Application of Ultraviolet Spectrophotometry to the Analysis of Fats and Derivatives[1]

907

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ULTRAVIOLET spectrophotometry was first employed in fat and oil analysis in 1934 by Dingwall and Thomson (12), who described a procedure for the determination of α- and β-eleostearic acids in mixtures. In this instance ultraviolet spectrophotometric measurements were possible because these acids absorb strongly and exhibit well-defined maxima in the near ultraviolet spectral region owing to the conjugated position of their double bonds. In most of our common fats and oils however the unsaturated acids have non-conjugated double bond systems which do not absorb in this spectral region. Hence ultraviolet spectrophotometric methods were not applicable to analysis of these oils until a procedure was found which would convert non-conjugated acids, such as linoleic and linolenic acids, to their conjugated isomers.

The first indication that such conjugation was possible is attributed to an observation made by Moore (31) in 1937. He found that prolonged heating of various fats and oils with alkali during saponification produced an increase in absorption in the ultraviolet spectral region and postulated that the absorption was caused by conversion of normal linoleic and linolenic acids to their conjugated forms. This observation prompted further investigations which not only substantiated Moore's findings but resulted in much more effective alkali treatment and consequently more complete conversion. In 1943 the first quantitative method for analysis of fats and oils based on this alkali treatment was proposed by Mitchell, Kraybill, and Zscheile (30). Subsequently substantial improvements and modifications have been incorporated in these methods, which today are being used extensively in the analysis of a wide variety of fats and oils. The present status, applications, and limitations of the more commonly used methods are discussed. This paper however is not intended as a complete review of all the work done in this field.

Instruments and Sources of Error

The Beckman quartz spectrophotometer, Model DU, and the Cary recording quartz spectrophotometer are the instruments most commonly employed for ultraviolet spectrophotometric analysis in this country. The principles involved in making spectrophotometric measurements will not be discussed; however it might be well to enumerate some of the sources of error that may be encountered. The wavelength scales of the commercially available instruments are generally accurate, but it is easily possible for an instrument to get badly out of adjustment and a continual check is strongly advised. Stray radiation is often a source of important error and may be caused by reflections from dust, scratches, and instrument parts or by scattering from optical parts. Slit width, lamp adjustment, and absorption cell imperfections also may be sources of error. There are available several good publications reviewing the cause, effect, and cure of such errors (16, 17). It should be remembered that only the most careful calibration and operation of the instrument will render such errors negligible.

One source of error that appears not to have been given sufficient attention in the analysis of fats and oils is the effect of slit width. The magnitude of slit width errors depends primarily upon two things: a) the effective width of the entrance and exit slits with a continuous spectrum source, and b) the curvature (not the slope) of the spectral distributions under consideration (16). It is apparent from Figure 1, curve X, that the narrower the slit width, the more nearly the observed value at C will approach the true value at D, but on curve Y, which is more linear, the slit width will have essentially no effect. The importance of this factor in fat and oil analysis was noted by Brice *et al.* (8) in a discussion of the determinations of linolenic acid. Recently Hammond and Lundberg (18) found that slit width was of great importance in the analysis of docosahexaenoic acid. In an analysis it would seem only necessary to use the slit width employed in the determination of the absorptivity[3] constants, but an example of the error that may occur by not employing optimum slit widths may be found from inspection of the curve of a pentaene-hexaene mixture. Isomerized pentaenoic acid has a maximum at 346 mμ which is employed in the analysis for this component, but when conjugated hexaenoic acid is present, the peak at 346 mμ may not be apparent because of the overlap of a principal maximum for the hexaene which occurs at 352.5 mμ. Therefore it is probable that, in an analysis of a mixture of these two acids, the observed absorbance at 346 mμ for pentaenoic acid will be determined on a slope rather than on a maximum. Thus a situation

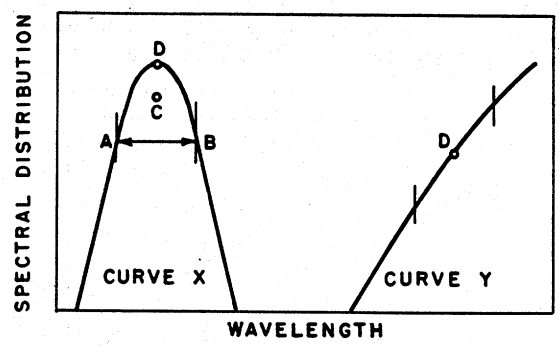

FIG. 1. Slit width error dependent on the curvature, not the slope, of the distribution curve under consideration. Spectrum band width extends from A to B. On curve X the observed value is at C and the true value at D. On curve Y the observed value and true value essentially coincide at D.

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[3] Absorptivity = A/bc where A = Absorbance, b = length of cell, and c = concentration in grams/liter. Suggested Nomenclature in Applied Spectroscopy. Anal. Chem., *24*, 1349 (1952).

occurs which is similar to that shown in Figure 1. The slit width employed in the analysis of the mixture (on a slope) is relatively unimportant, but the accuracy of the determination depends on the slit width employed in the determination of the absorptivity constant of the pure pentaenoic acid (on a maximum). Therefore it seems probable that further accuracy of the spectrophotometric method could be attained by establishing absorptivity constants of the reference standards at slit widths which give the greatest possible absorptivity.

For maximum absorptivity when analyzing docosahexaenoic acid with the Beckman spectrophotometer, a slit width of 0.15 mm. at 374 mμ has been recommended, and to obtain this slit width a tungsten lamp was used as the light source (18). In an investigation at this laboratory a relatively pure hexaenoic acid was isolated, and absorptivities were obtained that were in reasonably good agreement with those published by Hammond and Lundberg (18). It was found however that slit widths as low as 0.19 mm. could be obtained by using a hydrogen lamp. The absorptivities observed were equal to those determined when the tungsten lamp was employed. This is shown in Figure 2. The spectral region between 320 and

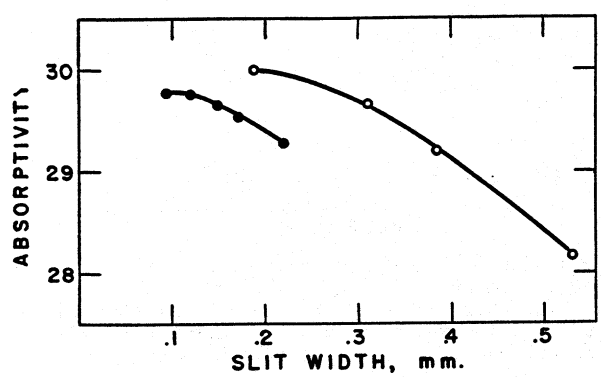

FIG. 2. Apparent absorptivity *vs.* slit width for methyl hexaenoate at 374 mμ after isomerization in 21% KOH-glycol at 180°C. for 15 min. ●-●-● tungsten light source; ○-○-○ hydrogen light source.

400 mμ is a region in which either light source is indicated. However since the hydrogen light source is necessary for the balance of the ultraviolet spectra, it is believed desirable not to change the light source in the middle of an analysis.

The data in Figure 2 were obtained from one of four fractions of methyl hexaenoate that appeared to be rather pure. In Table I the average absorptivities of these four fractions, after alkali treatment, are compared at the analytical wavelengths with those reported previously (18). Both samples were isolated from hog brain lipids.

TABLE I

Comparison of Hexaene Absorptivities

	233 mμ	268 mμ	315 mμ	346 mμ	374 mμ Hydrogen lamp	374 mμ Tungsten lamp
Hammond *et al.* (18)	40.0	49.4	27.8	26.2	.....	28.1
This Laboratory	39.7	47.4	30.0	27.0	29.3	29.1

Non-Conjugated Fatty Acids

Non-conjugated polyunsaturated acids when heated with alkali form conjugated isomers which have specific and well defined spectra. The extent of conversion of non-conjugated to conjugated isomers and the ratios of the various conjugated isomers produced are dependent upon the conditions employed in the alkali isomerization. The strength of alkali, solvent for alkali, temperature, time of heating, and size of sample are important factors in the isomerization procedure, and all of these factors must be held within the limits specified for the particular method used for analysis.

Method A—6.5% KOH-glycol. Probably the best known and most widely used method for the analysis of fats and oils is the American Oil Chemists' Society Tentative Method Cd 7-48 (2). This method employs 6.5% KOH-glycol and is essentially the procedure described by Mitchell, Kraybill, and Zscheile (30), which has been modified and improved by the addition of absorptivity constants for arachidonic acid (4), improvement of the transparency of the reagent by protection with an atmosphere of nitrogen (36), introduction of corrections for absorption by extraneous compounds, and increasing the sensitivity and accuracy in the determination of small proportions of conjugated and non-conjugated acids (7, 9, 39, 47). Probably the most notable improvement in recent years was the adoption of absorptivity constants based on pure polyunsaturated acids, isolated by physical means to replace the constants determined on acids prepared by the usual bromination-debromination chemical techniques (21, 45). It has been shown that chemically prepared acids contain isomeric acids which are not of the all *cis* type (29) and also that acids isolated by physical means have all the double bonds in the *cis* configuration, which is the configuration that occurs naturally in most fats and oils. It has further been shown that *cis* and *trans* isomers isomerize at different rates during alkali treatment resulting in quite different absorption intensities after a given time of heating (33, 34). Substantially greater accuracy was obtained when the constants from acids in their natural configuration were employed in the analysis of lard, cottonseed, soybean, perilla, linseed, and tobacco seed oils (8). A variation of this method was proposed by Hilditch, Morton, and Riley (23A) and later improved by including the absorptivity constants determined for natural polyunsaturated acids (23B).

Method B—11% KOH-glycerol reagent. A method that has attracted wide attention was proposed by Brice and Swain (7) and later improved to include more accurate constants (8). The method employs 11% KOH-glycerol as the isomerization reagent. This reagent offers the advantage of a transparent medium without using an atmosphere of nitrogen either in the preparation of the reagent or during the isomerization procedure. An extension of the utility of the method resulted from the determination of the absorptivity constants for pentaenoic acids when employing this reagent (19).

Method C—21% KOH-glycol reagent. Holman and Burr (24) studied the effect of various concentrations of alkali on polyunsaturated fatty acids prepared by bromination-debromination techniques. They found that maximum yields of conjugated diene from linoleic acid are produced over a wide range of alkali

concentrations, but to produce maximum conjugated triene, tetraene, and pentaene from their respective non-conjugated acids a strong alkali concentration is needed. Application of their optimum conditions of isomerization (18% KOH-glycol) more than doubled the absorptivity of arachidonic acid over that obtained by employing either method A or B. This effect of higher concentration of alkali was confirmed by Herb and Riemenschneider (19), who found however that 21% KOH-glycol was optimum for producing maximum tetraene conjugation of arachidonic acid. They also extended the investigation and determined the absorptivity constants with this reagent for linoleic, linolenic, arachidonic, and pentaenoic acids of natural configuration. Absorptivity constants for docosahexaenoic acid have recently been determined with the 21% KOH-glycol reagent (18).

Method D—21% KOH-glycol (micromethod). Investigators, particularly in biological and medical research, have been interested in a micromethod that would require but a few milligrams of sample, and considerable work has been published in this direction (6, 24, 35, 46, 54). For one reason or another these workers did not recommend their published constants but advised others to determine their own independently. The 21% KOH-glycol method, owing to its increased sensitivity, was found adaptable to the analysis of samples of fats and oils as little as one milligram. This was accomplished by reduction in the amount of reagent and a modification of the reaction tube (20). A procedure was also described in which a microanalytical balance was not necessary for weighing the sample. If as much as 10 to 30 milligrams of material is available, it can be diluted with lauryl alcohol and an aliquot weighed on a good analytical balance. The remainder of the lauryl alcohol solution can be used to determine iodine and saponification values by micromethods and thereby obtain rather complete analysis.

Method E—11% KOH-glycol (semi-micro). A semi-micro method for the analysis of the component acids of blood serum was proposed by Weise and Hansen (55). In this method 11% KOH-glycol was employed as the isomerizing reagent, and the amount of fat analyzed was between 7 and 15 milligrams. Unfortunately it is not clear if their reference compounds, linoleic, linolenic, and arachidonic, were prepared by the usual bromination-debromination techniques, but, if so, the method would suffer the same inaccuracy found in the A.O.C.S. Method Cd 7-48 before the adsorptivity constants for the natural acids were adopted.

Applications and limitations. Method E will not be discussed in this section since comparative data with other methods are not available. Methods A, B, C, and D are highly acceptable methods for the analysis of the polyunsaturated fatty acids found in the usual animal fats, vegetable oils, and purified fatty acids and esters where the unsaturation is due to linoleic, linolenic, and arachidonic acids. Method B may also be used when pentaenoic acids are present and methods C and D where pentaenoic and hexaenoic acids are found. Method D (micro) has wide application where the amount of sample is limited, such as in the analysis of small fractions obtained by distillation or chromatographic separations. The method has been employed to determine the composition of fat extracted from potatoes where, on the fresh whole basis, the fat content was only 0.02% (23). It has also been employed to determine the differences in composition of bone marrow fat of fed and fasting rabbits (14), and of the fat contained in several fractions of rat livers during a study of the onset of fatty acid deficiency (26, 27).

None of the methods apply in a strict quantitative sense to the analysis of oxidized fats, hydrogenated oils, certain drying oils, fish oils, or oils containing unusual acids or pigments whose interference is unknown or cannot be determined.

It is important when choosing a method for an analysis to select a method that provides absorptivity constants for all the polyunsaturated acids that will be present in the sample. In a recent publication (56) absorptivity constants were published for hexaenoic acid to be employed in method E; however pentaenoic acid constants were lacking. While the authors recognized this weak link, they made calculations for all other components and minimized the effect of the presence of pentaene acids. In their particular case this may not have been serious; however it is a pitfall that is found many times in the literature and should be avoided. An example of how this could lead to serious error is shown in the analysis of a sample in which the absorptivities at the analytical wavelengths indicate the presence of acids up to and including hexaenes. The absorptivities were obtained by both the 6.5% KOH-glycol and 21% KOH-glycol methods and are given in Table II to show the low absorptivity in the hexaene region and the great influence it exerts on the calculated composition. The calculations of composition employing these values are shown in Table III. The following assumptions are made: Calculation (A) no constants are available for pentaenoic or hexaenoic acids; Calculation (B) no constants are available for hexaenoic acid; and Calculation (C) all the constants necessary are available.

TABLE II

Absorptivities Determined by Two Different Methods of Alkali Treatment

Method	Absorptivities of sample				
	233 mμ	268 mμ	315 mμ	346 mμ	374 mμ
21% KOH-glycol	33.6	11.8	8.8	7.1	2.1
6.5% KOH-glycol	37.4	10.0	5.1	2.6	0.56

TABLE III

Calculation of Composition Showing the Importance of Employing All Necessary Absorptivity Constants

Component acid	Calc. A[1] %	Calc. B[2] %	Calc. C[3] %
Linoleic	27.7	27.4	26.9
Linolenic	5.3	6.6	4.0
Arachidonic	14.5	2.7	2.6
Pentaenoic	.....	10.3	7.4
Hexaenoic	.....	.....	7.3
Oleic	47.1	41.4	26.7
Saturated (by difference)	1.1	7.2	20.9
Saturated (Bertram)	17.8	17.8	17.8

[1] Calculation omitting constants for pentaene and hexaene acids.
[2] Calculation omitting constants for hexaene acids.
[3] Calculation including constants for all acids.

Significant differences are noted between the three analyses. A calculation of the total composition was

made in order to compare the calculated percentage of saturated acids with an independent determination of saturated acids by a modification of the Bertram procedure (42). While the agreement is not perfect, it can readily be seen that the saturated acid values are in much better agreement for the method which includes all the constants.

It was mentioned previously that none of the methods should be applied to the analysis of fish oils. A spectrophotometric method for the analysis of marine oils has been considered to have two principal drawbacks. The first is the lack of absorptivity constants for all the acids present, and the second is the unusual isomers and homologs reported present in such oils. The absorptivity constants are now available for pentaenoic and hexaenoic acids and would allow a reasonable estimation of the composition of these oils if the acids present were not such unusual isomers. It is this point that will be discussed further.

Farmer (15) has shown that 1,5-diene systems react very slowly under the conditions of alkali isomerization employed for linoleic and linolenic acids. Brice and Swain (7) have shown in linolenic acid that, once a double bond is separated by at least two methylenes, it appears to be very resistant to further isomerization to conjugated forms. Further two acids having an equal number of double bonds and differing only in chain length would be expected to have absorptivities that differ only by the ratio of their molecular weights. Paschke and Wheeler (41) have used this reasoning to determine the composition of the unsaturated fatty acids of the Alga Chlorella. They prepared the methyl ester, distilled into fractions containing substantially only one carbon chain length and then analyzed each fraction spectrophotometrically. A similar scheme of analysis would be expected to have value in the analysis of marine oils.

Fish oils are reported to have a number of unsaturated acids which have 1,5-diene systems as opposed to the usual 1,4-diene systems present in most land animals or plants. For example, 6,10,14-hexadecatrienoic (higaronic) acid has been isolated from sardine oil (50, 52) while 7,10,13-hexadecatrienoic acid has been isolated from rape leaf glycerides (22). A 4,8,12,16-eicosatetraenoic acid has been reported in sardine oil (51) and bonito oil (28) and the structure confirmed by permanganate oxidation procedures (48). Its counterpart in animal fat is 5,8,11,14-eicosatetraenoic (arachidonic) acid (13), and the position of the double bonds has also been confirmed (3, 32). Recently Toyama and Shimo-oka (49) isolated higaronic, moroctic, 4,8,12,16-eicosatetraenoic, eicosapentaenoic, and clupanodonic acids from sardine oil which reportedly contain 1,5-diene systems and examined these acids spectroscopically after alkali isomerization; surprisingly these acids exhibited absorption curves quite similar to isomerized acids which originally had the double bonds separated by only one methylene. In other words, after alkali isomerization similar curves are found for 6,10,14- and 7,10,13-hexadecatrienoic acid, and for 9,12,15-octadecatrienoic (linolenic) acid; for moroctic acid, 4,8,12,16-eicosatetraenoic acid and arachidonic acid; the two pentaenoic acids from sardine oil and the C_{20} and C_{22} pentaenoic acids isolated from beef adrenals. In Table IV comparisons are shown where absorptivities were determined by the same procedure, but the acids were isolated from different sources. The similarity of absorptivities between most samples whether from land or marine sources is apparent.

A little note of confusion may be added to this discussion. Bergmann and Swift (5) reported the isolation of a hexacosadienoic ($C_{26}H_{48}O_2$) acid from the lipids of sponges, which on oxidation by potassium permanganate produced oxidation products that indicated the double bonds were in the 17,20-position. However attempts to conjugate this acid by the usual alkali isomerization procedures were unsuccessful. We therefore have reports in the literature where acids whose double bonds are separated by one methylene will not conjugate and acids whose double bonds are separated by two methylenes will conjugate. The questions now are: a) has the position of the double bonds in the acids isolated from fish oils been proven beyond reasonable doubt; if so, b) is it true in all cases that double bonds separated by two methylenes conjugate with difficulty, or c) can these differences in absorption be explained on the basis of the particular geometrical isomers present? These questions must be answered in order to have a reliable method of analysis for marine oils. In spite of the conflicting data it appears likely that in the not too distant future these questions will be answered and a satisfactory spectrophotometric method for the analysis of marine oils will be developed.

TABLE IV

Absorptivities of Polyunsaturated Acids from Various Sources

Acid	Source	Absorptivities at Various Wavelengths (mμ)									
		Method [a]	233	235	268	270	315	316	346	348	349
7,10,13-Hexadecatrienoic	Rape leaf oil	A	61.7 [b]		49.0 [b]						
6,10,14-Hexadecatrienoic	Sardine oil	B	61.8		51.3						
		C	75.6		78.9						
9,12,15-Octadecatrienoic	Linseed oil	B	58.6		48.6						
		C	47.5		90.5						
4,8,12,15-Octadecatetraenoic	Sardine oil	B		88.0 [c]		75.0 [c]		45.0 [c]			
4,8,12,16-Eicosatetraenoic	Sardine oil	B		63.1		46.0		22.9			
5,8,11,14-Eicosatetraenoic	Beef adrenals	B	55.0		46.8		20.3				
Eicosapentaenoic	Beef adrenals	C	39.4		41.2		82.4		87.5		
Eicosapentaenoic	Sardine oil	C		59.0		54.6		75.0		87.8	
Eicosapentaenoic	Cod liver oil (1)	C	43.0		45.7		47.4		48.0		
Docosapentaenoic	Beef adrenals	C	43.5		46.0		56.9		50.4		
Docosapentaenoic	Sardine oil	C		60.1		48.7		53.2			39.0
Docosapentaenoic	Cod liver oil (1)	C	42.7		42.8		54.7		40.8		

[a] Method A, 6.5% KOH-glycol; Method B, 11% KOH-glycerol; Method C, 21% KOH-glycol.
[b] Values estimated from published curve (22).
[c] Values estimated from published curve (49).

Conjugated Fatty Acids

General (small proportions of conjugated acids). Conjugated acids are determined spectrophotometrically by simply diluting the sample in a suitable solvent and measuring the absorption in the analytical regions. However most spectrophotometric methods employ the absorptivities for the all *trans* acids which may lead to incorrect analysis since *cis* and/or *trans* isomers may be present and it is known that the absorptivity constants of these isomers differ. Method Cd 7-48 (2) also includes "background corrections" which are applied for greater accuracy when small proportions of conjugated acids are present but should not be applied when substantial quantities are present since this may lead to erroneous values (8).

Drying oils (large proportions of conjugated acids). The comments on spectrophotometric analysis of drying oils will be confined to tung oil and castor oil although the comments will apply equally as well to certain other oils. Tung oil is an important oil used in the coating industry. As mentioned previously, Dingwall and Thomson (12) first published a quantitative spectrophotometric method for the determination of mixtures of α- and β-eleostearic acids, which are the chief unsaturated acids present in tung oil. The method is actually based on the differences in the position of the maxima and differences in the absorptivities of *cis* and *trans* isomers. It has been shown that α-eleostearic acid is *cis* 9, *trans* 11, *trans* 13- and β- is all *trans* 9,11,13-eleostearic acid (10, 40). The original method has been modified to include determination of α- and β-isomers in tung oil (37), and determination of linoleic and conjugated dienoic acids in tung oil (38), also to determine the fatty acid composition of partially hydrogenated tung oil (43). An analyst employing these methods is readily able to duplicate his own values, but differences may be quite large from one analyst to another. Probably the cause for the discrepancies between analysts is the lack of specific information as to the slit width employed at each analytical wavelength. The acids in tung oil have very sharp absorption peaks, and as mentioned earlier, it is under these conditions that slit width errors are at a maximum.

Castor oil is another important oil used by the coating industry, and it is valuable because its properties are intermediate to the highly conjugated tung oil and the non-conjugated linseed oil. Ricinoleic acid which comprises about 90% of the acids present in the oil is converted by a dehydration process to predominately 9,11- and 9,12-octadecadienoic acids although small proportions of other acids have been reported (53). To determine the percentage of diene acids present in dehydrated castor oil, it is customary to determine the absorption of 233 mμ and divide the absorptivity of the sample by the absorptivity determined for pure *trans-trans* dienes and multiply the result by 100. However it has been shown that dehydration of castor oil produces a mixture of *cis* 9,*cis* 11-, *cis* 9,*trans* 11-, and possibly some *trans* 9,*trans* 11-conjugated acids and that the *cis-trans* isomers have an absorptivity of 95 to 98 (25, 33) as compared to 119 for the *trans-trans* acids. Therefore the use of the higher value would introduce an appreciable error in the calculation of the amount of conjugation. The absorptivity for *cis-trans* isomers is probably close to the correct one since *cis-cis* isomers are present in the mixture, and although their absorptivity is unknown, it is likely to be lower than the *cis-trans* value and would cancel out the effect of the *trans-trans* isomer.

Heat-bodied dehydrated castor oil is again another problem. Evidence has been presented that the conjugated *cis-trans* isomers of dehydrated castor oil isomerize on heating to the *trans-trans* form (25), but the action of heat on the *cis-cis* isomer remains unknown. The conjugated isomers present in heat-bodied dehydrated castor oil is a mixture of *cis-cis, cis-trans,* and *trans-trans* acids and the probable average absorptivity value would lie between 95-98 and 119 since there is present a higher proportion of the *trans-trans* isomer than is found in the dehydrated castor oil.

The consideration of the various isomers present in a sample is not new. It was recognized and employed by Privett *et al.* (44) in a study of the structure of hydroperoxides obtained from autoxidized methyl linoleate. It was also recognized by Desnuelle and Massoni (11) when they remarked that the spectrophotometric determination of the dienes of dehydrated castor oil is the best method available although not entirely satisfactory as the various dienes do not have the same absorptivity. However as information of the isomers and their absorptivities become known, it should be employed in correcting this shortcoming of the method.

Summary

There are several spectrophotometric methods available which allow a high degree of accuracy in the analysis of the common fats and oils. Further there are methods available for those fats and oils that contain unsaturated acids up to and including hexaenoic acid which allow good estimations of the composition. Several sources of error are discussed in an effort to point the way to their elimination.

Recently published spectrophotometric data on certain polyunsaturated acids isolated from marine oils are discussed in relation to the possibility of extending the method to include these oils.

More accurate spectrophotometric constants to be employed in the analysis of certain drying oils are suggested.

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