The Linoleic Acid Content of Seed Fats and the Isomerism of Linoleic Acid*

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Abstract

The linoleic acid content of a series of seed fats was determined by the thiocyanometric and the tetrabromide-precipitation methods in a search for isomeric linoleic acids. The results indicated the presence of only one form of linoleic acid. The tetrabromide number was shown to be affected by the pronounced solubility of alpha tetrabromostearic acid in the other bromides and in the solid acids, its determination therefore being of only limited value. The thiocyanogen numbers of pure linoleic and linolenic acids were found to be empirical values differing markedly from the theoretical constants, requiring a revision of the accepted equations for the calculation of the per cent concentration of the unsaturated acids in oils and mixed fatty acids.

OUR investigation of the nutritional importance of linoleic acid and its isomers in relation to their ability to cure the symptoms of the fat-deficiency syndrome (1) has raised anew the question concerning the number of linoleic acids occurring in natural fats. The opinion of some authorities (2,3) that at least two geometrically isomeric forms are found in seed fats and that still another octadecadienoic acid may be present in other fats (4) rests on conflicting conclusions based upon controversial evidence (5,6). However, several recent publications (6,7,8) have clarified the problem of the geometric isomerism of linoleic acid and the present paper is therefore concerned with a study of the possible number of isomeric linoleic acids in a wide variety of seed fats. In the interest of simplicity and accuracy, the investigation was limited to oils known to be free from linolenic acid, the absence of which was proven by subjecting aliquot portions of the free fatty acids of every oil used to bromination in ether solution. No hexabromide precipitation was observed in the case of any authentic and unadulterated oil.

Bromination of linoleic acid in cold ligroin precipitates a crystalline tetrabromide, m.p. 114-115° C., in quantities approaching 50% of the total yield of tetrabromostearic acids (7,8,9,10); the remainder is a soluble liquid tetrabromide. The simultaneous formation of these two derivatives, usually differentiated as the *alpha* and *beta* tetrabromides, is the basis for the supposition that the parent substance is a 1:1 mixture of two isomers, correspondingly designated as the *alpha* and *beta* linoleic acids, the former being completely precipitable as the crystalline *alpha* tetrabromide, and the other characterized by the liquid *beta* tetrabromide (11). However, Rollett (9), and more recently McCutcheon (7) and Riemenschneider, Wheeler, and Sando (8) have shown that the debromination of both tetrabromides regenerates identical octadecadienoic acids and they therefore rejected the view that linoleic acid is heterogeneous. They accounted for the simultaneous formation of two tetrabromides on the theoretical grounds that the bromination of the diethenoid linoleic acid should result in equal quantities of two stereoisomeric racemic compounds resolvable into optically active enantiomorphs, each of which should regenerate the same parent substance on debromination. Furthermore, Rollett (9), Brown and Frankel (10), Birosel (12) and others have demonstrated that the *alpha* tetrabromide is the only crystalline tetrabromostearic acid precipitable from seed fats containing linoleic acid.

Although this evidence indicates the identity of the regenerated acid with the natural form, it does not exclude the existence in seed oils of additional isomers characterized by the formation of only liquid bromides and undifferentiated by their tetrahydroxy derivatives (4,13), indications of which have been found in silkworm pupae (14), grass fats (15) and elsewhere. The presence of such isomers in any given seed fat should become evident by a comparison of the total linoleic acid content with the yield of *alpha* tetrabromide, for it follows from the preceding discussion that in the presence of isomers giving no precipitate with bromine, less than a 50% precipitation of the total linoleic acid as *alpha* tetrabromide must necessarily result. Conversely, where ordinary linoleic acid is the only isomer present, the yield of *alpha* tetrabromide should correspond to approximately 50% of the total linoleic acid, which could therefore be calculated readily by the multiplication of the tetrabromide number by an empirical constant, as suggested by Brown and Frankel (10). In the latter case, again, where analytical values for the erroneously differentiated *alpha* and *beta* linoleic acids have been reported, these would be expected to be approximately equal. The literature, on the contrary, presents analytical data showing disproportionate concentrations of the proposed isomers (16).

It was concluded that the analysis of a representative number of seed oils for total and bromide precipitable linoleic acid would establish whether such inequalities were due to the actual presence of isomers or merely to factors affecting the solubility of the *alpha* tetrabromide. The total linoleic acid was obtained by the thiocyanometric technique of Kaufmann (17), the experimental procedures and the constants used in the equations first being standardized with carefully purified oleic, elaidic, and linoleic acids and their mixtures.

Experimental

The officially accepted (18) procedures were followed in the determinations of the iodine numbers (Wijs, 100-150% excess of 0.2 N solution, ½ hour), and the thiocyanogen values (100-150% excess of 0.2 N solution, 24 hours, dry KI). The preparation of the reagents and the conditions of the determinations were exactly as specified, the thiosulfate solution being standardized against recrystallized potassium bichromate. The duplicate determinations invariably agreed within fractions of a unit.

The oleic acid was prepared by the low temperature cystallization of the acids of olive oil as described by Brown and Shinowara (19). Recrystallization of the fraction giving no precipitate from ten volumes of acetone at —20° C. was continued at —40° and —60° C. until the iodine number of the acid precipitating at the lower temperatures became constant at 89.6. Al-

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though this value closely approximates the theoretical limit of 89.9, the oleic acid was used only for investigating the behavior of mixtures of oleic and linoleic acids on bromination, and not for the standardization of the iodine and thiocyanogen values, since it is well known that complete removal of the solid and linoleic acids from oleic acid preparations is never beyond question. However, elaidic acid, being a relatively high melting substance of comparatively low solubility, appeared to be a more reliable standard for the determination of the precision of the iodine and thiocyanogen numbers of the monoethenoid acids. It was obtained by the action of nitrous acid on oleic acid (20). The product was repeatedly recrystallized from ligroin, methanol and acetone until the melting point remained constant at 44.4°. As shown in the accompanying table, its iodine and thiocyanogen numbers were 90.1 and 89.1 respectively. While this deviation from the theoretical values of 89.9 indicated a calculated purity of only 98%, with approximately 1% each of the contaminating linoleic and solid acids, it was assumed that the product was sufficiently pure to demonstrate the quantitative addition of the Wijs and thiocyanogen reagents to the double bond of oleic and elaidic acids, the slight discrepancies being considered as the probable errors of the determinations.

Linoleic acid was prepared according to the directions of Rollett (9), after careful purification of the tetrabromostearic acid obtained by the bromination of the fatty acids of corn oil in petroleum ether at 0°. The precipitated bromide was washed copiously on the suction funnel with petroleum ether, recrystallized with decolorizing charcoal from high-boiling ligroin (90-100° C.), dissolved in ethyl ether and reprecipitated with petroleum ether as suggested by McCutcheon (7), then recrystallized once more from ligroin (90-100° C.). The beautifully crystalline product, melting sharply at 115.2° C., was debrominated with zinc and 5 N methyl alcoholic sulfuric acid. The resulting ester was distilled at 2 mm. pressure in a stream of nitrogen and saponified in the cold by permitting it to stand overnight in ten volumes of a 7.5% solution of KOH in 95% ethyl alcohol in a completely filled flask. The solution was then poured into an equal volume of water, the fatty acid liberated with 20% sulfuric acid, extracted with petroleum ether, washed free of inorganic acid and dried over sodium sulfate. After removal of the solvent at the vacuum pump at 50° in a stream of nitrogen, the water-white linoleic acid had an iodine number of 181.0 (theory, 181.1). However, its thiocyanogen value proved to be 96.3 rather than the theoretical 90.5 used in Kaufmann's equations (18). Since the iodine number of linoleic acid and both the iodine and thiocyanogen numbers of elaidic acid agreed closely with the theoretical values, substitution of the empirical thiocyanogen value of linoleic acid for the accepted constant made a revision of the accepted calculations necessary, and the following equations were therefore devised:

$$\%\ \text{Linoleic Acid} = 1.180\ (\text{I.V.} - \text{T.V.})$$
$$\%\ \text{Oleic Acid} = 2.377\ \text{T.V.} - 1.265\ \text{I.V.}$$
$$\%\ \text{Solid Acids} = 100 - \%\ (\text{L.A.} + \text{O.A.})$$

The mixed fatty acids of the various oils were obtained by the quick saponification method of Moore (21). Five gram specimens of the oils were boiled for two minutes with alcoholic potash (2 gm. KOH, 1.4 ml. water, 10 ml. redistilled 95% ethanol). Unsaponifiable matter was removed from the soaps by three washings with ether. The fatty acids were then liberated as described previously and were sealed in highly evacuated tubes which were stored in the icebox until needed for the analyses.

Bromination was performed as follows: approximately one gram of fatty acids was dissolved in 25 ml. of purified petroleum ether (b. pt. 30-60° C.) in a weighed 50 ml. centrifuge tube and the solution was then cooled in an ice-hydrochloric acid bath. A slight excess of anhydrous bromine was added from a burette by permitting it to drain down slowly in a very thin stream along the inner surface of the cold centrifuge tube; this procedure, together with constant stirring of the solution, prevented any appreciable rise in the temperature of the reaction mixture. The tube was then set aside for 16-20 hours at 0° C. in the dark. The precipitated bromide was washed three times with 25 ml. portions of cold ligroin, the washings being separated from the precipitate by centrifuging and decanting; it was then dried for one hour in a vacuum oven at 60° C. and weighed. All brominations were performed in duplicate with excellent agreement. Blank determinations of the solubility of the *alpha* tetrabromide under the conditions of this procedure showed a loss of 14.5 and 13.4 mgm. per 100 ml. of ligroin. These values are well within the range of solubility reported by Lewkowitch (22) and the tetrabromide numbers were corrected accordingly.

The accompanying table summarizes the analytical data.

THE COMPARATIVE CONCENTRATIONS OF LINOLEIC, OLEIC, AND SATURATED ACIDS OF SEED FAT ACIDS AS DETERMINED BY THE THIOCYANOGEN AND BROMIDE-PRECIPITATION METHODS

Oil (free fatty acids)	Iodine Number (Wijs)	Thiocyanogen number (0.2N)	Saturated acids (calc.)	Oleic acid (calc.)	Linoleic acid (calc.)	Linoleic acid as tetrabromide ("alpha" linoleic	Ratio of "alpha" to calculated linoleic acid	Melting point of tetrabromide
			%	%	%	%	%	°C
Elaidic acid	90.1	89.1						
Linoleic acid	181.0	96.3			100	46.7	47.3	112
Mixed oleic and linoleic acids				80.3(d)	19.7(d)	5.1	28.8	113
Almond	101.2	83.5	8.7	70.4	20.9	7.7	39.5	113
Cocoa butter	37.2	34.3	62.1	34.5	3.4	(a)		
Coconut	9.2	7.9	91.4	7.1	1.5	(b)		
Corn (Mazola)	134.2	82.8	12.3	27.1	60.6	29.1	48.7	113
Cottonseed	118.5	71.5	24.4	20.1	55.5	26.9	49.7	113
Olive	88.3	76.5	16.0	70.1	13.9	3.0	26.7	110 112(c)
Peanut	101.4	75.1	18.8	50.2	31.0	13.1	44.0	111 114(c)
Poppyseed	144.2	86.3	9.0	22.7	68.3	32.9	48.7	113
Sunflower	128.1	83.5	11.0	36.4	52.6	26.0	50.7	106-112 112(c)

(a) The large quantity of saturated acids prevented the determination of tetrabromides by method outlined.
(b) Slight precipitate of tetrabromide redissolved during washing with ligroin.
(c) After one recrystallization from hexane.
(d) Weighed out.

Discussion

Consideration of the experimental data makes it evident that the ratio of the bromide-precipitable to the total linoleic acid of these oils approaches the limiting value of 1:2, deviations from this ratio being clearly related to the concentration of oleic and saturated acids which interfere with the complete precipitation of the *alpha* tetrabromide. This conclusion is verified by the roughly similar behavior of the synthetic mixture of oleic and linoleic acids and the fatty acids of a similarly constituted natural oil. Fluctuations of the expected ratio merely indicate that the determination of the tetrabromide number is only of limited value and that the constant proposed by Brown and Frankel (10) is applicable, at best as an approximation, only to oils very rich in linoleic acid. The reported concentrations of *beta* linoleic acid are consequently meaningless except as a measure of the linoleic acid corresponding to the liquid and dissolved solid tetrabromides, and in no way different from *alpha* linoleic acid.

The results strongly suggest, therefore, that in the series of seed fats studies, only one isomer exists in sufficient quantity to be detectable by the available analytical methods. This conclusion is, of course, predicated upon the assumption that the thiocyanogen values of the unknown linoleic acids are of the same order of magnitude as that of ordinary *alpha* linoleic acid. We have shown elsewhere (6) that this is true of at least three of the geometrically isomeric linoleic acids. Should the remaining geometric isomer and other position isomers prove to form only liquid bromides and to have a normal thiocyanogen value of 181, then their presence in this series of seed fats is obviously not excluded. In that case, deviations from the 1:2 ratio between the bromide-precipitable and total linoleic acids would be expected to occur in oils of higher iodine numbers rather than in those of low unsaturation as shown in the table. Furthermore, the characteristic melting point of the *alpha* tetrabromide obtained in every case, when considered in conjunction with the other data, lends additional support to the conclusion that *alpha* linoleic acid is the only octadecadienoic acid present in these seed fats.

The question of the true thiocyanogen value of linoleic acid and the optimum conditions for determining it calls for further consideration. Kaufmann (17), using 0.1 N and 0.05 N solutions, found the thiocyanogen number of a trilinolein having an iodine number of 169.1 (theory 173.3) to be 82.5, and the equations proposed for the calculation of the composition of fatty acid mixtures from their iodine and thiocyanogen numbers were consequently based on the premise that the thiocyanogen value of linoleic acid is very likely 90.5, or exactly one-half its iodine value; that is, thiocyanogen adds to only one of the two double bonds of the acid. However, Waterman and co-workers (23), apparently using reagents of the normality specified by Kaufmann, reported a value of 92.9 for pure linoleic acid. Again, in a very thorough and independent study published since the presentation of our paper, Riemenschneider and Wheeler (24) also showed that the thiocyanogen value of a methyl linoleate having an essentially theoretical iodine number rises with time to a level 2.4 to 2.7 points too high at the end of a twenty-four hour reaction period with 100-150% excess of the 0.1 N reagent. The linoleic acid used in our determinations gave under similar conditions comparable values of 92-93. With the same reaction period and excess of 0.2 N reagent, specified by the official methods of the American Oil Chemists' Society, we repeatedly obtained values of 96-97. Increasing the excess of the 0.2 N reagent to about 350% raised the value to 100 in 24 hours and to 105 in 48 hours. Other samples of linoleic acid prepared in our laboratory at various times and having iodine numbers ranging from 178.9 to 181.0 exhibited thiocyanogen values as follows (0.2 N reagent, 24 hours, *ca.* 125% excess): 94.3, 94.9, 95.3, 95.6, 95.7, 96.5. Similarly, crystallized linolelaidic acid (6), I. V. 178.8 (theory 181.1), gave thiocyanogen values of 94.8, 94.6, 94.6 under the accepted conditions. There can be little doubt, therefore, that the thiocyanogen number of linoleic acid is an empirical value depending upon the concentration and excess of reagent, the time of reaction, and which is appreciably higher than required by theory for half-saturation. Riemenschneider and Wheeler (24) have already pointed out the significant errors which appear in the calculation of the compositions of mixtures of fatty acids containing large quantities of linoleic acid when the theoretical constants are used. This inaccuracy is emphatically demonstrated by the recalculation of the compositions of the mixed fatty acids of corn, cottonseed, poppyseed, and sunflower oils on the basis of their iodine and thiocyanogen numbers as listed in the accompanying table and assuming the correctness of the accepted equations. The percentages of calculated linoleic acid drop to levels where their precipitable tetrabromides amount to appreciably more than 50%, which are obviously erroneous values in comparison with the theoretical and actual behavior of pure linoleic acid. Correct approximations of the true composition of fatty acid mixtures may be obtained only by shortening the reaction time with the 0.1 N reagent to a period of three hours at which the empirical thiocyanogen number of linoleic acid equals the theoretical, as proposed by Riemenschneider and Wheeler (24), or by retaining the accepted experimental conditions and modifying the equations as suggested in the discussion of our analytical data. The latter alternative appears preferable to us at present in that it will permit the inclusion of the analysis of drying oils in a uniform procedure. A preliminary investigation of the behavior of a linolenic acid having an iodine number of 270.5 (theory 273.7) showed its thiocyanogen value (0.2 N reagent, 250% excess, 24 hours) to be only 171 rather than the theoretical 182.5 required by two-thirds addition. Forty-eight hours' contact raised the value to 175.6. This increase with time is in harmony with the observations on linoleic acid and with the results obtained by Gay (25) on linseed oil, although Griffiths and Hilditch (26) found a slight drop after 24 hours, while Kaufmann and Keller (27) stated that there was little effect of either excess or time after 18 hours. It is apparent that the theoretical value for linolenic acid will probably be impossible to achieve and that here also an empirical constant will have to be substituted in the calculations, revision of which is now under investigation in our laboratory.

The remarkable correspodence between the constants obtained by Riemenschneider and Wheeler (24) for methyl oleate and by us for elaidic acid, in both cases the iodine numbers being very slightly higher and the thiocyanogen number a fraction of a unit lower than theoretical, may possibly suggest the need for revising the constants of oleic acid also, but here the discrepancies are so slight as to be within the limits of experimental error and to have the plausible explanation of contamination of the standards with traces of linoleic and saturated acids (28). In any case, such a revision would result in only an insignificant modification of the computations. However, the deviation of linoleic acid is real and unquestionably significant in the analysis of the semi-drying oils. It therefore appears desirable that in reporting thiocyanogen values, the exact experimental conditions should be indicated, and where the 0.2 N reagent is used the specified conditions the modified calculations proposed above should be used, while with the 0.1 N reagent, either the 3 hour reaction period and the theoretical equations, or the following equations based on an average value of 93 for the 24 hour period may be tentatively recommended for closer approximation to the true composition of mixed free fatty acids:

$$\%\ \text{Linoleic Acid} = 1.135\ (\text{I. V.} - \text{T. V.})$$

$$\%\ \text{Oleic Acid} = 2.287\ \text{T. V.} - 1.174\ \text{I. V.}$$

$$\%\ \text{Solid Acids} = 100 - \%\ (\text{L. A.} + \text{O. A.})$$

Summary

1. Only one form of linoleic acid, identical with the octadecadienoic acid regenerated from either *alpha* or *beta* tetrabromostearic acids, was found in a series of seed fats of widely different compositions.

2. The tetrabromide number was shown to be of questionable significance in that the precipitation of *alpha* tetrabromostearic acid is markedly affected by the component fatty acids of the oil.

3. Although the iodine numbers (Wijs) of pure linoleic and linolenic acids agreed closely with the theoretical, their thiocyanogen values determined according to the officially recommended methods were found to be 96.3 and 171 respectively, these values further varying with the conditions of the determination. The substitution of the empirical values for the theoretical constants in the accepted equations for the calculation of the concentration of unsaturated fatty acids was therefore suggested.

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The Use of Fatty Acid Ester Distillation Methods in Fat Analysis*

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THE first serious efforts to elaborate the composition of natural fats by distillation methods were made towards the end of the nineteenth century. They followed the laborious attempts of Heintz, Bömer, Klimont, Amberger and others to determine fat compositions by repeated crystallizations. As early as 1877 Krafft (1) attempted the distillation of castor oil but succeeded only in cracking the oil. In later studies between 1880 and 1903, Krafft and his students (2) used high vacuum techniques for the distillation of fatty acids and the derived aldehydes and esters. In the same year, 1903, both Krafft (2) and Kreis and Hafner (3) resorted to fractional distillation in order to obtain relatively pure palmitic and stearic acids for glyceride synthesis.

Haller and Youssoufian (4) described the fractional distillation of methyl esters of coconut oil fatty acids in 1906, but made no claim for quantitative results. These workers obtained methyl esters by methanolysis of the fat as described by Haller (5). Hilditch (6) has since pointed out that direct alcoholysis is an unfortunate method to use for the preparation of fatty acid esters in quantitative fat analysis because it offers no opportunity for an initial separation of the mixed fatty acids into saturated and unsaturated groups. The alkali required for the reaction inevitably causes some soap formation although soaps may be removed from the esters. Unchanged glycerides, however, cannot be removed by any known method.

Bull (7) contributed materially to the problem of fat analysis by ester distillation in 1906. His careful fractionation of the methyl esters of cod liver oil fatty acids was carried out with a heated column provided with total reflux.

Fractional distillation of coconut oil itself in high vacuum was undertaken in 1909 by Caldwell and Hurtley (8) with some degree of success. A similar attempt was described later by Bömer (9).

In 1911, both Smedley (10), in England, and Holland (11), in this country, recorded their observations on the composition of butter fat based on methyl ester fractionations. Smedley obtained evidence for the existence of lower members of the oleic acid series.

The percentage composition of fatty acids in coconut and palm kernel oils was published in 1913 and 1914 by Elsdon (12). The results were calculated from analytical data obtained for esters separated by fractional distillation.

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