A Study of the Tetrabromide Method of Estimating Linoleic Acid in Fatty Acid Mixtures

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IN previous reports (1, 2) we have studied the factors affecting the yields of polybromides from methyl arachidonate and of hexabromides from linolenic acid and have shown in both instances that the relationships are empirical. Interpolation curves based on our data were proposed for the estimation of methyl arachidonate in methyl ester mixtures and of linolenic acid in fatty acid mixtures. Analytical findings based on these curves are highly specific, unlike the results found with spectrophotometric and thiocyanometric technics. Both of the latter methods can also be used for the estimation of linoleic acid and have been widely used for that purpose, but, here again, the accuracy of the results depends on an assumption, namely, that linoleic acid (cis-cis 9,12-octadecadienoic acid) is the only diene unsaturation present in the mixture.

Only two specific tests for linoleic acid are known, i.e., the formation of two sativic acids upon cold permanganate oxidation and of an insoluble tetrabromostearic acid upon bromination in cold petroleum ether. The sativic acid reaction is not well adapted to a quantitative procedure. The formation of a tetrabromostearic acid is excellent from the standpoint of specificity, but its value is thrown in question by the early observation (3) that the yield of insoluble bromides is somewhat less than half of the theoretical yield, due to the simultaneous formation of soluble, non-crystalline bromides.

In 1938 Brown and Frankel (4) found the tetrabromide number (i.e., the per cent yield of petroleum ether insoluble bromides) of a specimen of a-linoleic acid (i.e., a specimen prepared by debromination of tetrabromides) to be 90.6 and proposed the following equation for estimating the linoleic acid content of fatty acid mixtures:

$$\text{linoleic acid, per cent} = \frac{\text{TbN} \times 100}{90.6}$$

where TbN is the tetrabromide number of the specimen in question and 90.6 is the TbN of a-linoleic acid. They stated further that "this method unavoidably will give low results if too small samples are used, or if the specimen contains relatively small amounts of linoleic acid, conditions which will exaggerate the unavoidable error of solubility in the wash liquid." Their suggestion of partly correcting this error by using petroleum ether which has been saturated with tetrabromides has not been further explored in our work for several reasons, but mainly because of the probable heterogeneous nature of the precipitated bromides.

Since 1938 there have been several reports dealing with various aspects of the bromide method for linoleic acid estimation. Special mention should be made of a study of the linoleic acid of seed oils by Hilditch and Jasperson (5), who brominated the mixed fatty acids of several of these oils and, in addition, specimens of a- and β-linoleic acids. They concluded, in part on the basis of tetrabromide yields, that the natural and a-linoleic acids were identical. Kass, Lundberg, and Burr (6) determined the linoleic acid contents of several oils both thiocyanometrically and from the tetrabromide yields and concluded that only one form of the acid was present in corn, cottonseed, peanut, poppyseed, sunflower, and olive oils. On the other hand, Frankel, Stoneburner, and Brown (7) noted that a linoleic acid concentrate of olive oil acids which had been isolated by low temperature crystallization gave a lower tetrabromide number than normal and concluded that more than one octadecadienoic acid was present in this oil. Kass et al. (loc. cit.) concluded that, as an analytical tool, the tetrabromide number is of questionable significance in that the precipitation of a-tetrabromostearic acid is markedly affected by the component fatty acids of the oil.

In 1941 Matthews, Brode, and Brown (8) by repeated recrystallization of a-linoleic acid isolated a product of tetrabromide number 102.9 and, on the basis of their results, proposed that the a-linoleic acid, as they had prepared it, is a mixture of isomers of which linoleic acid is the principal component. Their final crystal fraction was doubtless the purest specimen of linoleic acid prepared up to that time. The use of the factor 102.9 in place of 90.6 in the above equation naturally follows but does not correct for the inherent solubility errors, noted previously.

In the present work we had hoped to be able to report finding the comparatively simple relationships between amounts of linoleic acid brominated and tetrabromide yields such as we have found in studying similar relationships with methyl arachidonate and linolenic acid. With the former, the polybromide yields were relatively unaffected by the presence of other methyl esters, and with linolenic acid only a comparatively slight solubilizing effect of other fatty acids was noted. In the case of linoleic acid, on the other hand, we have found that the yield of tetrabromides is greatly affected by the presence of other acids so that, at first, no simple curve seemed possible to describe the empirical relationships which were found. The yield was even affected to a certain extent by variation in the brand of petroleum ether employed in the determination.

It finally occurred to us that the many variables affecting the tetrabromide yield can be accounted for in large part by brominating 1.0-g. specimens of linoleic acid and of linoleic-oleic acid mixtures and plotting the yield of bromides against the per cent of linoleic acid in the specimen brominated. The curve based on these data can be used as an interpolation method for determining the linoleic acid content of a fatty acid mixture, provided no appreciable amounts of linolenic and other polyethenoic acids are present in the mixture. Data are presented in Table III from which it is possible to draw curves based on 2.0, 1.0, and 0.5 g. samples of fatty acids.

Linoleic acid alone (Table II) shows a wide variation in tetrabromide number in that specimens of the pure acid varying from 2.0 g. to 10 mg. gave tetrabromide numbers of 103.7 to zero.

Linoleic Acid Brominated g.	Observed Tetrabromide Number					
	Mallinckrodt 30-60°	Merck 30-60°	General Chemical 20-40°	General Chemical 35-60°	Baker 30-60°	Skellysolve B
2.0	90.3*	90.0*	90.1*	84.3*	90.3*	....
1.0	84.7	85.7	88.2	82.4	84.2	83.2
0.5	69.6	74.6	78.3	83.1**	70.3	82.4

* Average of two determinations.
** Probably too high.

A large series of linoleic acid concentrates from corn oil, safflower oil, and butter fat were subjected to the proposed procedure of analysis and the results compared with linoleic acid contents as calculated from the iodine number. In a majority of the analyses the agreement is excellent. When results by the tetrabromide method are found to be lower than those by the iodine number, it is interpreted that the difference is due to the presence of isomeric diene acids.

Experimental

The Effect of Petroleum Ether Brand on the Tetrabromide Number of Linoleic Acid. Petroleum ether of 30-60° C. boiling range is a complex mixture of hydrocarbons. Different brands of this solvent represent products of different origin and composition. An immediate problem in studying tetrabromide yields is to select and always use an ether of constant composition. It is even important not to use a specimen of recovered solvent because a variable amount of low boiling material will unavoidably have been lost.

In investigating comparative tetrabromide yields in different brands of petroleum ether, we have used a specimen of linoleic acid prepared in this laboratory by Mrs. Betty M. Orians by low temperature crystallization of the fatty acids of corn oil. Its content of octadecadienoic acid calculated from the iodine number as a binary mixture of linoleic and oleic acids, was 94%. From our one-gram curve mentioned later, its linoleic acid content was found to be 87.5%. Amounts of this acid very close to 2.0, 1.0, and 0.5 g. were brominated in five different brands of reagent grade petroleum ether and in Skellysolve B.

The tetrabromide yields were determined by the use of the same method as we have previously described for methyl arachidonate and linolenic acid, with the exception, of course, of substituting petroleum ether for ether. Briefly, the brominations were made in tared 50-cc. centrifuge tubes in 35 cc. of cold (0-3°) solvent. After centrifugation, three thorough washings in 35-cc. portions of cold solvent were made, and the tube and residual bromides left overnight at about 60° C. for solvent to evaporate. In order to compare results with the five petroleum ethers, the tetrabromide yields were then corrected to 2.0, 1.0, and 0.5 g. respectively and expressed in Table I as the tetrabromide numbers.

Two points stand out in Table I, namely, the fair agreement between the values obtained with Mal-

linckrodt's, Baker's, and Merck's ethers, all of which are 30-60° fractions, and the tendency of the results with both General Chemical's fractions and with Skellysolve B to show less decrease in tetrabromide number with decrease in weight of sample brominated. None of the last three solvents has a comparable boiling range to the first three. Skellysolve B, for example, is a much more closely cut fraction (57-63°). The use of General Chemical's 35-60° ether and of Skellysolve B should be further explored. In the work which follows Mallinckrodt's petroleum ether (30-60°) was used exclusively.

The Tetrabromide Number of Linoleic Acid in Relation to the Amount of Acid Brominated. In Table II are shown the tetrabromide numbers of linoleic acid over a wide weight range of samples brominated. The specimen of linoleic acid used in this and later experiments was prepared in this laboratory by Dr. F. J. Baur (9), who repeatedly recrystallized a-linoleic acid by a simplified modification of the low temperature crystallization procedure described by Matthews, Brode, and Brown (8). The specimen, from which presumably most, if not all, of the isomeric diene acids had been removed, had an iodine number of 180.8 and melted sharply at —5.0°. Baur reported its tetrabromide number to be 106.0, but, upon redetermination of this value at the 2.0-g. level, we found it to be 103.7. From our 1.0-g. curve, noted later, Matthew's acid of tetrabromide number 102.9 was found to be 99.8% pure. It should be further noted that Matthew's acid, as analyzed spectrophotometrically by Brode, Patterson, Brown, and Frankel (10), contained 1.2% of conjugated unsaturation, mainly of the diene type.

The values in Table II were found by our empirical method of determining bromide yields.

The data in Table II require no discussion, except to point out the anomalous rise in tetrabromide number at the 0.05-g. level. Because the values in Table II are of little analytical use, this finding was not

Linoleic Acid g.	Tetrabromides g.	Tetrabromide Number
1.9963	2.0709	103.7
1.0032	0.9882	98.5
1.0002	0.9857	98.6
0.4996	0.4369	87.5
0.4994	0.4355	87.2
0.3509	0.2640	75.3
0.3497	0.2613	74.7
0.2498	0.1636	65.4
0.2498	0.1657	66.3
0.2498	0.1628	65.1
0.0999	0.0653	65.3
0.0999	0.0663	66.3
0.0999	0.0672	67.2
0.0500	0.0359	71.8
0.0500	0.0351	70.2
0.0500	0.0367	73.4
0.0300	0.0169	56.3
0.0300	0.0188	62.7
0.0200	0.0093	46.5
0.0200	0.0096	48.0
0.0200	0.0096	48.0
0.0150	0.0032	21.0
0.0150	0.0036	24.0
0.0100	Trace	......
0.0100	Trace	......

further investigated. As little as 10 mg. of linoleic acid is detectable by the method employed while 15 mg. of the acid, if no other acids are present, gives a tetrabromide number of more than 20. These findings should be compared to those listed in Table III in which it is shown that in a 1.0-gram mixture of acids nearly 200 mg. (20%) of linoleic acid must be present to be detectable by the test.

Tetrabromide Yields From Linoleic Acid and Linoleic-Oleic Aicd Mixtures When Two-, One-, and One-Half-Gram Specimens Are Brominated. After several series of exploratory experiments, the bromide yield

TABLE III

Tetrabromide Yields From Linoleic Acid and Linoleic-Oleic Acid Mixtures, With Data Corrected to Two-, One-, and One-Half-Gram Specimens

Composition of Mixture Brominated			Linoleic Acid Per Cent	Yield Tetrabromide g.	Yield Corrected g.*
Linoleic g.	Oleic g.	Total g.			
Two-Gram Samples					
1.9963	0.0	1.9963	100.0†	2.0709	2.0750
1.8793	0.1206	1.9999	94.0	1.8237	1.8237
1.0016	1.0000	2.0016	49.9	0.7498	0.7490
0.5015	1.5000	2.0015	25.1	0.2129	0.2128
0.3497	1.6500	1.9997	17.5	0.0280	0.0280
One-Gram Samples					
1.0032	0.0	1.0032	100.0†	0.9882	0.9850
1.0002	0.0	1.0002	100.0†	0.9857	0.9855
0.7643	0.2352	0.9995	76.5	0.7100	0.7104
0.7571	0.2444	1.0015	75.6	0.7055	0.7044
0.6037	0.4145	1.0182	59.3	0.5408	0.5311
0.4993	0.4997	0.9990	50.1	0.3901	0.3913‡
0.4934	0.4990	0.9924	49.7	0.4255	0.4288
0.4591	0.5356	0.9947	46.2	0.3357	0.3375
0.4542	0.5456	0.9998	45.4	0.3571	0.3572‡
0.4492	0.5512	1.0004	44.9	0.3808	0.3807
0.4074	0.5921	0.9995	40.8	0.3407	0.3409
0.4009	0.6203	1.0212	39.3	0.2992	0.2930‡
0.3537	0.6424	0.9961	35.5	0.2708	0.2719
0.3147	0.6836	0.9973	31.5	0.2411	0.2415
0.3056	0.6964	1.0020	30.5	0.2164	0.2160
0.2567	0.7690	1.0257	23.9	0.1290	0.1258
0.2550	0.7456	1.0006	25.5	0.1578	0.1577
0.2318	0.7790	1.0108	22.9	0.0890	0.0880
0.2054	0.8612	1.0666	19.3	0.0216	0.0204
0.2011	0.8000	1.0011	20.0	0.0155	0.0155
0.1993	0.8544	1.0537	18.9	0.0067	0.0065
One-Half-Gram Samples					
0.4996	0.0	0.4996	100.0†	0.4369	0.4373
0.4994	0.0	0.4994	100.0†	0.4355	0.4360
0.2466	0.2542	0.5008	49.2	0.1344	0.1342
0.1438	0.3563	0.5001	28.8	0.0334	0.0334
0.0998	0.4008	0.5006	19.9	Trace	Trace

* These yields are corrected to exactly 2.0-, 1.0-, and 0.5-g. samples brominated.
† However, Matthew's acid, prepared by a similar but longer procedure, contained 1.2 per cent of conjugated unsaturation (10).
‡ These values are seriously "off the curve."

was found to be greatly affected both by the amount of linoleic acid present in a mixture and by the amount of other acids present. Thus, there seemed at first to be almost an infinite number of variables. It finally occurred to us that most of these variables can be taken into account by the use of a curve in which is plotted the bromide yields from 1.0-g. specimens of linoleic acid and of a range of linoleic-oleic acid mixtures against the per cents of linoleic acid in these specimens. The linoleic acid content of a given mixture can then be ascertained by interpolation from this curve provided a 1.0-g. sample of unknown is brominated and the weight of tetrabromides found. Actually, in practice, it is only necessary to brominate an approximately 1.0-g. sample, and to correct the yield of bromides to the amount which will be obtained from 1.0 g. By plotting the tetrabromide yield, instead of the tetrabromide num-

ber, it is equally possible to prepare curves from data based on 2.0- and 0.5-g. samples, and to read by interpolation the per cent linoleic acid in a mixture if its tetrabromide yield has been determined.

In Table III data are recorded from which three such curves can be drawn. The linoleic acid employed in securing these data was again Baur's preparation. The oleic acid was prepared from olive oil, the C_{18} fraction from this was repeatedly crystallized to remove the last traces of linoleic acid and most of the stearic acid. Its iodine number, 88.2, is indicative of a content of about 2% of stearic acid.

Curves were drawn from the data in Table III and from them simplified data for the construction of curves for analytical use were read for five even percentage levels, as shown in Table IV.

TABLE IV

Simplified Data for Drawing Interpolation Curves for the Estimation of Linoleic Acid in Fatty Acid Mixtures From the Tetrabromide Yields

Linoleic Acid Per Cent	Tetrabromide Yield		
	2.0 g.	1.0 g.	0.5 g.
100	2.075	0.985	0.436
75	1.420	0.702	0.286
50	0.748	0.437	0.139
25	0.213	0.152	0.018
20	0.035	0.028	Trace

The limit of sensitivity of the procedure for the detection of linoleic acid in fatty acid mixtures is shown to be between 15 and 20%. This explains the previous failure to find linoleic acid in butter fat (11, 12) which usually contains about 5% of total octadecadienoic acids and probably also the failure to identify the acid in many other fats and oils which contain it in relatively small amounts. For example, linoleic acid is present in beef fat but is not detectable by the bromination test until a concentrate has been prepared by crystallization (13).

In the course of an extended crystallization study of the fatty acids of corn oil, Mrs. B. M. Orians, in this laboratory, isolated a series of linoleic acid concentrates with iodine numbers ranging from 180.7 to 111.4. In Table V we have compared the linoleic acid contents of these mixtures as evaluated from the curves mentioned above with the values as calculated from the iodine numbers, assuming only oleic and linoleic acids to be present in each mixture. This assumption is reasonably valid because by the crystallization procedures which were used it is easy to remove all except the last traces of saturated acids. Three linoleic acid concentrates from safflower oil and butter fat have also been assayed in Table V.

Of the 14 corn oil linoleic acid concentrates in Table V, Specimens 2 and 9-14, inclusive, show excellent agreement by the two methods of estimation. Specimens 1, 3, 5, and 8 gave appreciably lower linoleic acid contents by the tetrabromide method than from the iodine number calculation. This finding we believe to be due to the presence of appreciable amounts of isomeric octadecadienoic acids in these mixtures. This is also the case with the two concentrates from butter fat. This is the first authentic finding of linoleic acid in butterfat, about three-fourths of Specimen 16 and two-thirds of Specimen 17 consisting apparently of ordinary linoleic acid. This finding will be reported elsewhere in more detail (14).

TABLE V

The Linoleic Acid Contents of a Series of Concentrates of This Acid as Evaluated From the Iodine Numbers and From the Tetrabromide Yields

Spec. No.	Source	Iodine No.	Spec. Brominated g.	Tetrabromides (Corr.) g.	Linoleic Acid Per Cent From Iodine No.	T. B. Yield
1	Corn oil	179.6	2.0052	1.8511	98	93.7
			1.0004	0.8743	98	89.5
			0.4986	0.3805	98	91.5
2	Corn oil	179.4*	1.9972	1.9886	98	97.2
			0.9991	0.9564	98	96.8
			0.4987	0.4327	98	99.4
3	Corn oil	159.4	2.0014	1.1858	76	70.6
			0.9995	0.5420	76	61.0
			0.4999	0.2137	76	63.5
4	Corn oil	114.5	2.0051	0.5363	27	38.5
			1.0008	0.2641	27	33.7
			0.5025	0.1216	27	44.6
5	Corn oil	180.7	1.9997	1.8726	100	94.2
6	Corn oil	176.9	2.0039	1.9157	95	95.4
7	Corn oil	176.3	2.0032	1.9425	94	97.0
8	Corn oil	173.5	1.9941	1.5216	91	83.5
9	Corn oil	172.0	2.0031	1.7127	90	89.7
10	Corn oil	170.8	1.9956	1.8109	89	92.5
11	Corn oil	167.9	1.0036	0.7854	85	83.6
12	Corn oil	142.7	0.9987	0.5004	58	57.1
13	Corn oil	147.0	2.0087	1.0300	62	62.7
14	Corn oil	111.7	0.9996	0.1653	24	25.8
15	Safflower	148.4	0.9969	0.6551	62	72.4
16	Butter fat	153.2	2.0026	0.8294	69	54.2
17	Butter fat	165.0	1.0008	0.5206	82	58.0

* Specimen 1 crystallized four times.

Specimens 4 and 7 show higher results by the tetrabromide method. This can be explained either by assuming the presence of saturated acids in the mixture or by postulating the presence of another linoleic acid of higher tetrabromide yield than we have found for the allegedly pure specimen of Baur (and of Matthews). Specimen 15 from safflower oil would give the results found if it were composed of approximately 72.4 per cent of linoleic acid, 19.4 per cent of oleic acid, and 8.2 per cent of saturated acids.

Specimens 1-4 in Table V were assayed at all three bromination levels. Results agree well for Specimens 1 and 2, but rather poorly for 3 and 4.

Discussion of Results

The validity of the interpolation curves proposed in Table IV is based on the assumption first that the linoleic acid specimen employed in securing the data is entirely the cis-cis 9,12-octadecadienoic acid which we understand to be linoleic acid. The cis-trans and trans-cis modifications are known to yield petroleum ether-soluble, non-crystalline oils. Isomeric octadeca-dienoic acids have been previously shown to be present in varying amounts in α-linoleic acid (15). The data in Table V are indicative of the presence of isomeric diene acids in several of the linoleic acid concentrates from corn oil and in the two preparations from butter fat. The validity of the curves also depends on the assumption that solubilities of the tetrabromides in petroleum ether when oleic acid is in the bromination mixture are the same as solubilities in the presence of other fatty acids, as for example, hexadecenoic acid and the saturated acids. This objection cannot be answered, because the solubilizing effects of dibromo-stearic acid, of liquid tetrabromostearic acids, and of saturated acids seem likely

to be different. In spite of these objections we feel that the interpolation curves described above constitute a technic of fair accuracy and, so far as the results go, of high specificity in the examination of fatty acid mixtures. Results by this method will be made of more value when used along with iodometric, thiocyanometric, and spectrophotometric methods. The method is of little quantitative value when the mixture of acids under examination contains appreciable amounts of linolenic and other highly unsaturated acids.

One of the most serious limitations of the method as a precise quantitative technic is the effect of composition of the petroleum ether on the tetrabromide yield. However, as seen in Table I, the results in three 30-60° ethers were in fair agreement. To overcome this objection to the method we are undertaking to re-evaluate the main points on our curves by determining the tetrabromide yields in one of the pure normal hydrocarbons which have recently become available for experimental use, as for example, n-pentane or n-hexane.

Summary

1. When linoleic acid is brominated in cold petroleum ether, the yield of insoluble tetrabromides is empirical; it is affected by the composition of the petroleum ether and by the amounts of linoleic acid and of other fatty acids which may be in a given mixture.

2. The tetrabromide number of pure linoleic acid ranges from 103.7 when a 2-gram sample is brominated to practically zero with 10 mg. of the acid.

3. Data are presented describing the tetrabromide yields of linoleic acid alone and in various mixtures with oleic acid, when brominated at the 2.0-, 1.0-, and 0.5-g. levels of sample. From these data curves can be drawn by which it is possible to ascertain by interpolation the per cent of linoleic acid in a mixture.

4. Seventeen concentrates of linoleic acid from corn oil, safflower oil, and butter fat have been assayed from the curves, and the results compared with values calculated from the iodine number.

5. Several linoleic acid concentrates from corn oil were shown to contain appreciable amounts of isomeric dienoic acids.

6. Examination of two linoleic acid concentrates from butter fat shows for the first time the indubitable presence of linoleic acid in this fat.

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