FACTORS INVOLVED IN THE SPECTROPHOTOMETRIC ANALYSIS OF FATS

by

GEORGE CHRISTIAN POTTER

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INTRODUCTION

The advent of spectrophotometers such as the Beckmann model DUV, which are simple to operate and sturdy, did much to allow analysts to determine percentage composition of the fatty acids in fats and oils. The older methods (3) of chemical analysis were tedious and inaccurate. The use of iodine and polybromide numbers led to large errors in analysis. The thiocyanogen number procedure is quite accurate when relatively large amounts of the individual fatty acids are present but is of doubtful value for minor constituents.

The analysis of fatty acids by means of the absorption spectra has been the object of a great deal of work. Each fatty acid shows a certain characteristic maxima in the ultraviolet region. Part of this absorption can be attributed to the carboxyl group. However, the absorption peculiar to this chromophoric group would be of little use as compounds other than straight chain fatty acids, containing the carboxyl function, would exhibit this characteristic spectral pattern.

A system of conjugated bonds will exhibit characteristic absorption patterns in the ultraviolet region extending from 2000 Å to 3200 Å. From this it can be seen that ultraviolet analysis does not identify molecules as a whole but only the various chromophores which show absorption in the ultraviolet region.

When a double bond is introduced into an aliphatic acid

a characteristic shift in maxima occurs. Oleic acid having a single double bond will have stronger absorption in the region above 2000 Å than stearic acid, due to the ethylenic bond. Correspondingly greater absorption occurs with the addition of more ethylenic bonds in the aliphatic chain.

With the presence of more than one double bond, isomers can be formed in which the ethylenic bonds are in the isolated, cumulative, and conjugated positions. The absorption will become greater in the order conjugated>cumulative>isolated positions.

Dann and Moore (5) and Moore (10, 11) noted that prolonged alkaline saponification of unsaturated fatty acids produced marked changes in the ultraviolet spectra of fatty acids. When linseed oil was refluxed in alcoholic potash for more than 12 hours the maxima at 2300 Å and 2700 Å increased greatly. Some workers thought that the increase was due to cyclic products formed. However, Moore (11) and Kass and Burr (8) proved that the increased absorption was due to conjugation of the ethylenic bonds.

Kass and Burr (8) found that the use of high boiling point solvents such as anhydrous butanol and ethylene glycol would reduce the reaction time greatly. By catalytic partial hydrogenation of tung oil, Moore (11) established that the maxima at 2700 Å was reduced and that at 2300 Å it was increased. This substantiated the fact that the absorption at 2700 Å was due to triene conjugation, and that at 2300 Å due to

diene conjugation.

Mitchell et al. (9) standardized the time of alkaline conjugation and developed an empirical method for the analysis of fats. The specific absorption coefficients of pure fatty acids under the conditions given were determined and substituted into graphically derived formulas to give the percentage composition of various naturally occurring unsaturated fatty acids.

Fat extracts obtained from natural sources are very difficult to purify. Such fat-soluble substances as sterols, carotenoids, vitamin A and the other fat-soluble vitamins may also be present in varying amounts, depending on the source of the material. Such compounds contain chromophores which show absorption in the ultraviolet region and their presence may lead to erroneous results in fat analyses.

In the present study the effect of these various factors was evaluated. Fat extracts were purified by distillation, chromatography, fractional crystallization, and the formation of various derivatives that could be separated in a pure state. The natural materials present in impure fat extracts were then added to the pure fats and the results of such additions on the spectrophotometric method noted.

EXPERIMENTAL

Extraction and Purification of Fats

The Bloor (2) method of extracting the tissue was used. It consisted of treatment of fresh tissue slices with acetone, alcohol, and Skellysolve F. The fresh weighed tissue was minced in a Waring Blendor with acetone as a solvent. The minced material was then refluxed for two hours with acetone and filtered. The refluxing was repeated using fresh solvent. The tissue was treated similarly with alcohol and Skellysolve F. The acetone and Skellysolve F extracts were combined, washed at least three times with water, and dried over sodium sulfate. The alcohol fraction was dried over sodium sulfate and the alcohol removed under vacuum. The alcohol extract contained most of the phospholipids present in the original material. The Skellysolve-acetone extract was then added to the weighed flask which contained the alcohol extract. The solvent was then removed under vacuum and the total weight of fat determined.

Alkaline saponification using 30 per cent aqueous potassium hydroxide and subsequent extraction of the fatty acids was also used to some extent. The weighed tissue was placed in a beaker and covered with 30 per cent aqueous potassium hydroxide. The beaker was then heated on a steam plate until complete saponification occurred as indicated by the absence

of tissue. The alkaline extract was cooled, diluted with an equal volume of alcohol and this soap solution extracted three times by shaking in a separatory funnel with Skellysolve F. Most of the nonsaponifiable material was removed in this way. The soap solution was then carefully acidified using either hydrochloric acid (concentrated) or glacial acetic acid. The free fatty acids were then extracted by shaking in a separatory funnel with Skellysolve F. All Skellysolve extracts were combined and washed at least three times with water. The wash water was equivalent in volume to the combined extracts. The use of a bubbler tube for the water washing eliminated the formation of emulsions and subsequent loss of fatty acids. After washing the Skellysolve F fraction was dried over sodium sulfate and the solvent removed under vacuum.

A distinction must be made between purification and fractionation of fats. By purification is meant the removal of nonfatty materials that are soluble in the solvents used. Fractionation consists of separating mixed fats into their various components.

The Bloor extraction of fats results in extracts containing not only triglycerides and free fatty acids but also the nonsaponifiable portions of fats. The nonsaponifiable portion can be removed by alkaline saponification and extraction of the soaps to remove the nonsaponifiable portion. Acidification then results in quite pure fat extracts.

Vacuum distillation of esterified fatty acids can be used

as a means of purifying fat extracts. The esters are thus removed from sterols and most of the components of the non-saponifiable portion.

Preparation of Pure Fatty Acids

Pure linoleic, linolenic, and arachidonic acids were used as reference standards in the spectrophotometric analysis of fats. These acids were prepared by formation of bromo derivatives from the appropriate sources and recrystallization of the bromo derivatives until a constant melting point was reached.

Preparation of Methyl Arachidonate. Two and one-half kilograms of beef adrenal phospholipid[1] were saponified for three hours using five liters of four per cent alcoholic potassium hydroxide. The soaps were then cooled and acidified with hydrochloric acid and extracted with two liters of Skellysolve F using 200 to 300 ml portions at a time. The combined Skellysolve extracts were washed three times with an equal volume of water with the use of a bubbler tube to prevent emulsions. The washed extract was dried over night with sodium sulfate, the Skellysolve removed under vacuum, and the fatty acids taken up in acetone. The volume of acetone was adjusted until a 10 per cent solution resulted. The acetone solution was chilled over night at -18°C. and the solid fraction filtered

[1]Furnished by Parke-Davis Company, Detroit, Michigan.

off. The liquid portion was then fractionally crystallized by cooling in a special cold box-filtration system. The solid fractions were removed after every 10°C. drop in temperature until -60°C. was reached. The liquid portion at this temperature was considered to be arachidonic acid. Fractional distillation under 50 μ mercury failed to produce arachidonic acid of more than 60 per cent purity as shown by ultraviolet absorption data (Table 1).

Table 1. Fractional distillation of impure arachidonic acid.

Fraction II	6	7	8
I_2 value	176.2	193.9	207.2
Per cent arachidonic acid	46.1	47.2	61.7

The impure arachidonic acid fractions were then combined and dissolved in ether of sufficient volume to result in a 1:10 ratio by weight of fat extract to ether. This solution was then cooled to 0°C. and bromine slowly added with constant stirring. The bromine must be added slowly at first to prevent excessive local heating. When a slight excess of bromine was present the reaction mixture was removed from the cooling bath and kept over night at -18°C.

The highly colored ether solution was then decanted from the solid bromides and fresh ether added with vigorous shaking.

After eight washings with fresh ether the solid bromides were filtered by suction and dried in a vacuum desiccator over night. If all free bromine is not washed out, discoloration of the solid bromides will occur during drying. The melting point of the solid octabromide was taken in a capillary tube (M.P. 227-228°C., corr.; theoretical M.P. 227-228°C.) The yield of octabromide was 35 g.

The dried octabromide was suspended in 350 ml of methyl alcohol to which one drop of hydrochloric acid had been added. Powdered zinc was added and the mixture refluxed; from time to time fresh portions of zinc were added. After the reaction had ceased, as shown by no reaction when fresh zinc was added, the alcohol solution was cooled and filtered. The alcohol fraction was then extracted with Skellysolve F in a separatory funnel. The Skellysolve solution was washed three times with an equal portion of three per cent sodium carbonate and three times with an equal portion of water. The Skellysolve solution was dried over sodium sulfate and freed from solvent under vacuum. The residual oil weighed six grams; it was light yellow in color and had an offensive odor.

This yellow oil had an iodine value of 307.2. The theoretical iodine value for methyl arachidonate is 318.48, thus indicating that the preparation had a purity of 96.5 per cent. The colored acid was distilled in a pot-type molecular still under a pressure of 0.01 μ mercury. Three fractions were taken and the iodine value determined. These results are

tabulated in Table 2. Fraction No. 2 weighed two grams and was a water-clear odorless liquid.

Elementary analysis of the ethyl ester gave the following results:

Calculated for $C_{22}H_{36}O_2$: C, 79.46; H, 10.91; found C, 79.44; H, 10.81.

Table 2. Iodine values of distilled methyl arachidonate.

Fraction:	1	2	3
Iodine value	304.80	316.58	Residual gum

EXPERIMENTAL TECHNIQUES

Iodine Values

Approximately 0.1 g of fat was weighed into a tared glass-stoppered iodine flask. The fat was dissolved in five ml of chloroform and 15 ml of Wijs solution (13) added. The flasks were stoppered and placed in the dark for one hour. At this time 10 ml of 15 per cent potassium iodide were added with constant swirling to assure complete mixing. The stopper and neck of the flask were washed with 10 ml of distilled water and the solution titrated with N/10 sodium thiosulfate solution to a faint yellow color. At this stage a few drops of starch solution were added and the flask shaken well to free all

available iodine and titrated to a colorless end point. Two blank determinations were run along with the unknown.

Wijs Solution. Thirteen g of iodine were dissolved in one liter of glacial acetic acid. The solution was heated on a steam cone until all the iodine was dissolved; after cooling, chlorine gas was bubbled through the solution until it turned light brown. The titre of the solution should be twice that of the original iodine solution as compared with N/10 sodium thiosulfate solution.

Potassium Iodide Solution. Fifteen g of potassium iodide were dissolved in 85 ml of distilled water.

N/10 Sodium Thiosulfate Solution. Twenty-four and eight-tenths g of sodium thiosulfate were dissolved in distilled water and diluted to one liter and standardized by the following procedure.

Ten ml of N/10 potassium dichromate were measured into a 250 ml Erlenmeyer flask and five ml of concentrated hydrochloric acid added. Ten ml of 15 per cent potassium iodide solution were added and the mixture titrated at once to a dull green color. Several drops of starch solution were added and the titration continued to a brilliant green end point. The end point is rather sudden and care should be taken to avoid over-running the end point.

The formula used in calculating iodine values was:

$$\text{Normality factor} = \frac{126.9 \times \text{normality of dichromate}}{\text{ml thiosulfate used}}$$

$$\text{Iodine value} = \frac{\text{normality factor (blank titration - sample titration)}}{\text{sample weight}}$$

Acid Value

A sample of 0.5 g of fat was accurately weighed in a 125 ml Erlenmeyer flask, five ml of alcohol neutralized to a phenolphthalein end point was added and the solution heated to boiling. The sample was titrated with N/100 potassium hydroxide using phenolphthalein as an indicator. The end point was considered to be reached when a pink color was retained for one minute. The acid was recorded as percentage of oleic acid.

$$\text{Per cent oleic} = \frac{\text{ml KOH} \times \text{N KOH} \times .282 \times 100}{\text{sample weight}}$$

Spectrophotometric Analysis

Alkaline conjugation of the fats (1, 3, 9) was performed under controlled conditions of temperature and time. The temperature was kept at 180°C. ± 0.2° by means of a mercury thermostat and electronically controlled relay. The samples were weighed carefully into small glass cups of approximately one ml capacity and of such a diameter as to fall into a 25 x 275 mm pyrex test tube. The optimum weight of samples

was 0.1 g ± 0.01.

Ethylene glycol containing 12.5 per cent potassium hydroxide was used as the isomerizing solvent. Ten ml of the solvent were pipetted into the 25 x 275 mm test tubes and heated to 180°C. in the oil bath under a blanket of nitrogen. When the solvent reached 180°C. the small cups containing the sample were dropped in and the tube rotated to mix the sample and solvent. A 30-minute reaction period was used on all samples. At the end of 30 minutes the tubes were removed, the oil wiped off with a towel, and cooled in a beaker of cold water to stop the reaction.

After treatment the samples were quantitatively transferred to a 100 ml volumetric flask and made up to volume using redistilled 95 per cent ethanol. The diluted samples were kept over night at refrigerator temperature to precipitate any silica removed from the test tubes by the alkaline reagent.

The samples were then allowed to come to room temperature and filtered. Appropriate dilutions were then made to bring the optical density of the solution to values between 0.500 and 0.800. A 0.1 g sample of the original material was diluted to 100 ml and its optical density determined without treatment with alkali. All readings were made on a Beckmann Model DUV spectrophotometer using one cm silica cells.

The following formulas were used in calculating results. The formulas are adjusted to a dilution of 10 ml, from the

100 ml, made up to 250 ml. The primed k values indicate conjugated material and non-primed k values indicate non-conjugated material. The double primes indicate corrected values.

D = optical density

W = weight of sample

$$k_2 = \frac{D_{232} \times 10}{4 \times W} - 0.07$$

$$k_3 = \frac{2.8 \times 10}{4 \times W} \left[D_{268} - 1/2 \left(D_{262} + D_{274}\right)\right]$$

$$k_4 = \frac{2.5 \times 10}{4 \times W} \left[D_{316} - 1/2 \left(D_{310} + D_{322}\right)\right]$$

$$k_2' = \frac{D_{232} \times 10}{4 \times W} + 0.04$$

$$k_3' = \frac{4.1 \times 10}{4 \times W} \left[D_{268} - 1/2 \left(D_{274} + D_{262}\right)\right]$$

$$k_4' = \frac{2.5 \times 10}{4 \times W} \left[D_{316} - 1/2 \left(D_{310} - D_{322}\right)\right]$$

$$k_2'' = k_2' - k_2$$

$$k_3'' = k_3' - k_3$$

$$k_4'' = k_4' - k_4$$

Per cent linoleic acid = $1.125 \times k_2'' - 1.27 \times k_3'' + 0.04\ k_4''$

Per cent linolenic acid = $1.87 \times k_3'' - 4.43\ k_4''$

Per cent arachidonic acid = $4.43 \times k_4''$

Per cent oleic acid =

$$\frac{f_2 \times 100 - (181.5 \times \% \text{ linoleic acid} + 273.5 \times \% \text{ linolenic acid} + 333.5 \times \% \text{ arachidonic acid})}{90}$$

Per cent of saturate = 100 - (% oleic acid + % linoleic acid + % linolenic acid + % arachidonic acid)

RESULTS

Effect of Impurities Upon Analysis

All fats from natural sources contain impurities that may have the property of absorbing in the ultraviolet spectrum. Carotenoids, sterols, glycerol, phosphoric acid, choline, and possibly blood pigment fragments would all be present in varying amounts depending upon the source of the sample.

To determine the effect of such extraneous materials, a series of analyses were performed in which all suspected impurities were added in known amounts. Carotene, vitamin A and nonsaponifiable material were found to have an appreciable effect upon the analyses.

The method used to determine the effect of nonsaponifiable material upon the analyses is representative of the technique used in all analyses and will be explained in some detail.

Distilled methyl esters from beef liver extract were used as the source of fatty acids. One set of samples was isomerized and analyzed by means of the Beckmann spectrophotometer. Another set of samples was prepared using the purified esters with the addition of varying amounts of nonsaponifiable material. The results are tabulated in Table 3. The beef liver

Table 3. Spectrophotometric analysis of methyl esters plus nonsaponifiable materials.

Per cent nonsaponifiable material	Per cent linoleic	Per cent linolenic	Per cent arachidonic
0	16.51	0	20.70
19.2	16.78	0	22.80
39.0	16.54	0	23.00
59.5	16.00	0	18.99
100.0	7.26	0	2.56

was extracted by the Bloor solvent method using acetone, alcohol and Skellysolve F in the order named as solvents. The nonsaponifiable material was extracted from beef liver after saponification in 20 per cent potassium hydroxide. The esters were distilled under a pressure of 100 microns in a still modeled after that of Diemair and Schmidt (6). After several distillations the esters were white to pale yellow in color and were assumed to be quite free of nonsaponifiable material.

A definite change in the percentage of linoleic acid was shown by the increase between zero per cent and 19.2 per cent nonsaponifiable material. Arachidonic acid showed a steady increase up to 39 per cent nonsaponifiable material and then fell off to below the original percentage at zero per cent nonsaponifiable material.

Vitamin A is also found in liver extracts and a similar procedure was followed to determine the effect of vitamin A on spectrophotometric analyses. The vitamin A[2] which was used contained 200,000 U.S.P. units per gram of oil. If these units are calculated to a weight basis, one gram of the extract contained $\frac{200,000}{4,500,000}$ grams of pure vitamin A (12). This is equal to 0.044 g vitamin A per gram of extract. The methyl esters were procured by both the Bloor extraction and also by alkaline saponification of the liver tissues and extraction of nonsaponifiable material with Skellysolve F. The esters were then distilled under a pressure of 100 microns, using the still of Diemair and Schmidt as above.

The results of adding vitamin A to the methyl esters are tabulated in Table 4. In each case the percentages of linoleic

Table 4. Spectrophotometric analysis of methyl esters plus vitamin A.

Per cent vitamin A	Per cent linoleic	Per cent linolenic	Per cent arachidonic
	Alkaline extract		
0	21.02	0	23.12
0.85	21.99	0	20.58
	Bloor extract		
0	15.16	0	17.90
0.90	17.71	0	15.70

[2]Furnished by Distillation Products, Inc., Rochester, N. Y.

acid increased while that of arachidonic acid decreased. However, the results are anomalous in that the original materials do not show good correlation in spite of being extracted from the same beef liver which had been divided into two equal parts.

A sample of free fatty acids from turkey gizzard and skin fats was prepared by saponification and then converted to methyl esters. These esters were highly colored after three washings with water.

The esters were divided into two equal portions of which one portion was distilled under 100 μ mercury pressure and the other analyzed with no further preparation. The results of spectrophotometric analysis are tabulated in Table 5.

Table 5. Spectrophotometric analysis of distilled and nondistilled methyl esters from turkey skin and gizzard fats.

Per cent fatty acids:	Distilled	Nondistilled
Linoleic	19.89	22.64
Linolenic	1.32	1.65
Arachidonic	0	0.22

The possibility that phospholipids might have some influence on the above analysis was considered. A beef liver was extracted by solvents and fractionated into phospholipid

and nonphospholipid portions by cold acetone precipitation of the phospholipids. The results of spectrophotometric analysis of the original beef liver fats and phospholipid fractions were considered inconclusive and were rejected. The phospholipid fractions decomposed rapidly and changed from a clear white amorphous solid to a dark tarry material while being weighed for spectrophotometric analysis. The results of analysis indicated that 40 to 50 per cent of the arachidonic acid in liver fat was included in the phospholipid fraction. The rapid decomposition of phospholipids made it impossible to recombine the various fractions in an attempt to ascertain the influence of phospholipids on spectrophotometric analysis.

The next step was the chromatographing of highly colored fats from rats and linseed oil. Silica gel, alumina, magnesia, slaked lime, and powdered chalk were used as adsorbent materials. However, all except alumina were found to have insufficient adsorbing power to remove the pigments in the fats. The alumina (80-200 mesh) was standardized using the method of Brockmann and Schodder (4) to a value of IV. The fats were saponified and the resulting free fatty acids esterified with methyl alcohol and chromatographed. A large amount of solvent was needed to remove the adsorbed esters. The pigments and esters had very similar adsorption properties and a quantitative recovery from the column was not achieved. Six liters of Skellysolve F containing four per cent acetone failed to remove one g of esters from the columns. The method

was not considered feasible for purification of fats to be analyzed by spectrophotometric analysis. The results of chromatographing methyl esters of rat fat and linseed oil are given in Table 6. Spectrophotometric analysis of chromatographed linseed oil indicated that it was lower in diene and triene acids than the iodine value indicated it should be and as a result the value for the percentage of oleic acid was over 100 per cent.

Table 6. Spectrophotometric analysis of chromatographed methyl esters.

	Per cent			
	Oleic	Linoleic	Linolenic	Arachidonic
Chromatographed rat fat				
Group 3-A*	52.77	1.1	1.76	0.58
Group 7-B*	-	2.0	0.50	1.46
Original fat before chromatographing				
Group 3-A	70.32	2.09	1.49	3.41
Group 7-B	65.85	1.35	0.60	1.50
Chromatographed linseed oil**	-	15.35	10.02	4.25
Original linseed oil	30.45	15.88	45.50	0

* Silica (Brockmann IV) column 2.5 x 25 cm.
** Carbon black (Norite) column 2.5 x 25 cm.

The apparent percentage of tetraene acids increased when the fatty acids were chromatographed using active carbon as the adsorbing material. However, the increase was not sufficient to explain the high iodine values of the chromatographed fatty acids when the apparent decrease in diene and triene acids was considered. The product recovered from the activated carbon column when linseed oil was the starting material, was a clear liquid of high viscosity. The change in viscosity suggested that oxidation might occur due to the absorbed oxygen in activated carbon.

An attempt was made to gain more information by excluding oxygen from the reaction surface to the greatest possible extent. A closed system was constructed of pyrex tubing of 12 mm inside diameter. A 15 x 15 mm section was sealed in this system and filled with activated carbon. A filling vent was left through which nitrogen gas was blown while the carbon was heated to redness. After three 10-minute periods of heating and cooling the column was allowed to cool in an atmosphere of nitrogen. Pure linolenic acid was dissolved in purified Skellysolve F and forced into the column with nitrogen. The column was sealed after filling and the liquid circulated by means of an internal magnetic pump. After 15 days of constant cycling through the carbon column the system was opened and the solution removed. The solvent was removed under vacuum and the material stored under vacuum in sealed ampules. The spectrophotometric data and iodine values of

both the original and treated materials are given in Table 7.

Table 7. Spectrophotometric analysis and iodine values of treated and untreated methyl linolenate.

	Iodine no.	Per cent			
		Oleic acid	Linoleic acid	Linolenic acid	Arachidonic acid
Treated methyl linolenate	256.8	0	0	98.25	0.49
Untreated methyl linolenate*	244.0	0	0	94.69	0.11

*Prepared by Winston H. Wingerd of this laboratory.

The data in Table 7 did not show the gross changes between chromatographed and nonchromatographed materials that linseed oil exhibited. The only change of any magnitude was in the percentage of linolenic acid. The apparent percentage of arachidonic acid increased but the method of analysis is only indicative of magnitudes and not absolute values at such low concentrations.

Elementary analysis for carbon and hydrogen of the treated methyl linolenate gave the following results: Calculated: C, 78.04; H, 11.03; found C, 77.18; H, 11.00.

The results from low pressure distillation and chromatographing were disappointing from an analytical viewpoint. Distillation in the ultra high vacuum range was next resorted to in an attempt to remove impurities without altering the

fatty acid composition appreciably.

A pot-type molecular still was constructed after the plans of Gould et al. (7). This still was operated at 0.01 of mercury pressure which was attained by a butyl phthalate diffusion pump.

The fats extracted from the skins of chickens were used as the test material in the molecular still. These fats were saponified, neutralized and the free fatty acids esterified. After washing with three per cent sodium bicarbonate and water, the esters were still highly colored. One portion of the esters was spectrophotometrically analyzed without any further treatment. The remainder was distilled in the molecular still and analyzed using the spectrophotometer. The results of these analyses are given in Table 8.

The distilled methyl esters were clear to faintly yellow liquids while a deep red residue remained in the still pot. No evidence of carbonization was apparent in the still pot or residues.

The results from this work were essentially the same as those obtained when nonsaponifiable materials were added to purified methyl esters. However, the correlation between the phospholipid fractions was very poor and it was felt that decomposition had occurred during the preparation of the materials for analysis.

Table 8. Spectrophotometric analysis of distilled and nondistilled chicken fatty acids.

Distilled methyl esters	Per cent Linoleic acid	Linolenic acid	Arachidonic acid
Group 35	21.55	1.07	2.79
Group 23	21.81	2.11	0 phospholipid fraction
Group 26	30.60	1.87	0.62 phospholipid fraction
Nondistilled methyl esters			
Group 35	21.84	0.69	1.33
Group 23	32.85	1.17	1.20 phospholipid fraction
Group 26	28.09	1.15	0.90 phospholipid fraction

DISCUSSION

The use of the spectrophotometer for the analysis of fats and oils by ultraviolet absorption gives excellent results when the constituents sought are present in relatively large amounts but the method suffers a sharp decrease in both precision and accuracy when minor constituents of fats are sought.

The presence of extraneous constituents that absorb ultraviolet light can lead to quite large errors in the analysis of minor components. Fats from such sources as livers

and whole body fats are usually highly colored. Saponification of such fats and the extraction of nonsaponifiable material does not remove much of the color unless protracted extraction procedures such as continuous liquid-liquid extractors are used. Such long procedures may cause decomposition of the materials to be analyzed.

The deep red to maroon color of fat extracted from livers is due possibly to porphyrin rings from the blood which was present in this organ. These pigments are not removed by water-washing of the fats. In most cases the washing of either fats or fatty acids must be done with care or emulsions will form that are very difficult to dissipate. The failure to wash out the red pigments led to the belief that such pigments are absorbed on the fat. This belief was further substantiated by the behavior of such pigmented fats when chromatographed. The red pigments do not separate on the adsorbent material and are eluted by the same solvents that elute the fats.

Skin fats of poultry do not exhibit the red color of liver fats. The percentage of nonsaponifiable material in skin fats is relatively low as compared to those from livers. The nonsaponifiable material from chicken skin fat formed crystals when allowed to stand in cold Skellysolve B. The crystals were yellow but did not have the characteristic absorption spectrum of beta-carotene and other carotenoid pigments. These crystals when dissolved in Skellysolve F and

chromatographed on magnesia columns did not form any colored bands. The Liebermann-Burchard color test for sterols indicated the presence of cholesterol.

The possibility that riboflavin was adsorbed on the crystals was considered and fluorometric analysis performed on samples of nonsaponifiable. The results of such analyses were negative.

The skin and gizzard fat of poultry fed a diet containing alfalfa had a strong yellow to green fluorescence. This fluorescent material was removed by an alumina chromatograph column. The eluate had high absorption peaks at 225 and 250 mμ. A few crystals of this highly absorbing material were isolated but have not been identified. A pigment having the same ultraviolet absorption behavior has been isolated from alfalfa lipids. This fluorescent material has not been observed in skin and gizzard fats from birds on diets not containing alfalfa.

The sterols present in skin fats act as inert materials and must be corrected for if absolute values are to be obtained. The predominant part of nonsaponifiable materials from skins and gizzards consists of sterols and it was found quite permissible to consider all nonsaponifiable material from such sources as inert to ultraviolet absorption.

The procedure used to correct for such inert materials in the absence of the previously mentioned fluorescent material was to subtract the total weight of the nonsaponifiable

fraction from the weight used for analysis. For instance, if the sample of fat contained four per cent nonsaponifiable then 96 per cent of the sample weight was used in calculating the K values from the spectrophotometric data.

Fats obtained from liver may contain as much as 40 to 50 per cent nonsaponifiable material and the above procedure of subtracting the nonsaponifiable fraction was not considered feasible. Liver may contain appreciable quantities of vitamin A, tocopherol, and carotenoids. The nonsaponifiable material was removed from the fats before subjecting them to spectrophotometric analysis.

When the fats are saponified and acidified free fatty acids are released. The removal of glycerol from the fats must be compensated for if the results are to be compared with other analyses based on the original glycerides. All analyses based on free fatty acids can be corrected to the triglyceride basis by using the factor 1.046 (1).

In this laboratory two methods of extraction were used in preparing fat samples: one, the solvent and two, the saponification method. The possibility that the analyses might be affected by the type of extraction used was considered. Analyses performed on samples extracted from beef liver showed that this factor must be considered in evaluating the analyses. The removal of nonsaponifiable material from triglycerides which had been extracted with solvents resulted in analyses that checked very well with the values obtained

by the saponification method. However, samples containing high percentages of nonsaponifiables could not be correlated by applying corrections for the nonsaponifiable material. This was due to the extraneous absorbing materials present from such sources. In this case the method of choice was removal of nonsaponifiable material before spectrophotometric analysis.

The possibility that conjugation of isolated bonds might occur during alkaline saponification was of considerable importance since an alkaline conjugation method was used in preparing fats for spectrophotometric analysis. However, no appreciable conjugation was found to occur under the conditions of such extractions.

Chromatographic techniques for purification of fats did not prove feasible. The possibility of purifying by distillation was therefore thoroughly investigated. It was found that esters only could be distilled with any degree of success by normal procedures. All of the long chain, polyunsaturated acids decomposed to a considerable degree when distilled under a vacuum of 100μ of mercury. However, the distillation of esters in the vacuum attainable by a commercial model butyl phthalate diffusion pump gave little or no decomposition to the point of carbonization. This method proved particularly valuable in preparing pure reference compounds.

The change in ultraviolet absorption exhibited by arachidonic acid upon molecular distillation is shown clearly in

Fig. 1. The broken line is the spectral absorption of impure arachidonic acid prepared from octabromoarachidic acid. The spectrophotometric analysis of this acid indicated an acid of only 62.21 per cent purity. The preparation was a yellow oil having an offensive odor.

The solid line indicates the spectral absorption of the arachidonic acid after several distillations. The distilled product was a clear, colorless and odorless liquid. The iodine value of this preparation was theoretical for the pure acid. A marked change in the absorption peaks was shown; these changes were most apparent in the triene and tetraene regions. The irregularities of the broken line were nearly removed by molecular distillation.

When these pure reference compounds were analyzed by the spectrophotometer and calculated by the accepted formulas anomalous results were obtained. The analysis of pure arachidonic acids always amounted to more than 100 per cent as the percentage of acid present.

The specific extinction coefficients obtained for linoleic and linolenic acids prepared in this laboratory checked very well with those found in the literature (1, 2, 9). However, the specific extinction coefficient obtained for arachidonic acid did not agree with the values found in the literature (3). In all cases the specific extinction coefficients are those obtained upon alkaline conjugation using 12.5 per cent potassium hydroxide in ethylene glycol, heated

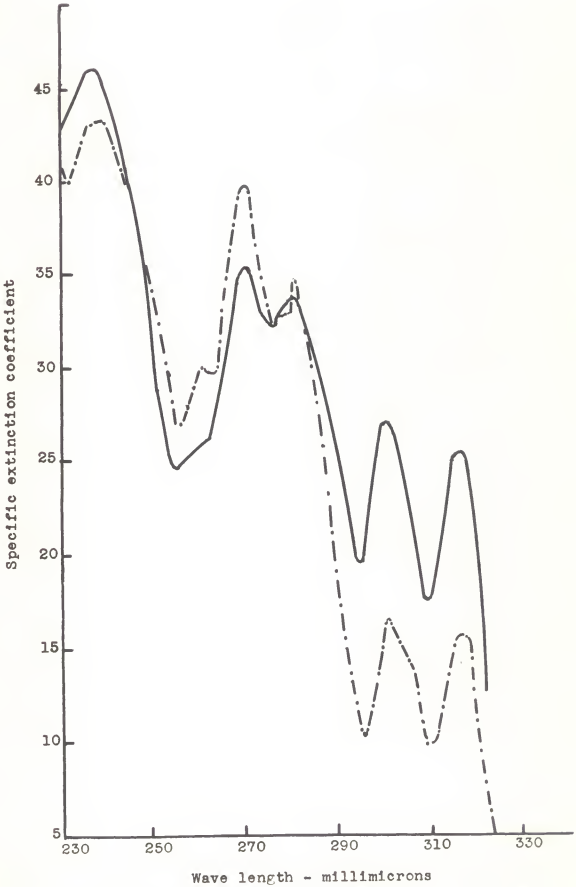

Fig. 1. Absorption spectra of distilled and nondistilled ethyl arachidonate.

to 180°C. for 30 minutes.

The specific extinction coefficient of a mixture of linoleic, linolenic and arachidonic acids is the sum of their respective theoretical extinction coefficient multiplied by the concentration of each present in the mixture. This is shown in equations I, II, and III.

I. $86.0x + 53.20y + 48.03z = k_2$

II. $53.20y + 36.64z = k_3$

III. $27.74z = k_4$

k_2, k_3, k_4 = specific extinction coefficient of the mixture

86.0 = specific extinction coefficient of linoleic acid at 232 mμ

53.2 = specific extinction coefficient of linolenic acid at 268 mμ

27.74 = specific extinction coefficient of arachidonic acid at 316 mμ

x = per cent of linoleic acid

y = per cent of linolenic acid

z = per cent of arachidonic acid

Since there are three equations and three unknowns the above equations can be solved simultaneously. The results of the solution are given in equations IV, V, and VI.

IV. $x = 1.16\ k_2 - 1.33\ k_3 - 0.26\ k_4$

V. $y = 1.88\ k_3 - 2.48\ k_4$

VI. $z = 3.60\ k_4$

Fats prepared from biological sources contain extraneous components that absorb ultraviolet light. Equations VII, VIII, and IX correct for this absorption by substances other than unsaturated fatty acids.

$$\text{VII. } k_2 = \frac{D_{232}}{W \times l} + 0.04$$

$$\text{VIII. } k_3 = \frac{4.10}{W \times l} \left[D_{268} - 1/2 \left(D_{262} + D_{274}\right)\right]$$

$$\text{IX. } k_4 = \frac{2.55}{W \times l} \left[D_{316} - 1/2 \left(D_{310} + D_{322}\right)\right]$$

The constants 4.10 and 2.55 are evaluated by graphical means and are the ratio of total height of absorption peak to the height from an arbitrarily selected base line. For k_3 the base line is drawn from the point at 262 mμ to the point on the curve at 274 mμ. The base line for the k_4 values extends from the point at 310 mμ to that at 322 mμ.

Equations IV, V, and VI are based on purer reference standards than those described in the literature and are suggested for use in spectrophotometric analysis of fatty acids.

Using the standards tabulated in Table 9, the equations give excellent results for the tetraene acids. However, in all cases the formulas give appreciable percentages of diene acids. No explanation is advanced at this time for this result. Further work is to be done on the specific extinction coefficients for the diene and triene components.

Table 9. Specific extinction coefficients used in calculations.

Linoleic 232	- 86.00[1]
Linolenic 232	- 60.90[1]
Arachidonic 232	- 47.85[2]
Linolenic 268	- 53.2[1]
Arachidonic 268	- 36.64[2]
Arachidonic 316	- 27.74[2]

[1]Values reported by Beadle (1).

[2]Values from this investigation.

SUMMARY

The effect of various naturally occurring impurities in fat upon its spectrophotometric analysis has been investigated. Such extraneous materials can affect the analysis markedly when they are present in relatively large concentrations. Liver fats contain a high proportion of pigments which do not absorb ultraviolet light in the region used for the spectrophotometric analysis of fats and therefore act only as inert dilution factors. Skin fats of poultry, on the other hand, contain pigments that absorb quite strongly even in relatively small amounts.

The method of extraction of the fats can change the apparent composition of the fats to a considerable degree. As a result of this investigation, it is felt that saponification of the fats and thorough extraction of the nonsaponifiable material gives the most reliable data.

A method was devised for preparing pure arachidonic acid as a reference compound. Molecular distillation was found to give very pure preparations of this acid. The pure arachidonic acid was used to evaluate new constants for use in the analysis of fats and fatty acids.

Further investigation of reference standards for diene and triene acids is needed to explain the anamalous results obtained for pure preparations of arachidonic acid.

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LITERATURE CITED

1. Beadle, Bernard W.
   Applied ultraviolet spectrophotometry of fats and oils. Oil and Soap 23:140-145. 1946.

2. Bloor, W. R.
   Biochemistry of the fatty acids. New York: Rheinhold Pub. Co. 387 p. 1943.

3. Brice, Boyd A. and Margaret L. Swain.
   Ultraviolet absorption method for the determination of polyunsaturated constituents of fatty materials. Jour. Opt. Soc. America 35:532-544. 1945.

4. Brockmann, H. and H. Schodder.
   Aluminumoxyd mit abgestuften adsorptionvermögen fur Chromatographischen adsorption. Ber. der Deutsch. Chem. Gesell. 74:73-78. 1941.

5. Dann, William J. and Thomas Moore.
   The absorption spectra of the mixed fatty acids from cod liver oil. Biochem. Jour. 27:1166-1169. 1933.

6. Diemair, W. and W. Schmidt.
   Erfahrungen bei de Fraktionierten Distillation von Phosphatidfettsauren. Biochem. Zeit. 294:348-352. 1937.

7. Gould, C. W. Jr., G. Holzman, and C. Niemann.
   Distillation equipment suitable for centigram and decigram quantities. Anal. Chem. 20:361-364. 1948.

8. Kass, J. P. and G. O. Burr.
   Pseudo eleostearic acid. Jour. Amer. Chem. Soc. 61: 3292-3294. 1939.

9. Mitchell, J. H., H. R. Kraybill, and F. P. Zscheile.
   Quantitative spectral analysis of fats. Indus. and Engin. Chem. Anal. Ed. 15:1-3. 1943.

10. Moore, Thomas.
    Changes in the absorption spectra of various fats induced by treatment with potassium hydroxide. Biochem. Jour. 31:138-141. 1937.

11. Moore, Thomas.
    Spectroscopic changes in fatty acids. VI. General. Biochem. Jour. 33:1635-1638. 1939.

12. Rosenberg, H. R.
Chemistry and physiology of the vitamins. New York: Interscience. 678 p. 1945.

13. Yasuda, M.
The determination of the iodine number of lipids. Jour. Biol. Chem. 94:401-402. 1939.