (50% C_{20}-50% C_{22})	3.5	5.3[6]
Methyl esters[5] from adrenal lipids	Linoleic	24.1	22.9
	Linolenic	4.9	4.1
	Arachidonic	15.9	16.5

[1] All results are reported as percentage of acid in sample.
[2] Standard method may be either 6.6% KOH glycol or 11% KOH glycerol; where data were available by both methods the average values are given.
[3] Saturated esters removed by low temperature crystallization.
[4] Concentrate of polyunsaturated components obtained by low temperature crystallization and high vacuum distillation.
[5] Fraction obtained by adsorption separation on silicic acid.
[6] Calculated from coefficients given in Table I.

Summary

Optimum conditions for production of maximum conjugation of methyl arachidonate were determined. These comprise heating the sample in 21% KOH glycol for 15 minutes at 180°C. The optimum conditions of isomerization have also been applied to methyl linoleate, methyl linolenate, methyl eicosapentaenoate, and docosapentaenoate prepared by physical methods. These conditions greatly increased the sensitivity of the spectrophotometric method for all the polyunsaturated acids except linoleic, for which the sensitivity was unchanged.

Analyses of a series of fats and oils isomerized under optimum conditions and also under standard conditions were in good agreement. Constants are given for use when pentaene acids are present as well as for acids of less unsaturation.

Spectroscopic evidence strongly indicates that pentaene acids are present in lard.

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A Study of the Spectrophotometric Method for Polyunsaturated Fatty Acids in Cottonseed Oils and A Comparison with Chemical Methods[1]

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IN the evolution of the American Oil Chemists' Society's spectrophotometric method, Cd 7-48, for determining polyunsaturated acids (1) in fats and oils attention was given to the development of a general method. The purpose of the present investigation was to determine how much simplification could be made in the complicated equation specified for the calculation of linoleic acid content in the case of cottonseed oils without adversely affecting the precision and the accuracy of the results. In addition, an attempt has been made to evaluate the use of the spectrophotometric method for the analysis of cottonseed oils by a comparison of the results obtained for unsatu-

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