Standardization of Spectrophotometric Methods for Determination of Polyunsaturated Fatty Acids Using Pure Natural Acids[1]

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IN the nine years since Mitchell, Kraybill, and Zscheile (1) published a quantitative spectrophotometric method for determination of linoleic and linolenic acids in fats and oils, considerable attention has been devoted to extension of the method, to improvements in sensitivity, and to evaluation and application of methods. Beadle and Kraybill (2) extended it to include determination of arachidonic acid. Brice *et al.* (3, 4, 5, 6) improved the sensitivity and accuracy in respect to determination of small proportions of the conjugated and nonconjugated acids by proposing measurement of absorption before and after isomerization in KOH-glycerol, a medium more transparent than KOH-ethylene glycol in air, and by applying corrections for preformed conjugation and extraneous background absorption. O'Connor *et al.* improved the purity of standards by excluding oxygen in their preparation (7) and greatly improved the transparency of the KOH-ethylene glycol isomerization medium by protection with an atmosphere of nitrogen (8). Other methods, with variations in procedure designed primarily to effect improvements in sensitivity, have been described by Hilditch *et al.* (9), Baldwin and Longenecker (10), Holman and Burr (11), Berk *et al.* (12), and others. Privett and Lundberg (13) have studied the nature and extent of interferences by autoxidized fatty acids in the application of spectrophotometric methods and have suggested means for their removal. Typical applications of spectrophotometric methods have been discussed by Beadle (14). The Spectroscopy Committee of the American Oil Chemists' Society (15) has been conducting collaborative tests on spectrophotometric analyses of fats and oils for polyunsaturated fatty acids in an effort to establish a suitable standard method of analysis for the Society.

The present status of spectrophotometric methods as applied to fats and oils may be summarized as follows: a) They are highly specific, within limits for the common polyunsaturated constituents. b) They provide a means of determining both conjugated and nonconjugated fatty acids over a wide range of occurrence in mixtures. c) They are far more sensitive than the thiocyanometric and other methods in detecting small proportions of nonconjugated acids and in the range below about 5% far more accurate. d) They are capable of high reproducibility in experienced hands. In the latter connection it must be emphasized that the method (for nonconjugated acids) is highly empirical since the procedure must be standardized within close limits for temperature and time of isomerization, alkali concentration, sample size, and type of reaction vessel. Usually the analyst does not have satisfactory purified standards of his own and must rely on published procedures and constants and use of a spectrophotometer preferably of the same manufacture. However it has been our experience that, even under these conditions, high reproducibility and satisfactory inter-laboratory comparisons are readily obtained if all details of a standard procedure are closely followed.

We are now in a position to examine the spectrophotometric method for accuracy and to improve the accuracy. In our early work (4) on tallows and greases appreciable discrepancies were noted between spectrophotometric and thiocyanometric analyses for linoleic acid. These were not particularly disturbing, however, since the values were obtained assuming the presence of linoleic, oleic, and saturated acids only; moreover the accuracy of the thiocyanometric method is not high for fats having less than 10% of polyunsaturated acids. When the method was applied to a number of seed oils and concentrates having high contents of linoleic or linolenic acids, serious discrepancies between spectrophotometric and thiocyanometric analyses were encountered (4). For example, with perilla oil the result for linolenic acid (glyceride basis) was 58.1% by the spectrophotometric method and 65.2% by the thiocyanometric method; and with tobacco seed oil the result for linoleic acid (glyceride basis) was 78.3% by the spectrophotometric and 73.0% by the thiocyanometric method. It was established that the spectrophotometric method was at fault in these cases since iodine values calculated from the spectrophotometric results, after separate determination of the saturated acids, did not agree with observed iodine values.

In studying these discrepancies between spectrophotometric and chemical results, the possibility of the existence in these rather rare oils of non-conjugating isomers in which the double bonds are separated by more than one methylenic group, perhaps similar to those produced on hydrogenation of linolenic acid, was first considered. Experience with other more common oils such as linseed, however, showed that the spectrophotometric results for linolenic acid were quite generally low when the content of this acid was high enough to make a comparison with the chemical methods reliable. Since it seemed improbable that the intensive chemical studies carried out on linseed oil would have failed to reveal the presence of substantial amounts of anomalous isomers of linolenic or linoleic acids, an alternative explanation of the discrepancy was sought.

The spectrophotometric standards used heretofore have been highly purified linoleic, linolenic, and arachidonic acids or their esters, prepared by bromination-debromination procedures. Such preparations are known (16) or suspected to contain substantial proportions of geometric isomers other than the natural all-cis variety. Strong evidence has been obtained in this laboratory (17) that differences in

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geometrical configuration result in significant differences in ultraviolet absorption characteristics after alkali-isomerization. The main points of this evidence are that: a) methyl esters of natural[4] polyunsaturated acids prepared by chromatographic methods (17) differed significantly from debromination esters in their ultraviolet absorption intensities after the analytical alkali-isomerization procedure; b) methyl linoleates prepared by chromatography and by low temperature fractional crystallization (18) were not significantly different in these ultraviolet absorption intensities; c) 9 cis, 12 cis linoleic acid isomerized with alkali 20 times faster than the trans-trans isomer and produced a mixture of dienoic conjugated isomers in the analytical alkali-isomerization procedure having a specific extinction coefficient at the maximum more than five times that of the latter (19); and d) restandardization of the spectrophotometric method, using methyl esters of natural linoleic and linolenic acids as standards, resulted in improved accuracy of spectrophotometric analyses for perilla oil, linseed oils, and tobacco seed oils. The latter results were presented before the Society in 1948 (20). More recently Hilditch, Patel, and Riley (21) have presented evidence that the analytical extinction coefficients of linolenic acid prepared by physical methods differ appreciably from those of linolenic acid prepared by debromination, thus confirming work at this laboratory (17). They found no appreciable difference, under their conditions of isomerization however, for the two types of linoleic acid.

The primary purpose of the present paper is to present complete results on a restandardization of spectrophotometric methods for the determination of polyunsaturated fatty acids in natural fats and oils, using as standards the purified methyl esters of natural linoleic, linolenic, and arachidonic acids prepared by crystallization and chromatographic methods by Riemenschneider *et al.* (17, 22). The paper includes results previously reported (20) on the restandardization of spectrophotometric methods with natural linoleic and linolenic acids; an extension of the investigation to include natural arachidonic acid; and application of the restandardized methods of analysis to a wide selection of fats, by which it is shown that higher accuracy is obtained.

[4]The word "natural" is used throughout this paper to mean the natural geometrical forms of polyunsaturated acids.

Restandardization

Natural methyl linoleate was prepared chromatographically as previously described (fraction 5, reference [17]) from tobacco seed oil, with the following characteristics: iodine value 172.8 (theory, 172.5); melting point −35° to −34°C.; $n_D^{25°}$ 1.45932; preformed conjugation, by spectrophotometric analysis, dienoic 0.1%, trienoic 0.00%, tetraenoic 0.00%. An additional preparation had similar characteristics, with an iodine value 172.2. All iodine values reported in this investigation were determined by the ½ hour Wijs method.

Natural methyl linolenate, prepared chromatographically from linseed oil as previously described (fraction 3, reference [17]), had the following characteristics: iodine value 260.2 (theory, 260.5); melting point −46.5° to −45.5°C.; $n_D^{20°}$ 1.47074;[5] preformed conjugation, dienoic 0.2%, trienoic 0.00%, and tetraenoic 0.00%. Two additional preparations had similar characteristics, with iodine values shown in Table I.

Natural methyl arachidonate, prepared from beef suprarenal glands by chromatographic and fractional distillation procedures (22), had the following characteristics: iodine value 316.1 (theory, 319); $n_D^{20°}$ 1.47986; preformed conjugation, by spectrophotometric analysis, dienoic 0.9%, trienoic 0.02%, tetraenoic

[5]This value was erroneously reported in reference (17) as having been determined at 25°.

TABLE I

New Standardization Data. Average Observed Specific Extinction Coefficients (Adjusted to Acid Basis and Referring to Solutions in Absolute Methanol) for Methyl Esters of Purified Natural Polyunsaturated Fatty Acids After Alkali Isomerization at 180°C. Under Various Conditions

Acid	Sample No.	Iodine Value (Ester)	λ (mμ)	Specific Extinction Coefficients After Isomerization							
				In 11% KOH-Glycerol[a]				In 6.5% KOH-Ethylene Glycol			
				30 minutes		45 minutes		25 minutes		45 minutes	
				Air	N_2	Air	N_2	Air[b]	N_2[c]	Air[b]	N_2[c]
Linoleic	1	172.8	233	91.1	.....	93.0	.....	91.9	92.2	93.4	93.2
	2	172.2		.....	.....	94.4	.....	.....	.....	.....	94.9
Linolenic	3	260.2	233	60.1	.....	58.5	.....	62.2	61.4	58.5	58.3
	4	259.6		60.2	.....	.....	.....	.....	61.8	...	59.1
	5	259.1		.....	.....	58.6	.....	.....	61.5	.....	59.2
	3		268	49.3	.....	48.5	.....	51.4	50.6	49.1	49.3
	4			49.2	.....	.....	.....	.....	50.4	.....	49.3
	5			.....	.....	48.6	.....	.....	50.9	.....	49.4
Arachidonic	6	316.1	233	56.9	56.4	55.7	55.4	.....	57.0	.....	54.6
	7	314.9		57.0	.....	54.9	55.1	57.3	.....	.....	54.9
	8	315.7		.....	.....	.....	54.5	.....	56.5	.....	53.8
	9	318.0		.....	.....	.....	54.5	.....	.....	.....	.....
	6		268	53.4	53.3	47.1	47.7	.....	52.5	.....	44.4
	7			53.4	.....	46.5	47.2	52.5	.....	.....	45.2
	8			.....	.....	.....	47.2	.....	53.8	.....	45.0
	9			.....	.....	.....	44.8	.....	.....	.....	.....
	6		315	18.4	19.7	19.9	20.6	.....	21.1	.....	22.7
	7			19.2	.....	20.4	20.6	22.0	.....	.....	22.1
	8			.....	.....	.....	20.6	.....	21.8	.....	24.2
	9			.....	.....	.....	20.1	.....	.....	.....	.....

[a] Six-inch reaction tubes, swirled 1 minute out of bath after introduction of sample. Brice *et al.*, references (3) and (4).
[b] Six-inch reaction tubes, swirled at 3 one-minute intervals out of bath after introduction of sample; conditions similar to Mitchell *et al.*, reference (1).
[c] Ten-inch reaction tubes, blanketed with nitrogen. Am. Oil Chem. Soc. Tentative Method Cd7-48; J. Am. Oil Chem. Soc., *26*, 399 (1949).

0.00%. Four additional preparations had similar characteristics, with iodine values shown in Table I.

For purposes of comparison a sample of methyl arachidonate prepared by bromination-debromination procedures, having an iodine value 321 (theory, 319) and an octabromide number 100.6 was available to us through the courtesy of J. B. Brown of Ohio State University. This preparation was previously used by us as a spectrophotometric standard (23), but detailed data on its specific extinction coefficients after isomerization have not heretofore been published, except for its absorption curve (Fig. 1, page 273 of reference 6). Preformed conjugation, by spectrophotometric analysis was: diene, 0.3%; triene 0.00%; tetraene 0.5%.

These purified methyl esters were subjected to alkali isomerization for various lengths of time by the glycerol-air method previously described by Brice *et al.* (3, 4). The standardized conditions of this method include: adding an accurately weighed sample (approximately 0.1 gram) of the ester to 11 grams of glycerol solution containing 11.0% KOH by weight and about 0.1% pure stearic acid (to hasten saponification of the ester), and heating at 180°C. in a covered 6 x 1 inch test tube.

The absorption spectra of methyl esters of the natural acids after alkali isomerization are shown in Fig. 1. Curves relating time of heating and specific extinction coefficients[6] in absolute methanol at the wave lengths[7] used for analytical purposes are shown in Figures 2, 3, and 4 for the three natural esters (adjusted to acid basis). For comparison, curves are shown for bromination-debromination acids (3) under similar conditions.

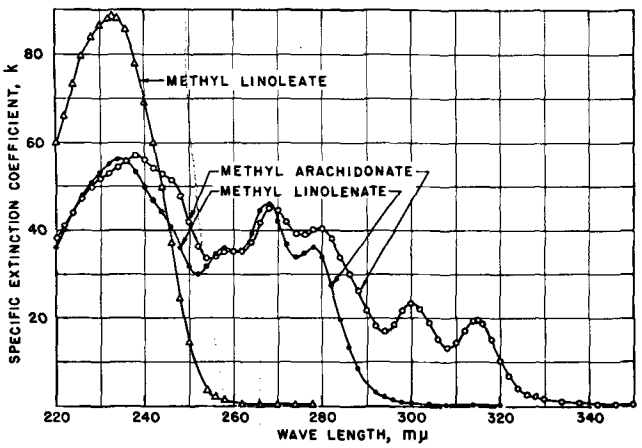

FIG. 1. Ultraviolet absorption curves for methanol solutions of purified methyl esters of natural acids after alkali-isomerization in KOH-glycerol solution for 45 minutes at 180°C.

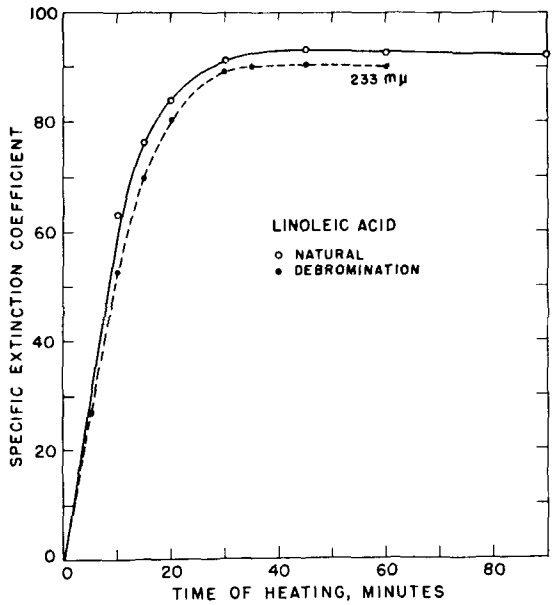

FIG. 2. Specific extinction coefficients after isomerization at 180°C. in KOH-glycerol-air for various lengths of time. Natural methyl linoleate calculated to acid basis (absolute methanol solvent); bromination-debromination linoleic acid (reference 3, sample 7, absolute ethanol solvent).

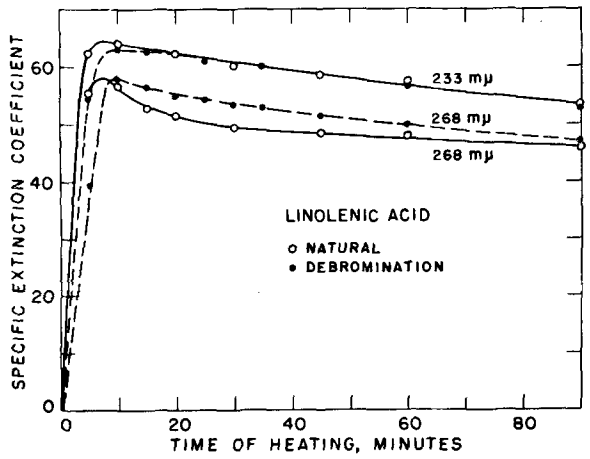

FIG. 3. Specific extinction coefficients after isomerization at 180°C. in KOH-glycerol-air for various lengths of time. Natural methyl linolenate calculated to acid basis (absolute methanol solvent); bromination-debromination linolenic acid (reference 3, sample 8, absolute ethanol solvent).

Inspection of these curves reveals significant differences between the natural and the bromination-debromination acids in the initial rates of conjugation, in the rates of destruction of conjugation (except for the dienoic components), and in the absorption intensities of the conjugated components at a given time of isomerization. For example, at 30 minutes time, the specific extinction coefficient at 233 mμ for the natural linoleic acid is higher, and that for the natural linolenic acid at 268 mμ is lower, than the coefficients for the corresponding bromination-debromination acids. These differences are in a direction which would account for the discrepancies discussed above in the spectrophotometric analyses of tobacco seed oil and perilla oil, in which bromination-debromination acids were used as standards. While the differences for arachidonic acid are significant, they are relatively unimportant in the analysis of common animal fats which contain 1% or less of this acid.

[6] Specific extinction coefficient is defined as $k = D/bc$ where D is the excess spectral density of solution over solvent in an equal cell, c is the concentration in grams per liter, and b is the internal thickness of the cell in centimeters. All data were obtained on a Beckman Model DU spectrophotometer with concentrations and cell lengths adjusted to give spectral densities in the range 0.2 to 0.8.

[7] Wave lengths 233 and 234 mμ are the positions of the absorption maxima for the conjugated dienoic products of isomerized linoleic and linolenic acids, respectively; 268 mμ, the position of the principal absorption maximum for the conjugated trienoic product of isomerized linolenic acid; and 315 mμ, the longest wave length absorption maximum for the conjugated tetraenoic product of isomerized arachidonic acid. Wave lengths 233 and 315 mμ are to be preferred over wave lengths 232 and 316 mμ, previously used (3, 4).

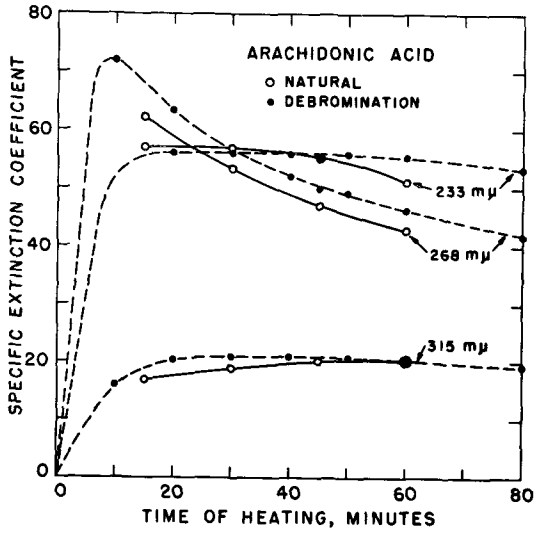

FIG. 4. Specific extinction coefficients in absolute methanol after isomerization at 180°C. in KOH-glycerol-air for various lengths of time. Natural methyl arachidonate; bromination-debromination ester (sample 11); both calculated to acid basis.

Fig. 5 shows similar rate curves at selected wave lengths for some typical natural oils and fats. It is highly significant that the 268 mμ curve for linseed oil parallels that for the natural linolenic acid rather than that for the bromination-debromination acid. Shape differences are less critical in the case of cottonseed oil and lard. In all three cases however a reasonably constant proportion of polyunsaturated acid is calculated by using the data for a sample and the natural acid at each of the various times of isomerization.

It is apparent, particularly from Figures 2 and 5, that the optimum time of isomerization selected for analytical purposes is longer than 25 minutes (1, 2, 15) or 30 minutes (3, 4) as previously used. It was pointed out in a previous publication (3) that 45 minutes was more favorable. At 25 or 30 minutes the contribution to the extinction coefficient at 233 mμ by linoleic acid is increasing (Figures 2 and 5) while that due to linolenic acid is decreasing (Fig. 3). Variations in the rapidity of saponification of a sample, or variations in the total time of isomerization due to initial manipulations, would result in errors of analysis or in poor reproducibility, particularly for linoleic and linolenic acids. At 45 minutes however where k_{233} for linoleic acid has reached a maximum and the total time of isomerization is longer, the possibility of error is reduced, and both accuracy and reproducibility should be greater.

Standardization was carried out with the new preparations for several sets of conditions of isomerization so that some of the various methods proposed or in current use could be compared when applied to actual samples. Average observed specific extinction coefficients for several preparations are shown in Table I. Inspection of these data indicates no significant differences, where comparisons are available, for isomerization in air and in nitrogen. Adopted standard values are assembled in Table II. These data comprise averages of replicate determinations and averages of data in air and nitrogen for the new preparations of Table I, and a tabulation of data for bromination-debromination acids.

Methods of Calculation

The data of Table II were used in deriving the equations of Table III for the simultaneous determination of linoleic, linolenic, and arachidonic acids in oils and fats (percentage of acid in sample) for the conditions indicated. The coefficients k'_2, k'_3, and k'_4 refer to observed specific extinction coefficients, after isomerization of the sample, at wave lengths 233, 268, and 315 mμ unless corrections for extraneous background absorption are to be applied as discussed below.

Brice *et al.* (3, 4) introduced the use of corrections for extraneous background absorption in the spectrophotometric determination of small proportions of polyunsaturated fatty acids in various materials. The various corrections proposed were designed to improve the accuracy of analyses for *small proportions* of conjugated and nonconjugated polyunsaturated

TABLE II

Adopted Specific Extinction Coefficients in Absolute Methanol for Purified Natural Polyunsaturated Fatty Acids After Alkali Isomerization at 180°C. Under Various Conditions. Data for Bromination-Debromination Acids Under Similar Conditions Are Listed for Comparison

Acid	λ	KOH-Glycerol-Air				KOH-Ethylene Glycol (Air or N_2)				
		30 Minutes		45 Minutes		25 Minutes			45 Minutes	
		Nat.	Debrom.	Nat.	Debrom.	Nat.	Debrom.		Nat.	Debrom.
		(b)	(c)	(b)	(c)	(b)	(c)	(d)	(b)	(c)
Linoleic	232	90.6	88.9	93.4	90.1	91.7	.....	.....	93.5	.....
	233[a]	91.1	89.2	93.9	90.3	92.1	.....	.....	93.9	.....
	234	90.2	88.6	93.3	89.8	91.5	.....	86.0	93.4	.....
Linolenic	232	59.5	60.0	58.2	57.9	60.7	.....	.....	58.5	.....
	233[a]	60.2	60.5	58.6	58.5	61.6	.....	.....	59.0	.....
	234	60.9	61.0	59.2	59.1	62.2	.....	60.9	59.8	.....
	268[a]	49.3	53.4	48.6	51.3	50.7	.....	53.2	49.3	.....
Arachidonic	232	56.1	55.8	54.5	55.3	56.1	55.4	.....	53.8	52.9
	233[a]	56.8	56.5	55.0	56.0	56.9	56.2	.....	54.5	53.7
	234	57.6	57.2	55.8	56.8	57.8	57.1	59.3	55.5	54.4
	268[a]	53.4	56.3	46.8	50.3	52.8	54.4	53.4	44.8	44.6
	315[a]	19.1	21.1	20.3	21.0	21.5	23.9	.....	22.9	24.2
	316	18.2	19.6	19.6	19.8	20.6	22.6	22.6	22.0	22.5
Background Factors	F_3[e]	4.08	4.14	4.08	4.30	4.05	.....		4.05	.....
	F_4[f]	2.06	2.03	2.06	2.12	2.06	2.20		2.12	2.20

[a] Wave-lengths recommended for analysis.

[b] Average of all observed values for preparations listed in Table I.

[c] The data for arachidonic acid are new; data for linoleic and linolenic acids are Brice *et al.*, J. Opt. Soc. Am., *35*, 532 (1945).

[d] Data of Beadle and Kraybill, J. Am. Chem. Soc., *66*, 1232 (1946).

[e] $k_{268}/[k_{268} - \frac{1}{2}(k_{262} + k_{274})]$ for linolenic acid.

[f] $k_{315}/[k_{315} - \frac{1}{2}(k_{308} + k_{322})]$ for arachidonic acid.

TABLE III

Equations for the Simultaneous Determination of Percentages of Linoleic (x), Linolenic (y), and Arachidonic (z) Acids for Various Conditions of Isomerization at 180° C., for Natural Acid Standards and Debromination Acid Standards. Subscripts 2, 3, and 4 Refer to Measurements at 233, 268, and 315 mμ Unless Otherwise Noted. For Vegetable Oils k'_4 Is Assumed to be Zero

Natural Acids		Debromination Acids	
30 Min. Glycerol-Air			
$x = 1.098\ k'_2 - 1.340\ k'_3 + 0.48\ k'_4$	(1)	$x = 1.121\ k'_2 - 1.270\ k'_3 + 0.39\ k'_4$	(13)
$y = 2.028\ k'_3 - 5.67\ k'_4$	(2)	$y = 1.873\ k'_3 - 5.00\ k'_4$	(14)
$z = 5.24\ k'_4$	(3)	$z = 4.74\ k'_4$	(15)
45 Min. Glycerol-Air			
$x = 1.065\ k'_2 - 1.284\ k'_3 + 0.08\ k'_4$	(4)	$x = 1.107\ k'_2 - 1.263\ k'_3 + 0.07\ k'_4$	(16)
$y = 2.058\ k'_3 - 4.74\ k'_4$	(5)	$y = 1.949\ k'_3 - 4.67\ k'_4$	(17)
$z = 4.93\ k'_4$	(6)	$z = 4.76\ k'_4$	(18)
25 Min. Ethylene Glycol (Air or N_2)			
$x = 1.086\ k'_2 - 1.319\ k'_3 + 0.37\ k'_4$	(7)	$x = 1.16\ k'_2 - 1.33\ k'_3 + 0.09\ k'_4$	(18)[a]
$y = 1.972\ k'_3 - 4.84\ k'_4$	(8)	$y = 1.88\ k'_3 - 4.43\ k'_4$	(19)[a]
$z = 4.65\ k'_4$	(9)	$z = 4.43\ k'_4$	(20)[a]
45 Min. Ethylene Glycol (Air or N_2)			
$x = 1.065\ k'_2 - 1.274\ k'_3 - 0.04\ k'_4$	(10)	....	
$y = 2.028\ k'_3 - 3.97\ k'_4$	(11)	....	
$z = 4.37\ k'_4$	(12)	$z = 4.13\ k'_4$	

[a]Am. Oil Chem. Soc. Tentative Method Cd 7-48, subscripts 2, 3, and 4 referring to 233, 268,and 316 mμ. The constants used in this method are derived from data of Beadle and Kraybill, J. Am. Chem. Soc., *66*, 1232 (1944) which refer to 234, 268, and 316 mμ.

fatty acid components in such materials as animal fats and their soaps, partly hydrogenated animal fats and their soaps, and preparations of oleic and saturated acids. These corrections were based primarily on observations that in animal fats: a) an appreciable proportion of conjugated dienoic material is present before alkali-isomerization and this material is stable to heat (see reference 6, Fig. 4, curve T and Th); b) before and after alkali-isomerization an appreciable portion of the observed absorption near 233 mμ may be due to COOR groups (3); and c) before and after the isomerization an appreciable proportion of the observed absorption near 268 and 315 mμ is due to an approximately linear extraneous background. Although some assumptions and uncertainties are involved in these detailed corrections, their application makes possible an unquestionable increase in the accuracy of determining small proportions of conjugated and nonconjugated polyunsaturated constituents.

Some attempts have been made (3, 4, 15) to apply this detailed method, with its numerous corrections, to the spectrophotometric analysis of common vegetable oils. Experience has shown however that such application is unnecessarily laborious for most purposes and, in fact, can lead to erroneous results for linolenic acid if present in substantial proportion. The method of correction for background absorption in the 268 mμ region (see Figure 3 and equation [8] of reference 3) involves absorption measurements on pure linolenic acid after alkali-isomerization at its relatively sharp maximum near 268 mμ and its relatively sharp minima near 262 and 274 mμ. The value of the "background factor" (F_3 in Table II) calculated from these measurements are, as expected, sensitive to slit-width changes and to wave length errors, much more so than k_{268} itself. The data for the values of F_3 in Table II apply to spectrum band widths of approximately 2.5 mμ (slit width 0.85 mm.) in this region. In some illustrative tests with a sample of linolenic acid the value of F_3 decreased by 7% when the slit widths on a given instrument were varied from 1.1 mm. to 0.6 mm., while k_{268} increased by only 1%; on two different spectrophotometers set for slit widths 0.85 mm., the values of F_3 differed by 6.5%, while the values for k_{268} differed by only 1.2%. Such differences are attributable to slit width and small wave length errors. They are of minor importance in dealing with the determination of small proportions of linolenic acid but illustrate the danger of attempting to apply the background correction method to such samples as linseed oil and soybean oil. In the latter cases background absorption is probably negligible.

Experience has shown that a number of simplifications in the calculations previously proposed (3, 4) for a complete spectrophotometric analysis can be made without sacrifice of accuracy. The following recommendations for calculation retain the useful features of background corrections where small proportions of polyunsaturated fatty acids are present; omit determination of trace and minor preformed conjugated constituents; omit determination in vegetable oils of tetraenoic constituents as arachidonic acid since tetraenoic absorption in such cases has been shown due to oxidation of linolenic acid (6); and retain maximum simplicity in the calculation of results for vegetable oils.

In the following, k'_{233}, k'_{268}, k'_{315} are observed specific extinction coefficients after isomerization and k_{233} the coefficient before isomerization, subscripts denoting the wave lengths; k'_2, k'_3, k'_4, and k_2 are the foregoing observed coefficients after correction for any extraneous absorption, the subscripts denoting the number of conjugated double bonds involved; and x, y, z, and C_2 are the percentages of linoleic, linolenic, arachidonic, and conjugated dienoic acids in the sample.

Linoleic acid:

A. Small proportions. If k'_{233} is less than 20 (examples are animal fats and preparations of oleic and

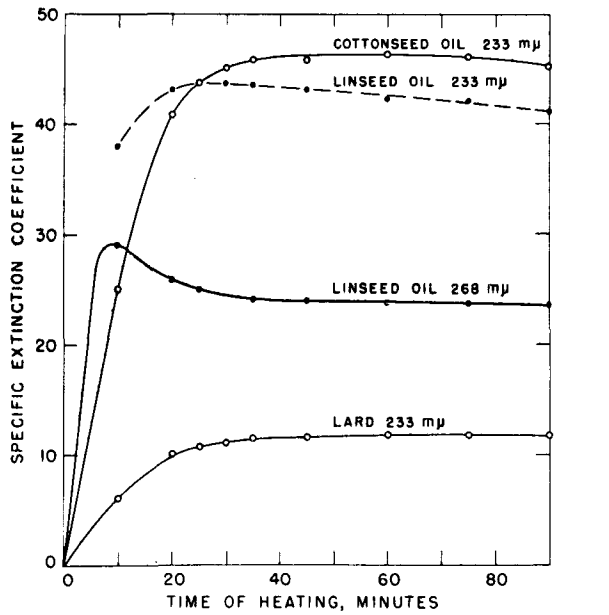

FIG. 5. Specific extinction coefficients in absolute methanol after isomerization at 180°C. in KOH-glycerol-air for various lengths of time, for cottonseed oil, linseed oil, and lard.

TABLE IV

Spectrophotometric Analysis of Miscellaneous Samples of Known Iodine Value and Content of Saturated Acids. Comparison of Results Using Natural Fatty Acid Standards and Bromination-Debromination Acid Standards; and Results Using Various Conditions of Isomerization. All Results Reported on Basis of Percent Acid in Sample

Samples	Sample No.	Conditions[a]	Conj. Diene, %	Linoleic, %		Linolenic, %		Arachidonic, %		Oleic[h], %		Saturated, %	Iodine Value				
													Calculated		Observed (Wijs)	Calc.-Obs.	
				Nat.	Debrom.	Nat.	Debrom.	Nat.	Debrom.	Nat.	Debrom.		Nat.	Debrom.		Nat.	Debrom.
Methyl Esters from Cottonseed Oil[i]	10	25′ EGN	0.08[b]	72.8[c]	77.6[b]	0.00[f]	0.00[b]	.....	0.00[b]	22.4	17.5	0.0	151.8	156.3	151.9	−0.1	4.4
		45′ EGN	.....[c]	71.8[c]	.....	0.00[f]	.....	.....	.....	23.4	.....		151.0	.....		−0.9	....
		45′ GA	.....[c]	71.2[c]	74.0[e,c]	0.00[f]	0.00[f]	.....	.....	24.0	21.2		150.5	153.0		−1.4	1.1
Cottonseed Oil	11	25′ EGN	0.32[b]	52.8[c]	56.0[b]	0.00[f]	0.00[b]	.....	0.00[b]	18.2	14.7	24.7[j]	112.0	115.1	111.9	0.1	3.2
		45′ EGN	.....[c]	53.4[c]	.....	0.00[f]	.....	.....	.....	17.6	.....		112.4	.....		0.5	....
Cottonseed Oil	12	25′ EGN	0.42[b]	51.6[c]	54.5[b]	0.00[f]	0.00[b]	.....	0.00[b]	20.0	16.7	24.1[j]	111.4	114.4	110.1	1.3	4.3
		45′ EGN	.....[c]	51.4[c]	.....	0.00[f]	.....	.....	.....	20.2	.....		111.2	.....		1.1	....
Cottonseed Oil	13	25′ EGN	0.43[b]	49.8[c]	52.5[b]	0.00[f]	0.00[b]	.....	0.00[b]	20.4	17.3	25.5[k]	108.4	111.4	107.8	0.6	3.6
		25′ EGA	.....[c]	49.4[c]	.....	0.00[f]	.....	.....	.....	20.8	.....		108.1	.....		0.3	....
		30′ GA	.....[c]	49.5[c]	50.5[e,c]	0.00[f]	0.00[f]	.....	.....	20.7	19.7		108.2	109.1		0.4	1.3
		45′ GA	.....[c]	48.8[c]	50.7[e,c]	0.00[f]	0.00[f]	.....	.....	21.4	19.5		107.5	109.3		−0.3	1.5
Fatty Acids from Tobacco Seed Oil[i]	14	25′ EGA	.....[c]	89.6[c]	96.6[b]	0.4[f]	0.4[b]	.....	.....	10.0	3.0	0.0	172.2	178.6	172.1	0.1	6.5
Soybean Oil	15	25′ EGN	0.18[b]	52.6[c]	56.8[b]	8.27	6.98[b]	.....	0.00[b]	20.8	17.7	14.0[j]	136.5	138.2	136.0	0.5	2.2
		45′ EGN	.....[c]	52.7[c]	.....	8.32	.....	.....	.....	20.7	.....		136.8	.....		0.8	....
Soybean Oil	16	25′ EGN	0.17[b]	52.3[c]	56.5[b]	8.30	7.11[b]	.....	0.02[b]	21.2	17.9	13.9[j]	136.5	138.4	136.0	0.5	2.4
		45′ EGN	.....[c]	52.1[c]	.....	8.35	.....	.....	.....	21.3	.....		136.4	.....		0.4	....
Soybean Oil	17	25′ EGN	0.19[b]	50.7[c]	54.4[b]	8.45	7.68[b]	.....	0.03[b]	23.0	19.8	13.6[k]	135.6	137.7	135.2	0.4	2.5
		25′ EGA	.....[c]	49.6[c]	.....	8.29	.....	.....	.....	24.2	.....		134.2	.....		−1.0	....
		30′ GA	.....[c]	50.8[c]	52.2[e,c]	8.90	8.22[e]	.....	.....	22.4	21.7		136.4	136.2		1.2	1.0
		45′ GA	.....[c]	50.6[c]	52.9[e,c]	8.63	8.18[e]	.....	.....	22.9	21.0		135.8	137.0		0.6	1.8
Methyl Esters from Soybean Oil[i]	18	25′ EGN	0.07[b]	62.6[c]	67.7[b]	9.37	8.08[b]	.....	0.0[b]	23.2	19.3	0.0	159.8	162.2	159.5	0.3	2.7
		45′ EGN	.....[c]	63.3[c]	.....	9.45	.....	.....	.....	22.4	.....		160.6	.....		1.1	....
		45′ GA	.....[c]	62.0[c]	64.7[e,c]	9.53	9.02[e]	.....	.....	23.7	21.5		159.5	161.0		0.0	1.5
Methyl Esters from Linseed Oil[i]	19	25′ EGN	0.26[b]	16.7[c]	20.8[b]	50.0	45.9[b]	.....	0.0[b]	28.5	28.2	0.0	192.7	188.9	191.5	1.2	−2.6
		45′ EGN	.....[c]	16.6[c]	.....	50.3	.....	.....	.....	28.3	.....		193.3	.....		1.8	....
		45′ GA	.....[c]	16.4[c]	18.7[e,c]	49.4	46.8[e]	.....	.....	29.4	29.7		191.3	188.6		−0.2	−2.9
Methyl Esters from Linseed Oil[i] (partially fractionated by adsorption)	20	25′ EGN	0.26[b]	42.6[c]	47.5[b]	32.8	29.9[b]	.....	0.0[b]	19.8	17.5	0.0	184.7	184.0	182.3	2.4	1.7
		45′ EGN	.....[c]	42.6[c]	.....	32.8	.....	.....	.....	19.8	.....		184.7	.....		2.4	....
		45′ GA	.....[c]	41.0[c]	43.6[e,c]	32.4	30.7[e]	.....	.....	21.8	20.9		182.0	181.7		−0.3	−0.6
Linseed Oil	21	25′ EGN	0.31[b]	14.4[c]	16.6[b]	49.7	47.7[b]	.....	0.03[b]	22.6	22.1	9.0[k]	182.3	181.1	181.8	0.5	−0.7
		25′ EGA	.....[c]	14.6[c]	.....	48.0	.....	.....	.....	24.1	.....		179.4	.....		−2.4	....
		30′ GA	.....[c]	14.9[c]	17.6[e,c]	50.8	46.9[e]	.....	.....	21.0	22.2		184.9	180.3		3.1	−1.5
		45′ GA	.....[c]	15.3[c]	17.6[e,c]	49.4	46.8[e]	.....	.....	22.0	22.3		182.7	180.0		0.9	−1.8
Perilla Oil	22	30′ GA	.....[c]	12.9[c]	16.1[e,c]	62.0	57.3[e]	.....	.....	14.6	14.4	6.3[l]	206.1	200.3	207.3	−1.2	−7.0
Lard	23	25′ EGN	0.22[b]	10.2	11.8[b]	0.68[f]	0.61[b]	0.30[g]	0.35[b]	49.4	47.8	34.9[k]	66.1	67.7	66.6	−0.5	1.1
		25′ EGA	0.18[d]	11.2	.....	0.56[f]	.....	0.17[g]	.....	48.7	.....		66.5	.....		−0.1	....
		30′ GA	0.18[d]	11.2	11.5[e]	0.74[f]	0.72[e]	0.38[g]	0.34[g]	48.3	48.1		67.4	67.4		0.8	0.8
		45′ GA	0.18[d]	11.4	11.9[e]	0.74[f]	0.74[e]	0.35[g]	0.35[g]	48.2	47.7		67.4	67.9		0.8	1.3
Tallow	24	25′ EGN	0.57[b]	1.18	1.30[b]	0.50[f]	0.43[b]	0.06[g]	0.07[b]	45.6	45.5	47.8[k]	45.7	45.7	43.9	1.8	1.8
		25′ EGA	0.56[d]	1.38	.....	0.38[f]	.....	0.04[g]	.....	45.5	.....		45.6	.....		1.7	....
		30′ GA	0.56[d]	1.34	1.39[e]	0.56[f]	0.53[e]	0.10[g]	0.09[g]	45.3	45.3		46.0	46.0		2.1	2.1
		45′ GA	0.56[d]	1.31	1.42[e]	0.55[f]	0.46[e]	0.09[g]	0.09[g]	45.4	45.4		46.0	46.0		2.1	2.1

[a] EGN—Ethylene Glycol-Nitrogen; EGA—Ethylene Glycol-Air; GA—Glycerol-Air. See footnotes of Table I and equations of Table III.

[b] Am. Oil Chem. Soc. Tentative Method Cd 7-48 (see also J. Am. Oil Chem. Soc. *26*, 399-404 [1949]). Preformed conjugated triene and tetraene components not reported.

[c] Conjugated diene neglected since k'_{233} is greater than 20.

[d] Brice and Swain, reference 3, equations (1) and (4).

[e] Brice and Swain, reference 3, with constants for 45 minutes from data used for plotting Fig. 5 of reference 3.

[f] Background correction applied since k'_{268} is less than 1.

[g] Background correction applied since k'_{315} is less than 1.

[h] Calculated by difference; see equation (25).

[i] Saturated acids eliminated by fractional distillation and low temperature crystallization.

[j] Average of determinations by crystallization method at Eastern (ERRL) and Northern (NRRL) Regional Research Laboratories, and determinations by Bertram method at SRRL and NRRL; A.O.C.S. Spectroscopy Committee 1950 samples.

[k] Average of determinations by crystallization and Bertram methods, J. Am. Oil Chem. Soc., *25*, 144 (1948), page 145; A.O.C.S. Spectroscopy Committee 1946-47 samples.

[l] Determined by Bertram method.

stearic acids) preformed conjugated diene may not be negligible. C_2 should be determined by the following equation:

$$C_2 = 100\,k_2/119 = 0.84\,k_2 \qquad (21)$$

In this expression $k_2 = k_{233} - k_o$, where k_o is 0.07 for fats and esters, and 0.03 for acids and soaps. The corrected coefficient k'_2 should be calculated by the following equation:

$$k'_2 = k'_{233} - k_2 - 0.03 \qquad (22)$$

These two equations are essentially those presented in a previous publication (3).

B. Substantial proportions. If k'_{233} is 20 or more (examples are common nondrying and semi-drying vegetable oils), C_2 need not be determined and k'_{233} need not be corrected.

C. Special cases. In oils and fatty materials known to contain substantial proportions of dienoic conjugated acids or other constituents having appreciable absorption near 233 mμ which is not destroyed by the alkali-isomerization (such as dehydrated castor oil, tall oil, crude cottonseed oil, or sesame oil), special corrections and calculations will be necessary.

Linolenic acid:

A. Small proportions. If k'_{268} is less than 1 (examples are animal fats and some vegetable oils such as tobacco seed oil), the observed specific extinction coefficient must be corrected for extraneous background absorption using equation (8) of reference (3):

$$k'_3 = F_3\,[k'_{268} - \tfrac{1}{2}\,(k'_{262} + k'_{274})] \qquad (23)$$

The appropriate value of F_3 is taken from Table II. The result is not necessarily of high accuracy since F_3 is dependent on slit width and since background absorption in this region may not be strictly linear.

B. Substantial proportions. If k'_{268} is 1 or more (examples are linseed oil, soybean oil, and special concentrates of animal fats) background absorption may be ignored and $k'_3 = k'_{268}$.

C. Special cases. Oils known to contain appreciable proportions of trienoic conjugated acids (such as tung oil, oiticica oil, and certain cucurbit oils) or other constituents having appreciable absorption near 268 mμ which is not destroyed by the alkali-isomerization will require special corrections and calculations.

Arachidonic acid:

A. Small proportions. If k'_{315} is less than 1 (animal fats and oils), the observed specific extinction coefficient must be corrected for extraneous background absorption using the method of equation *(10)* of reference (3):

$$k'_4 = F_4\,[k'_{315} - \tfrac{1}{2}\,(k_{308} + k_{322})] \qquad (24)$$

The appropriate value of F_4 is taken from Table II. For vegetable oils k'_4 and k'_{315} are assumed to be zero since arachidonic acid is not present.

B. Substantial proportions. If k'_{315} is 1 or more (special concentrates of animal fats) background absorption may be ignored and $k'_4 = k'_{315}$.

C. Special cases. Animal fats or concentrates containing appreciable proportions of fatty acids of higher unsaturation than arachidonic acid will require special corrections and calculations.

Analysis of Samples

A number of samples of vegetable oils and animal fats were subjected to spectrophotometric analysis in order to establish whether more reliable results were obtained, using the natural fatty acids as standards. Samples having known content of saturated fatty acids, or having been freed of saturated acids by low temperature crystallization, were used for this purpose. This permitted determination of oleic acid in the sample by difference:

$$w = (100/f) - (s + C_2 + x + y + z) \qquad (25)$$

where w is the percentage of oleic acid and s the percentage of saturated acids in the sample; and f = 1 if the sample is fatty acids, 1.050 if methyl esters, and 1.045 if glycerides. The iodine value of the sample, I_s, was then calculated from the analysis by the relation

$$I_s = [I_1 w + I_2(C_2 + x) + I_3 y + I_4 z]/100 \qquad (26)$$

where $I_1 = 89.9$, $I_2 = 181.0$, $I_3 = 273.7$, and $I_4 = 333.7$, the iodine values of the pure fatty acids. The calculated iodine value was then compared with the observed iodine value of the sample.

Average results for duplicate spectrophotometric analyses and for calculated and observed iodine values and their differences are shown in Table IV. Many of the samples were run under different conditions as well as for different standards. Calculations were made in accordance with the recommendations outlined in the preceding section, except for those made by A.O.C.S. Tentative Method Cd 7-48.

The differences between calculated and observed iodine values are averaged and summarized for the various sample types in Table V. It will be noted in Tables IV and V that the calculated-observed iodine values for the debromination acid data of Brice *et al.* (3, 4) are smaller than those for data calculated from A.O.C.S. Tentative Method Cd 7-48 (15), which is based essentially on debromination acid data

TABLE V

Average Differences, Using Natural and Debromination Acid Standards, Between Calculated and Observed Iodine Values (Data Calculated From Table IV)

Type of oil or fat	No. Samples	No. Analyses	Iodine Value			
			Range	Average Calc.-Obs. Values		
				Natural	Debromination	
					Brice *et al.*[a]	Cd 7-48[b]
Perilla	1	1	207	−1.2	−7.0	....
Linseed	3	10	182-192	0.9	−1.7	−0.5
Soybean	4	11	135-160	0.4	1.4	2.4
Cottonseed	4	11	108-152	0.1	1.3	3.9
Tobacco seed	1	1	172	0.1	....	6.5
Lard	1	4	67	0.3	1.1	1.1
Tallow	1	4	44	1.9	2.1	1.8

[a] See Tables II and III. Preparations of reference (4).
[b] Am. Oil Chem. Soc. Tentative Method Cd 7-48.

of Beadle and Kraybill (2). The explanation for this is probably due to the fact that the former preparations were subjected to distillation and crystallization after debromination. They should therefore differ less from the natural acids than the preparations of Beadle and Kraybill, which were not so treated.

Discussion and Conclusions

Study of Table IV indicates that when the natural acids are used as standards: a) for all samples the results for linoleic acid are appreciably lower and those for linolenic acid appreciably higher than when debromination acid standards are used; and b) with few exceptions the observed iodine value is satisfactorily accounted for and the difference between calculated and observed iodine values are larger when debromination acid standards are used. The first observation could of course have been predicted from the standardization data of Table I and by itself furnishes no criterion for deciding the question of proper standards. However in combination with the data for individual (Table IV) and average (Table V) differences between calculated and observed iodine values, there is little doubt as to the superior accuracy of the natural acid standards.

It should be pointed out that a number of difficulties are attendant on attempting to demonstrate the superiority of the natural acid standards by this method. The spectrophotometric method is highly empirical, and any experimental error either in the standardization or in the analysis of the sample which affects the result for linoleic or linolenic acid, particularly the latter, may affect the calculated iodine value materially. For example, in the case of linseed oil 21 the difference between calculated and observed iodine values for the natural acid standards was 3.2 units for the 30-minute GA technique, and −2.7 units for the 25-minute EGA technique. This is probably the only serious inconsistency of this nature obtained however. Another source of error lies in the necessity for separate determination of saturated acids. In order to reduce this source of error samples were included which had been carefully freed of saturated acids, but any superiority of these concentrates for the purpose is not apparent from the data.

Probably the most serious difficulty in attempting to use the difference between calculated and observed iodine value as a criterion is present in cases where both linoleic and linolenic acids occur in substantial proportions, as in the linseed oil types and to a lesser extent in the soybean oil types. As stated above, the use of the natural acid standards results in lower values for linoleic and higher values for linolenic acid as compared with use of the debromination standards. In such cases there will be some compensation of errors in the calculated iodine value. Hence the difference between calculated and observed iodine value should under the circumstances be a relatively poor criterion when linoleic and linolenic acids are both present in substantial proportions. It should however be a good criterion for samples free of or low in linolenic acid and high in linoleic acid (e.g., tobacco seed fatty acid concentrate 14), or vice versa (e.g., perilla oil 22). Inspection of Tables IV and V bear out this point. The iodine value differences for the two types of standards are larger and more clear-cut for perilla oil, tobacco seed oil fatty acid concentrate, cottonseed oil and methyl esters, and soybean oil and methyl esters, in approximately the order given. Results for iodine value differences are less clear-cut for the linseed oil types which contain high proportions of linolenic acid and substantial proportions of linoleic acid; also for tallows which contain only low proportions of the polyunsaturated fatty acids and for which the iodine values of the unsaponifiables were not determined. It is concluded that the use of natural fatty acids as standards in the spectrophotometric method leads to substantially greater accuracy in the analysis of natural fatty materials for polyunsaturated acids than does the use of bromination-debromination acids.

A brief discussion of the results obtained under different conditions of isomerization is appropriate. Agreement between analyses of a given sample under different conditions (Table IV) are in general quite satisfactory in spite of the highly empirical nature of the method and the limited standardization data available in some cases. Reasons were given above, in a discussion of Figures 2-5, for expecting higher reproducibility with the longer isomerization time of 45 minutes. Calculation of the average percentage of difference between duplicate determinations for the vegetable oil type samples of Table IV indicates a somewhat better reproducibility for the 45-minute isomerization time. For linoleic acid the average percentage of difference for 45 minutes was 0.51, and for 25 and 30 minutes, 0.95; for linolenic acid the average percentage of difference for 45 minutes was 0.59, and for 25 and 30 minutes, 0.71. In this laboratory the 45-minute glycerol-air method is preferred for general use because of its simplicity and high precision; the 45-minute ethylene-glycol-nitrogen method is a close second because of the greater transparency of reagent blanks. All of the methods examined should however give satisfactory results with proper care.

Summary

Spectrophotometric methods of analysis for the polyunsaturated constituents of oils and fats have been carefully restandardized for several conditions of alkali-isomerization, using purified methyl esters of linoleic, linolenic, and arachidonic acids prepared by physical rather than by chemical means. A number of vegetable oil and animal fat samples were subjected to spectrophotometric analysis, and the results based on natural and on bromination-debromination fatty acid standards were compared. The natural fatty acid standards lead to significantly higher accuracy and their use in the spectrophotometric analysis of natural fatty materials is strongly recommended. Results obtained under different conditions of isomerization were in satisfactory agreement. An isomerization time of 45 minutes is recommended rather than 25 or 30 minutes. The glycerol-air technique is preferred for general use because of its simplicity and high precision. The ethylene-glycol-nitrogen technique is a close second choice because of the greater transparency of reagent blanks.

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The Chemistry of Polymerized Oils. I. Characteristics of Some Pilchard Stand Oil Fractions

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THE work reported here is a part of a long-term research being undertaken by this Laboratory into the organic and physical chemistry of thermally polymerized oils. In order to place this and the future communications of the series into their proper perspective it is first desirable to discuss briefly some of the main aspects of present day knowledge relating to the composition of stand oils against the general background of polymer chemistry.

Current Views on Stand Oil Chemistry

The growth of polymer molecules during the thermal treatment of triglyceride oils results from the presence of olefinic double bonds which are situated near the center of the fatty acid chains in most vegetable oils but are fairly evenly distributed along the more highly unsaturated portion of most fish oil fatty acid chains. Although the structures of the thermally formed linkages between chains are not yet known with absolute certainty, two differing hypotheses have been advanced and discussed in detail. According to the first hypothesis (17, 22), which commands the support of the majority of authors in this field, Diels-Alder reaction takes place by the addition of a double bond in one chain to the ends of a conjugated diene system already present or formed by heating in another chain so that the linkage consists of a cyclohexene ring substituted in four neighboring positions. According to the second hypothesis (28) a hydrogen atom detached from a methylene group flanked on one or both sides by double bonds adds to one end of a double bond in another chain with the result that the two chains become linked by a single carbon-carbon bond. It has been pointed out (29) that it is unnecessary to assume, as most authors have, that the alternative structural hypotheses are mutually exclusive and, in fact, it is not impossible that both may occur together. It is reasonable to expect that the position of the formed linkages with respect to the length of the chains concerned will have an important influence on the viscous and other physical properties of a given stand oil and furthermore that the structure of the linkages themselves will considerably influence physical properties. In this connection one may quote the fact that comparatively minor alterations in chemical structure alter considerably the physical properties of many polymer types (5). Apart from these structural considerations it is to be expected that the molecular weight distribution of a given stand oil will largely determine its physical properties.

Molecular Weight Distribution of Stand Oils

Theoretical Considerations. The theory of molecular weight distribution in polycondensation reactions between pure monomers has been developed by Flory (19), and the degree of deviation, which exists in practice from the mathematically necessary basic assumption that no intramolecular reaction occurs, has been tested by comparing observed and calculated gel points. Thus in the cases of the glycerol/phthalic acid system and of the pentaerythritol/adipic acid system the observed extents of reaction at the gel points were found to be somewhat greater than the calculated figures, indicating a minor but definite degree of intramolecular reaction so that the number average molecular weights, weight average molecular weights, and complete molecular weight distributions calculated by using Flory's equations in conjunction with the measured extents of reaction at any given time deviate, but not considerably so, from those which actually exist in the cases studied by Flory.

The relevance of Flory's work to the case of stand oils and other surface coating materials has already been recognized (24, 32) although Bradley who early recognized (12) the importance of functionality considerations and who has published widely on the stand oil problem (12-15), differs from Flory at some points (17). Flory's theory may be very usefully applied in qualitative terms to the interpretation of several important physico-chemical aspects of stand oil formation. For example, the viscosity/time relationships observed during thermal polymerization are of the same general type as those Flory records for polyesterification reactions, which have been interpreted by him in terms of his general theory. Again, the