AN IMPROVED METHOD FOR THE DETERMINATION OF THE HYDROXYLATION OF FATS AND FREE FATTY ACIDS

by

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AN IMPROVED PROCEDURE FOR THE DETERMINATION OF THE HYDROXYLATION OF FATS AND FREE FATTY ACIDS

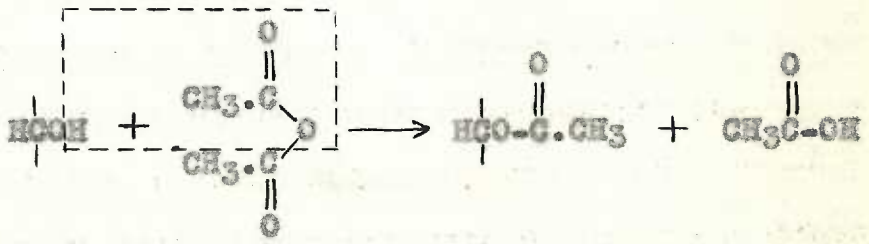
Introduction

Fats and oils comprise a large group of widely distributed biological materials, complex and variable in composition but chemically related. Except for small amounts of sterols, coloring matter, odorous substances, etc., the fats and oils consist chiefly of the triglycerides of a series of monobasic acyclic acids, known as the fatty acids. The large number of naturally occurring fatty acids in combination with glycerol as both simple and mixed triglycerides makes possible innumerable variations in the composition of fats and oils.

A strict chemical investigation of fats and oils should include the separation and identification of the various triglycerides and the isolation of the individual fatty acids. Up to the present, triglycerides have been separated in only a few cases and by methods which cannot be adapted to routine analysis. The isolation of individual fatty acids has met with more success, but the technique is complicated and the separation is by no means sharp. There are, however, a number of simple analytical procedures which give an insight into the chemical constitution of fats and oils. Although they

do not furnish absolute values with respect to any single constituent, they do yield average values which are important in establishing constants useful in the technical analysis of fatty materials. One of these constants, known as the saponification value, serves to indicate the mean molecular weight of the fatty acids. Another, the iodine value, is used to determine the degree of unsaturation.

The acetyl value, which is the subject under consideration in this dissertation, indicates the presence of glycerides of hydroxylated fatty acids. The determination of this value is based on the acetylation of the hydroxyl group by means of acetic anhydride, according to the reaction:



The several proposed methods fall into two groups employing somewhat different procedures. In one, a weighed amount of acetylated fat is hydrolyzed or saponified and the liberated acetic acid is determined. In the other, a known amount of fat is allowed to react with a definite quantity of acetic anhydride, the excess of the latter is decomposed with water and the acetic acid formed is titrated in the presence of the acetylated product.

These different procedures have led to different definitions of acetyl value. According to one of the earliest definitions, proposed by Lewkowitsch (12), the acetyl value is the number of milligrams of potassium hydroxide required for the neutralization of the acetic acid obtained on saponifying one gram of the acetylated fat. Holland (9) suggested that the acetyl value be expressed as the number of milligrams of potassium hydroxide required to saponify the acetyl taken up by one gram of the fat on acetylation. West (19) defines the acetyl value as the number of milligrams of acetyl taken up by one gram of the fat.

The true acetyl value of a fat is an indication of the degree of hydroxylation of its constituent fatty acids. Acetyl values as determined on fats and oils do not always represent the true hydroxylated fatty acid content. Several factors are involved in this error.

The first and most important of these factors is rancidity, the result of a series of slow chemical changes directly due to the action of certain enzymes, water, oxygen and light. One change consists in the partial or complete hydrolysis of the glycerides with the formation of mono- or di-hydroxy-glycerides or glycerol and fatty acids. It is apparent that fats which have undergone this process will exhibit an abnormally high acetyl value due to the hydroxyl groups of the glycerol or partially hydrolyzed glycerides.

A second change leading to rancidity and increased fatty acid hydroxylation is due to auto-oxidation of free unsaturated fatty acids. This oxidation takes place at the double bonds with the formation of peroxides, hydroxylated acids, ketones, aldehydes, and fatty acids of lower molecular weight. The relative number of hydroxyl groups arising by this process is small; hence, the increase in acetyl value is also small.

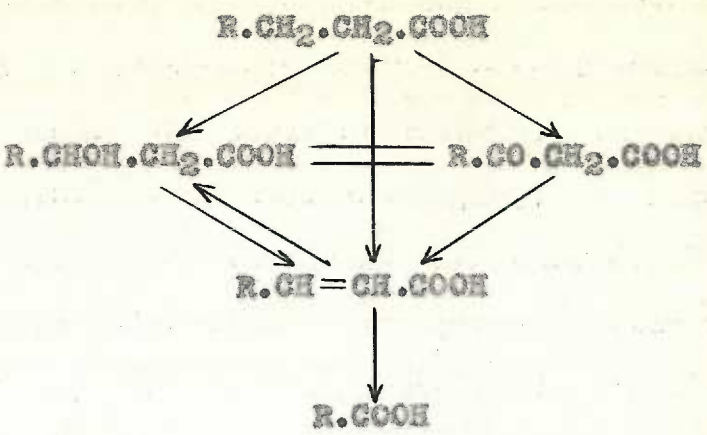
The second factor tending to augment the acetyl value of a fat is the presence of certain higher alcohols, the sterols. These substances are widely distributed in plant and animal tissues and are almost certain to contaminate fats and oils in which they are quite soluble. It is obvious that this factor, as well as the ones mentioned previously, should be carefully considered in the interpretation of acetyl values.

In consideration of these facts it became evident that determination of acetyl values upon the separated fatty acids would eliminate the more serious errors. Previous methods of analysis are incapable of doing this satisfactorily, and attempts were made, successfully we believe, to develop a method for this purpose. Such a procedure should prove valuable not only in establishing constants for technical analysis but also in affording a method for the study of intermediary fatty acid metabolism.

In all probability, hydroxylated fatty acids play an important role as intermediates in the degradation of fatty acids in the body. The earliest work throwing some light on this mechanism was that of Knoop (10). By feeding animals phenyl derivatives of fatty acids and recovering hippuric and phenaceturic acids in the urine, he was able to show that the breakdown of the fatty acid chain takes place by the successive removal of pairs of carbon atoms.

Similar experiments were performed by Dakin (4) who confirmed Knoop's work and found, in addition to the hippuric and phenaceturic acids, certain hydroxy- and keto-acids. In a later study in which he perfused a surviving liver with caproic acid, $\alpha\beta$-hexenic acid, β-hydroxy-caproic acid and β-ketocaproic acid, Dakin (5) showed that there exists a definite relationship in the metabolism of these acids. As a result of this work he postulated a possible mechanism by which fatty acids may be oxidized and which is concisely summarized in the following diagram taken from his monograph (5).

If the experimental work of Dakin has been interpreted correctly, one would expect to find increased amounts of hydroxylated fatty acids in the tissues of animals deriving most of their energy from fat oxidation. In testing this supposition, a satisfactory method of determining the hydroxylation of fatty acids isolated from tissues is imperative.


R.CH2.CH2.COOH
R.CHOH.CH2.COOH
R.CO.CH2.COOH
R.CH=CH.COOH
R.COOH

Discussion of Methods of Determining the Hydroxylation (Acetyl Values) of Fats and Fatty Acids

Of the many methods for the estimation of hydroxyl or acetyl groups in organic compounds, only a few have been devised for use with fats and fatty acids. The first was proposed in 1887 by Benedikt and Ulzer (2) for application to free fatty acids. It consists in the determination of first, the alkali equivalent to the free carboxyl groups and the acetic acid split off by saponification of the acetylated acids; and second, the alkali equivalent to the free carboxyl groups only. The difference between these two quantities gives the acetyl value. This procedure, however, is subject to error, as pointed out by Lewkowitsch (11). Fatty acids of high molecular weight form fairly stable anhydrides when boiled with acetic anhydride. This reduces the number of free carboxyl groups, which obviously would increase the acetyl value.

Ten years later, Lewkowitsch (12) published two methods for the analysis of acetylated fats. In the first, known as the distillation process, the acetylated fat is saponified by boiling with alcoholic potash. The mixture is acidified with dilute sulfuric acid and the acetic acid distilled off in a current of steam and titrated with alkali. Volatile fatty acids, however, are also carried over in the distillation,

and to correct for these, an estimation of the volatile acids must be made.

The other procedure, the filtration method, is carried out as follows: The acetylated fat is saponified with a known quantity of standard alkali. An exactly equivalent amount of dilute sulfuric acid is then added. The liberated fatty acids are filtered from the aqueous solution and carefully washed, and the filtrate containing the liberated acetic acid is titrated with alkali. Although this procedure is simpler than the distillation process, it is inaccurate if soluble fatty acids are present. In both methods the difficulty of titrating a small amount of acid in a large volume is encountered.

Andre (1) has applied the principle of the procedure of Benedikt and Ulzer (op. cit.) to fats and oils. The acetyl value is calculated from the saponification values of the acetylated fat and the untreated fat. It will be noticed that the difference between these values does not represent the true acetyl value, since the glycerides in one gram of acetylated fat do not contribute as much to the saponification value as do the glycerides in one gram of the untreated fat. Andre has derived a formula, later simplified by Cook (3), which correctly expresses the acetyl value in terms of the two saponification values.

An experiment performed by us and described in the following section demonstrates that this method gives inaccurate

results if the sample being analyzed contains free soluble fatty acids. Other disadvantages in the procedure are also pointed out.

Friedrich and Rapaport (7) have described a micromethod for the determination of the acetyl group. Analyses of a number of compounds showed that experimental values agreed well with the theoretical. Fürth, Kaunitz and Stein (8) tried the procedure on various acetylated fatty acids and found that purified ricinoleic acid gave nearly theoretical values. The procedure is as follows:

The acetylated product is hydrolyzed by means of 25% p-toluene sulfonic acid and the acetic acid is distilled off in a vacuum. During the hydrolysis and distillation, there is slight decomposition of the p-toluene sulfonic acid with the production of sulfur dioxide. This is carried over into the distillate and would lead to serious errors were corrections not made. For this reason the distillate is collected in a standard iodine solution, which oxidizes the sulfurous acid to sulfuric acid, thus:

$$H_2SO_3 + I_2 + H_2O \longrightarrow H_2SO_4 + 2HI$$

After titrating the excess iodine with N/100 thiosulfate, the quantity of sulfuric acid (and also hydriodic acid) can be calculated. A solution of iodide and iodate is now added. The acetic, sulfuric and hydriodic acids liberate from this

mixture an equivalent quantity of iodine which is titrated with standard thiosulfate. From the total titration volume is subtracted the volume equivalent to the sulfuric and hydriodic acids, the result being the acetic acid equivalent.

Although this method has not been tested by the writer, the complexity of the apparatus and procedure makes it undesirable.

In 1932, Roberts and Schuette (15) published a procedure based on a principle entirely different from any employed in the methods just discussed. A sample of the fat with a weighed amount of standardized acetic anhydride is heated at 120° in a sealed tube for an hour. The contents of the tube are transferred to a flask with water, refluxed at reduced acidity, and then titrated with aqueous alkali. Numerous faults have been found in the method, as will be described later. The procedure is especially tedious and time-consuming.

Verley and Bölsing (17) showed that quantitative acetylation of alcohols and phenols can be obtained by treatment of the substance with a mixture of acetic anhydride and pyridine. The pyridine promotes the reaction by removing the acetic acid (produced in the acetylation) as pyridine acetate. After the reaction is complete, the product is treated with water to decompose excess anhydride and the total acidity is titrated with standard alkali. The difference between a blank titration and the sample titration represents the acetyl bound by the compound used. The presence of the

pyridine in no way interferes with titration of the acetic acid, since sodium hydroxide readily displaces pyridine from combination with acetic acid.

These principles have been applied to the determination of hydroxyl groups in sugars and sugar derivatives by Peterson and West (14). In 1934, West, Hoagland and Curtis (19) reported that the same basic procedure, with several modifications, could be very satisfactorily used in the analysis of fats. Moreover, by the analysis of ricinoleic and stearic acids, they showed the applicability of this method to free fatty acids. The method, in brief, is as follows:

Into three 250 cc. glass-stoppered Pyrex flasks are placed the following substances:

1. 5 cc. of an acetic anhydride-pyridine mixture (1:7 by volume).
2. Sample + 5 cc. of acetic anhydride-pyridine mixture.
3. Sample + 5 cc. of pyridine.

The stoppered flasks are now heated on a steam bath about 45 minutes (or allowed to stand at room temperature for 24 hours or longer). 5 cc. of water are added and the flasks heated 1.5 to 2 minutes, after which they are allowed to cool. The flasks are then rinsed down with 25 cc. of butyl alcohol and the solutions titrated with 0.3 to 0.35 N alcoholic alkali with phenolphthalein. The titrations are all converted

to cc. of 0.1 N alkali. The acetyl value, defined by West as the milligrams of acetyl taken up per gram of substance, is calculated from this data.

Smith and Bryant (16) have recently described a method in which acetyl chloride in the presence of pyridine is used as acetylating agent. Although the method is satisfactory as worked out on alcohols and phenols, it is tedious and would require modification before being applicable to fats and fatty acids. The acetic anhydride-pyridine method, moreover, was found to be more satisfactory from the standpoint of precision than the acetyl chloride method.

An Improved Method for Determining the Hydroxylation (Acetyl Value) of Fats and Fatty Acids

Development of the Method

The method of West, Hoagland, and Curtis (op. cit.) offers several advantages over other methods in that it is simple, rapid, and applicable to free fatty acids. It was pointed out by Dr. West, under whose direction this work has been done, that the method as outlined needed further study not only to improve the analytical technique but also to establish optimum conditions for the reactions. A series of experiments was therefore performed and these, we believe, disclose the desired information. They are as follows:

1. Comparison of acylating agents.
2. Determination of the optimum concentration of acetic anhydride.
3. Determination of reaction rates at various temperatures.
4. Limitations of sample size.
5. Determination of the amount of water for decomposing acetylating mixture (1:7).
6. A study of the formation and decomposition of higher fatty acid anhydrides.
7. The effect of the use of condensers in the reaction tubes on the precision of the method.

Comparison of Acylating Agents. Until the recent introduction of acetyl chloride as a quantitative acetylating

agent, acetic anhydride has been used exclusively. Although acetyl chloride does show a higher activity than acetic anhydride, it has the disadvantage of a low boiling point which favors errors by volatilization. Acetyl chloride, as other acyl chlorides, also forms an insoluble compound with pyridine which complicates its use in this procedure. It was considered that some other reagent might be preferable for introducing an acyl (RC$\overset{O}{\diagdown}$) or other equivalent group into a hydroxylated compound.

The compounds examined for this purpose were the following: propionic, butyric, benzoic and phthalic anhydrides, the sulfonic chlorides of benzene, toluene and naphthalene, chlorsulfonic acid and phosphorus oxychloride. Castor oil, which is predominantly the triglyceride of ricinoleic acid, a monohydroxy-acid, was chosen for the tests, since it has a very high acetyl value and should test the activity of the reagent satisfactorily. Determinations were run using 1.25 molar solutions of the reagents in pyridine (dioxane in the case of chlorsulfonic acid). The technique of West, Hoagland, and Curtis was used except that Pyrex test tubes (25 x 200 mm.) covered with glass bulbs were substituted for glass-stoppered flasks. Unless otherwise indicated, the tubes were heated on a steam bath for one hour.

The observed acetyl values of castor oil with the various reagents are given in Table I. Phosphorus oxychloride

produced an insoluble product which could not be titrated. The unusually high values with chlorsulfonic acid are probably due to the addition of the reagent at the double bonds in the ricinoleic acid. The remaining values are all below the ones obtained with acetic anhydride. It is evident, therefore, that acetic anhydride is the only one of the reagents satisfactory under the conditions used.

Table I

Comparison of Acylating Agents

(1.25 molar solutions in pyridine)

Reagent and conditions	Weight of Castor Oil	Acetyl Value
Acetic anhydride 1 hr. at 100° C.	0.8612	124.0
	0.8501	124.1
	0.8534	125.8
Propionic anhydride 1 hr. at 100° C.	0.5097	117.3
	0.6686	117.3
	0.7957	115.5
	0.8898	115.3
Butyric anhydride 1 hr. at 100° C.	0.5120	109.8
	0.6705	107.2
	0.7929	105.1
	0.9087	103.2
Benzoic anhydride 1 hr. at 100° C.	0.6736	43.7
	0.7942	42.9
	0.7777	42.6
Phthalic anhydride 1 hr. at 100° C.	0.5157	122.1
	0.7660	122.0
	0.9550	118.8

Table I (cont'd.)

Reagent and conditions	Weight of Castor Oil	Acetyl Value
Phthalic anhydride (cont'd) 2 hr. at 100° C.	0.5258	122.2
	0.6844	121.0
	0.7970	120.6
	0.9482	122.0
1.5 hr. at 110-115° C.	0.5610	121.2
	0.6906	119.2
	0.8325	117.2
	0.9415	114.1
1.5 hr. at 120-125° C.	0.5573	121.8
	0.7081	----
	0.8368	120.9
	0.9630	120.6
Benzene sulfonic chloride 1 hr. at 100° C.	0.6556	112.3
	0.6716	111.7
	0.6760	111.3
p-Toluene sulfonic chloride 1 hr. at 100° C.	0.6541	112.0
	0.6170	112.0
	0.6480	111.1
Naphthalene sulfonic chloride 1 hr. at 100° C.	0.6725	116.1
	0.6610	115.0
	0.6444	115.4
Chlorsulfonic acid 20 hrs. at 20° C in dioxane	0.6727	205.2
	0.8054	174.8
	0.9233	148.0

Determination of the Optimum Concentration of Acetic Anhydride. This was done, as before, by using castor oil as the test material. Tubes were set up with weighed samples of castor oil and acetic anhydride-pyridine mixtures (of various ratios) and allowed to react 24 hours before decomposition with water and titration. The results in Table II show no great differences in values obtained with the reagents of various concentrations. The precision of the determinations was greatly decreased when the more concentrated reagents were used. A mixture of one part anhydride with 5 parts of pyridine has about the maximum concentration for accurate work.

Table II

Optimum Concentration of Acetic Anhydride

(Reaction mixtures allowed to stand 24 hours at room temperature.)

Ratio (by volume) Acetic anhydride:Pyridine	Acetyl Value of Castor Oil	Number of Determinations
1:7	123.0 ± 0.3	10
1:6	123.4 ± 0.4	10
1:5	123.9 ± 0.4	10
1:4	123.7 ± 1.1	16
1:3	123.3 ± 3.3	12

Determination of Reaction Rates at Various Temperatures.

A number of tubes were set up with castor oil and 5 cc. of the acetylating mixture (1:5) and allowed to stand at room temperature. At various intervals, 5 cc. of water were added to a tube and the mixture titrated. The same procedure was repeated with the exception that a 1:7 mixture was used and the tubes were heated on the steam bath. Table III shows that in the cold, the reaction is practically complete in 24 hours, while at 100° the time is reduced to 1-1½ hours. Moreover, values for the samples that were heated are slightly higher than the others indicating that the elevated temperature has driven the reaction more nearly to completion.

Table XXI

Reaction Rates at Various Temperatures

Temperature--20° C.
Acetylating mixture--1:8

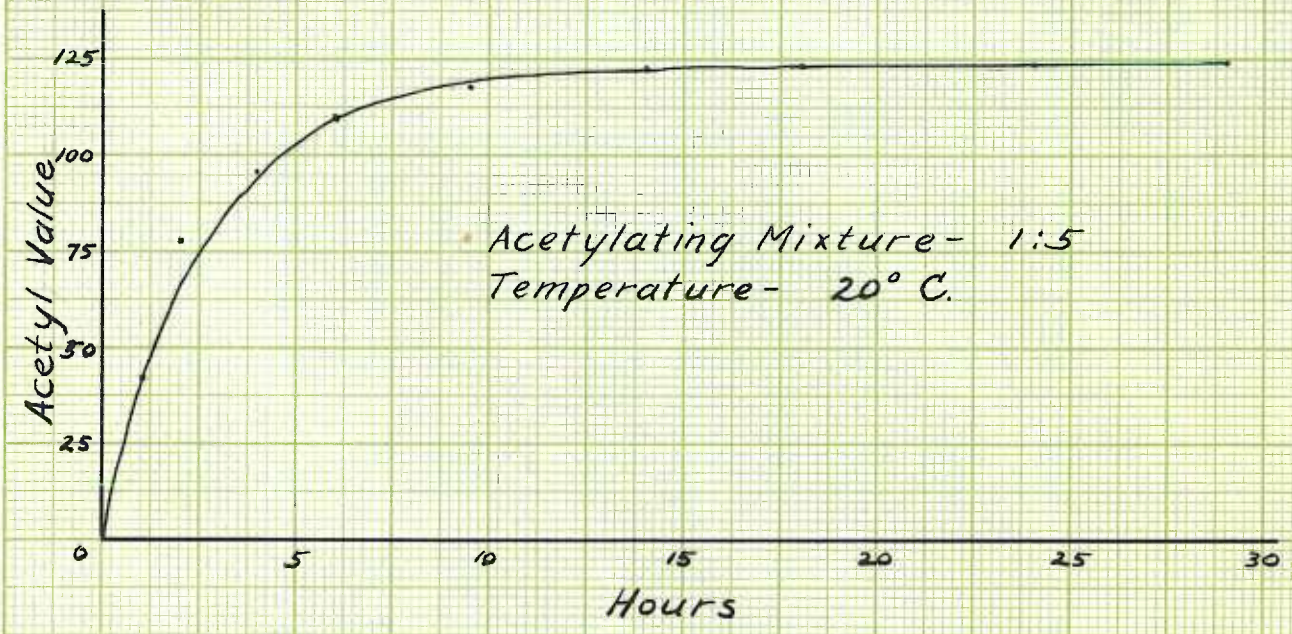
Time (hours)	Acetyl Value
0.5	22.3
1.0	42.3
2.0	78.6
4.0	96.5
6.0	110.7
9.5	118.4
14.0	122.6
18.0	122.8
22.0	123.3
24.0	123.4
29.0	124.0

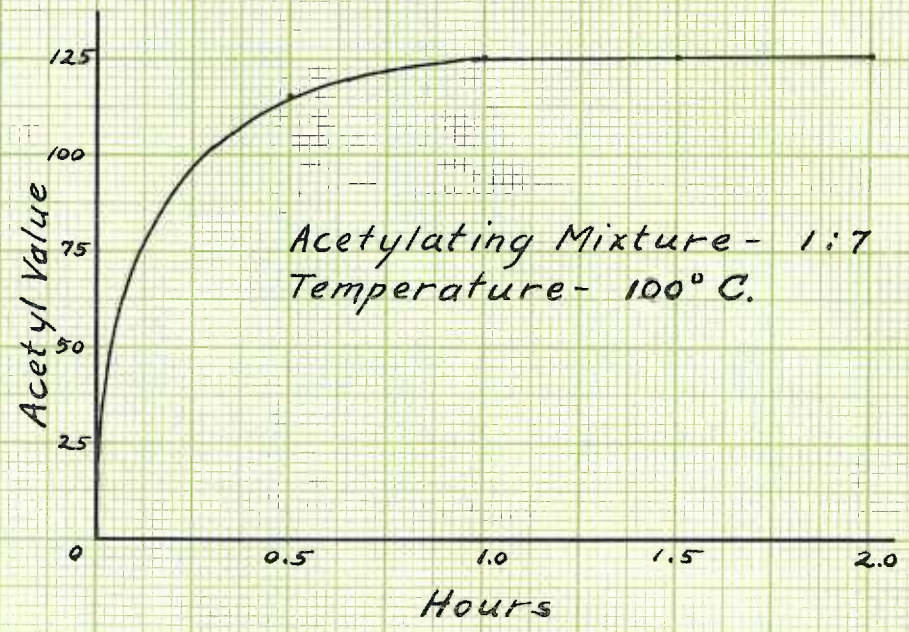
Temperature--100° C.
Acetylating mixture--1:7

Time (hours)	Acetyl value
0.50	115.4
0.75	122.1
1.00	125.4
1.50	125.5
2.00	126.0

Figure I

REACTION RATE OF ACETYLATING MIXTURE





Limitations of Sample Size. The lower limit of sample size is determined by the magnitude of titration difference; the upper limit by the acetylating capacity of the reagent. The latter was estimated by running acetyl determinations on increasing samples of castor oil using a 1:7 acetic anhydride-pyridine mixture at 100° C. The maximum sample weight for castor oil under these conditions is about 0.9 g. as shown by data in Table IV.

Table IV

Limitations of Sample Size

Test material--castor oil
Acetylating mixture--1:7
Heated at 100° C. for 1 hour

Wt. Castor Oil	Acetyl Value
0.6131	125.5
0.7442	125.5
0.8826	125.7
1.0157	123.1
1.1567	122.1

Amount of Water Required for Decomposition of the Anhydride-Pyridine Mixture (1:7 by volume). Volumes from 5 to 15 cc. gave identical results. Low values (for castor oil) were obtained when 3 cc. of water were used. The volume of 5 cc. was chosen as standard since with this quantity there is minimum dilution of the titration mixture.

A Study of the Formation and Decomposition of Higher Fatty Acid Anhydrides. In the course of an experiment on oleic acid, in which this substance was treated with a 1:7 acetylating mixture for an hour at 100° C., two peculiar phenomena were noticed (Table V):

(1) A rather high acetyl value, in spite of the fact that oleic acid is not hydroxylated;

(2) A steady decrease in acetyl value as the time between the decomposition of the excess reagent with water and the titration of the mixture increased.

No doubt, the apparent acetyl value was due to the formation of anhydrides of oleic acid which were gradually hydrolyzed on standing with water.

Table V

Acetyl Value of Oleic Acid

Acetylating mixture--1:7
Mixture treated with water at room temperature after heating at 100° C. for 1 hour.

Time* (min.)	Wt. sample	Acetyl Value
10	0.6829	15.7
20	0.9691	9.4
30	0.9246	8.5
40	0.9281	7.6

* Between addition of water and titration.

In another series of experiments one group of tubes was heated 10 minutes after adding water to decompose the excess of reagent; another group was not heated. The latter again gave evidence of considerable anhydride formation. The group which had been heated gave a low acetyl value as the data in Table VI shows.

Table VI

Acetyl Value of Oleic Acid

Acetylating mixture--1:7
Heated at 100° C. for 1 hour

Treatment after addition of water	Wt. sample	Acetyl value
Not heated	0.6858	14.1
	0.6634	13.7
	0.6696	16.6
Heated 10 min.	0.7371	2.5
	0.7449	2.8
	0.7378	2.1

The fact that the oleic acid gave an acetyl value of about 2 might be interpreted to mean that not all of the anhydrides had been decomposed. We have reason, however, to believe that this value is due to oxidation products in the oleic acid, since neutral equivalent determinations on this sample indicated its molecular weight to be 291 against a theoretical of 282.

Similar experiments were performed on palmitic acid with prolonged heating after decomposing the reagent. Palmitic acid showed the same general phenomenon (Table VII), but a much lower acetyl value. Undecomposed anhydrides, apparently cannot be responsible for these results for two reasons; first, during the period of heating following the addition of water, the acetyl value decreased to and remained at a minimum; and second, the palmitic acid was contaminated, since its molecular weight was 262 instead of a theoretical 256.

Table VII

Acetyl Value of Palmitic Acid

Treatment after addition of water	Wt. sample	Acetyl Value
Not heated	0.5800	32.2
	1.4995	17.9
Heated 15 min.	0.7970	0.8
	1.0242	1.0
Heated 30 min.	0.9047	0.9
	0.7934	1.1

The Effect of the Use of Condensers in the Reaction Tubes on the Precision of the Method. A considerable improvement in the precision of the method was obtained by replacing the glass bulbs used to cover the reaction tubes with simple condensers. These were made from 18 x 150 mm. Pyrex test tubes with rims. By filling these tubes with water and suspending them in the reaction tubes, the escape of acetic anhydride or acid was effectively prevented. If the rims of the condenser tubes are not wide enough to keep them suspended properly, a rubber ring placed around the top of the condenser serves the purpose.

Table VIII

Effect of the Use of Condensers in the Reaction Tubes on the Precision of the Method

Test substance--castor oil
Acetylating mixture--1:7
Tubes heated at 100° C. for 1 hr.

	With glass bulbs	With condensers
Acetyl Value Sample #1	124.0	124.7
	124.1	124.5
	125.8	124.3
	125.2	124.6
	125.0	124.3
	Ave. 124.8 ± 0.6	124.5 ± 0.1
Acetyl Value Sample #2	124.2	125.2
	125.8	125.2
	126.3	125.5
	126.4	125.5
	125.2	125.7
	Ave. 125.6 ± 0.7	125.4 ± 0.2

The Improved Method

Reagents

(1) Acetic anhydride--C. P. grade; Redistilled, the fraction boiling between 135-137° being collected.

(2) Pyridine--Mallinckrodt's medicinal grade; dried with "Drierite" and then distilled, the fraction passing over above 114° being used.

(3) Commercial normal butyl alcohol (Commercial Solvents Corporation).

(4) Alcoholic KOH--0.5 N aldehyde-free alcoholic potassium hydroxide is prepared according to the method of Malfatti (13) thus: 28 g. of the best grade of KOH and 45 g. of pure granulated calcium oxide are ground in a mortar to a fine powder. This mixture is transferred to a flask with 1000 ml. of 95% alcohol, rinsing the mortar with several portions of the alcohol. Mix thoroughly until the KOH is in solution. Cover the vessel and allow the solution to stand until the calcium hydroxide has completely settled and then filter. The alkali is standardized against 0.5 N acid using phenolphthalein and the titration apparatus described below. The solution should be kept in Pyrex glass.

Apparatus

(1) Pipettes for measuring acetic anhydride-pyridine mixture--Good grade Ostwald-Folin blood pipettes (with well ground tips) to deliver 5 ml. are very satisfactory.

(2) Burette--A good grade, narrow bore, 25 ml. burette graduated in 0.05 ml. is preferable. One graduated in 0.1 ml. may be used by exercising care in reading. The tip of the burette is fitted with a 20 gauge stainless hypodermic needle either by inserting the tip of the burette to the bottom of the hub of the needle and fastening securely with a tightly fitting piece of pure gum tubing or else by grinding the needle to the burette with fine carborundum and oil. This tip delivers about 0.007 ml. per drop of the alcoholic alkali solution. A small hand lens is helpful in reading the burette accurately. After use the solution should be drained from the burette, the latter rinsed with water and inverted until used again.

(3) Reaction tubes--Pyrex glass test tubes, 25 x 200 mm.

(4) Condenser tubes--Pyrex glass test tubes, 18 x 150 mm. (with rim). The condenser tubes are filled with water and suspended in the reaction tubes. If necessary, a rubber ring may be placed around the top of the condenser tube to prevent its slipping down into the reaction tube.

(5) Steam bath--A simple and inexpensive steam bath for heating the reaction tubes can be made by covering a hot water bath with a suitable board through which has been bored a number of 1 1/8 inch holes. The board is painted with a heat-resistant paint (Fischer's Plicote) and is reinforced by two iron strips screwed along the edges at

right angles to the grain. The bottoms of the reaction tubes are exposed to the steam by suspending the tubes about 5 cm. through the holes in the board. The tubes are prevented from slipping into the bath by means of rubber rings cut from large rubber tubing and placed around the tubes at the proper point.

Titrations

The burette is mounted on a stand fitted with a clamp to hold the reaction tubes so that the needle tip extends a short distance into the top of the tube. A thin stirring rod with a 15 mm. loop on the lower end and a right-angle bend at the top is used to stir the solution while titrating. The writers uses an easily constructed and inexpensive mechanical stirrer described by E. S. West (18). While excellent titrations can be done by hand stirring, the mechanical stirrer saves much labor when a large number of determinations must be run.

Procedure

Three dry reaction tubes are charged as follows: Two samples (0.5 to 1.0 gm. of materials with high acetyl values and 1.0 to 1.5 of those having low values) are weighed into reaction tubes (1) and (2), tube (3) being left blank. 5 ml. of the acetylating mixture (1 volume of acetic anhydride plus 7 volumes of pyridine) are carefully pipetted into tubes (1) and (3). To tube (2), 5 ml. of pyridine only are added. The condenser tubes are carefully filled with water (outside of tubes must be dry) and suspended in the reaction tubes

which are then placed in the holes of the board on the steam bath and heated 1½ hours. The condensers are slightly raised and 5 ml. of water are added to each reaction tube, the condensers replaced and the tubes heated 15 min. longer with occasional careful shaking. After cooling, the condensers and reaction tubes are rinsed down with 15 ml. of butyl alcohol and the titrations carried out as described above with the alcoholic KOH using 4 drops of 1 per cent phenolphthalein as indicator. The titrations of duplicate blanks should not vary more than 0.02 ml. The endpoints are exceedingly sharp.

Calculations

According to the West definition, the acetyl value equals the milligrams of acetyl bound per gram of substance. Calculations are as follows: Titration values are converted to cc. of 0.1 N alkali. Let

A = acidity (cc. of 0.1 N) of 5 ml. of acetylating mixture (tube 3)

B = acidity (cc. of 0.1 N) of 5 ml. of acetylating mixture plus sample (tube 1)

C = acidity (cc. of 0.1 N) of the sample in tube (1) calculated from titration of sample in 5 ml. pyridine (tube 2)

D = acidity equivalent of acetyl bound by the sample
= A - (B - C)

E = weight of sample

Then, acetyl value = $\frac{D \times 4.3}{E}$

Comparison of Several Methods

In a comparative study of methods of determining acetyl values of fats and oils, analyses were made of four oil samples supplied for the purpose by the Association of Official Agricultural Chemists. The methods under investigation were the following: the official method of the A. O. A. C. which consists in acetylating the material according to the directions of Lewkowitsch and analyzing the acetylated product by the Andre-Cook procedure; the Roberts-Schuette method; and the improved method described in this paper.

Analyses of the four oil samples by the three methods gave discordant results which in one case disagreed as much as 25%. Such wide variations may have been due to incomplete acetylation or some other factors inherent in the methods. On the other hand, the presence of considerable amounts of free fatty acids in the samples may have been the cause. The latter possibility was easily tested by running analyses on a known mixture containing a high proportion of free acids. Any errors introduced by this factor would under such conditions be magnified and easily recognized.

For this purpose, a mixture of castor oil, oleic acid and butyric acid was made containing 57.1, 28.6 and 14.3 per cent by weight of the constituents, respectively. Knowing the acetyl values of castor oil as determined by the different methods and the percentage composition of the mixture, the theoretical acetyl value of the mixture for a given method was

then compared with that obtained by actual analysis of the mixture. In Table IX, which follows, are listed the results of such analyses.

Table IX

Comparison of the Methods of Roberts-Schuette, Andre-Cook, and the Improved Method when Applied to a Known Mixture of Castor Oil, Oleic Acid, and Butyric Acid

Method	Acetyl Value of Castor Oil	Theoretical Acetyl Value of Mixture	Acetyl Value of Mixture as Determined
Andre-Cook	126.3	72.1	14.6
Roberts-Schuette	127.1	72.5	78.8
Improved Method	125.6	71.6	71.9

Excellent agreement with the theoretical value was obtained only with the improved method. In the Andre-Cook procedure, the loss of butyric acid in the washing process after acetylation probably constituted the main source of error. Two factors very likely affected the results of the Roberts-Schuette method: first, incomplete decomposition of higher fatty acid anhydrides; and second, difficulties in the titration of insoluble fatty acids in an aqueous medium. In addition to the more reliable results obtained by the improved method, this method also offers advantages over the others in its simplicity and rapidity. No preliminary treatment of the sample is necessary. The operations of the procedure are restricted to weighing, pipetting and titrating, without the transfer of the weighed sample from one vessel

to another. Finally, duplicate or triplicate determinations can be run simultaneously without greatly increasing the time of analysis and with no increase in the amount of apparatus outside of a few test tubes.

The Acetyl Values of Fats and Free Fatty Acids

The acetyl values of a number of fats and oils were determined and about seven months later the determinations were repeated. These values, together with the acidity of the fats and oils are tabulated in Table X. It is interesting to note that an increase in acidity is usually accompanied by an increase in the acetyl value. This phenomenon is probably due to the fact that when fatty acid molecules are split from the fat molecule by hydrolysis, glyceryl hydroxyl groups are produced, which increases the acetyl value.

The next subject investigated was the acetyl values of free fatty acids prepared from various fats. The principles employed in the preparation of the fatty acids consist of the saponification of the fat, extraction of the soap solution with ether to remove sterols, liberation of the fatty acids from the soap by acidification with hydrochloric acid, and washing the free fatty acids with water to remove glycerol and mineral acid. The procedure in detail is as follows:

Redistilled alcohol (70 ml.)and KOH (8 gm.) are placed in a 250 ml. flask and warmed. When the KOH is dissolved, 25 gm. of fat are added and the mixture is refluxed for 1½ hours, after which it is transferred to a separatory funnel with 250 ml. of water. The soap solution is extracted* successively

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* The procedure for the extraction of the soap solution is based on Wilkie's method for determination of unsaponifiable matter in fats (20).

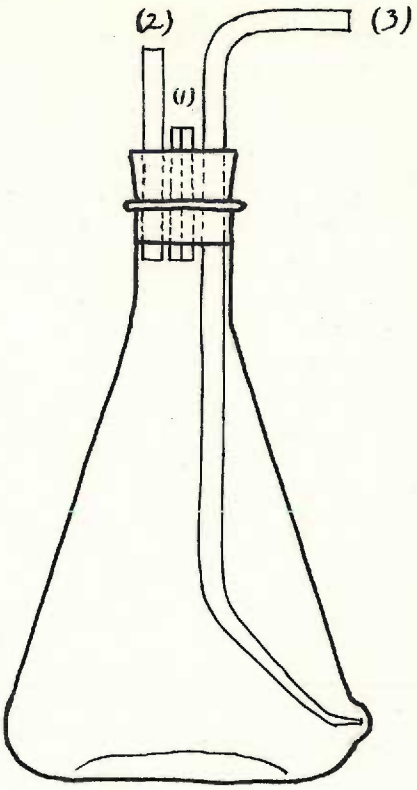
with a 200 ml. and three 150 ml. portions of peroxide-free ether. The combined ether extract is run into 100 ml. of water without shaking, and the water is run off. The ether extract is then further washed successively with 10, 25, and 75 ml. of water with vigorous shaking. The combined aqueous extract is added to the main portion of the soap solution and placed in a liter distilling flask.

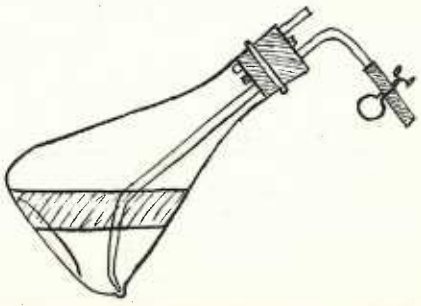
The solution is now treated with an excess of conc. HCl (15-16 ml. are sufficient) with vigorous shaking in order to liberate the fatty acids. The flask is connected with a condenser and the ether distilled off on a water bath with occasional shaking. A slow stream of air, CO_2, or N_2 and slightly reduced pressure speed up the distillation. When the ether has been removed, the aqueous layer is separated from the fatty acids, which are then washed with three 200 ml. portions of hot water to remove the mineral acid. This operation may be conveniently carried out in a simple apparatus constructed as follows: (see Figure II)

A 500 ml. Erlenmeyer flask is fitted with a 3-hole rubber stopper bearing (1) a fine capillary vent tube; (2) a short large-bore tube extending just through the stopper; and (3) a tube drawn to a small tip and bent so as to extend into a small bulge blown into the wall of the flask near the bottom. The acids are placed in this flask and are vigorously shaken with hot water. When the water and the acids have separated into two sharp layers, the flask is tilted at an

Figure II

Separatory Flask





Position for draining off bottom layer

angle with the bulge downward, a small positive pressure is applied to tube (2), which forces the water out through tube (3), the flow being regulated by a pinch clamp on a short piece of rubber tubing fitted on tube (3). When the water has been drained to the bottom of the bulge, the flow is momentarily stopped and the flask given a quarter turn to bring the tip of the tube above the level of the liquid. In this position, the water remaining in the tube may be blown out.

The acids, separated from water as thoroughly as possible, are dissolved in 50 ml. of dry, peroxide-free ether and dried with a few grams of anhydrous sodium sulfate. After filtration, the ether solution is transferred to a small distilling flask and the ether distilled off on a water bath, removing traces of ether under vacuum.

An interesting phenomenon was noticed in the marked increase of the acetyl values of free fatty acids which had been exposed to air for some length of time, as shown in Table XI. This increase in hydroxylation was no doubt the result of a process of auto-oxidation. This immediately raised the question as to the possibility of auto-oxidation taking place during the preparation of the acids. A number of acids were therefore prepared both in the presence and absence of air, and the acetyl values compared. The first three columns of Table XII show no great differences with regard to this point. A few preparations exclusively in nitrogen showed the variations to be within the limits of experimental error. It was concluded,

therefore, that the process of auto-oxidation is slow and does not affect the values of freshly prepared acids.

The final point investigated was the extent of the esterification of hydroxylated fatty acids and its effect on acetyl values. If a hydroxylated acid is esterified with another fatty acid, the hydroxyl group is blocked, with the result that the acetyl value does not indicate the true hydroxylation. If, however, the calculation of acetyl value is based on the saponifiable acidity of the fatty acids (determined by heating the acids with an excess of standard alcoholic solution of alkali and then titrating the excess with standard acid), rather than the simple titratable acidity, the error resulting from esterification is corrected.

In Table XIII, comparing values in columns A_1 and A_2, which give the neutral equivalents of acids (prepared in air) calculated on the basis of both titratable and saponifiable acidity, we see the following variations between the two methods:

4 samples -- 0.0% variation
2 samples -- 0.4% "
2 samples -- 0.7% "
1 sample -- 1.0% "
3 samples -- 1.4% "

Since two thirds of the values show either no differences or differences within the limits of experimental error, we must conclude that esterification probably takes place only to a very limited and insignificant extent. The greater differences in a few cases may have been due to other contaminating substances present in the acids. The acids which were prepared in the presence of carbon dioxide all showed considerable variations and these, we feel, were caused by the carbon dioxide which was not all eliminated from the fatty acids before titration.

Table X

Acetyl Values and Acidity Values* of Fats and Oils

Fat or Oil	Acetyl Value		Acidity Value	
	First Analysis	Analysis after 7 mo.	First Analysis	Analysis after 7 mo.
Butterfat	2.9	2.7	0.32	0.32
Castor Oil #1	124.5	-	0.10	-
Castor Oil #2	125.4	-	0.40	-
Coconut Oil	1.8	1.8	0.04	0.07
Cod Liver Oil	2.4	4.1	0.32	0.50
Corn Oil	3.0	3.6	0.09	0.15
Cottonseed Oil #1	4.0	5.2	0.04	0.19
Cottonseed Oil #2	4.4	-	0.11	-
Lard	0.9	1.5	0.23	0.34
Linseed Oil Raw	4.6	5.3	0.44	0.54
Linseed Oil Boiled	6.0	7.9	0.73	0.88
Neatsfoot Oil	7.2	9.0	2.11	2.24
Olive Oil #1	4.1	13.4	0.54	1.25
Olive Oil #2	3.1	-	0.17	-
Peanut Oil	2.6	2.6	0.14	0.19
Salmon Oil	3.7	-	0.66	-

* The acidity value is defined as the number of cc. of N/10 NaOH required to neutralize the free fatty acids present in 1 gm. of the fat.

Table XI

Auto-oxidation of Fatty Acids

Comparison of Acetyl Values of Fatty Acids Determined Immediately After Preparation and After Standing Two Months

Source of Acids	Acetyl Value Fresh	Acetyl Value Two Mo. Old
Butterfat	1.4	12.2
Coconut Oil	0.4	1.7
Corn Oil	2.0	13.4
Cottonseed Oil	3.3	20.3
Lard	1.2	6.5
Linseed Oil Raw	4.7	*
Linseed Oil Boiled	5.5	*
Neatsfoot Oil	5.4	5.2
Olive Oil	1.7	9.9
Peanut Oil	2.4	19.8

* Samples marked thus darkened to such an extent that titration was impossible.

Table XII

Acetyl Values of Free Fatty Acids

Source of Acids	Acetyl Values Calculated on basis of acidity of acids after heating with pyridine		
	A	B	C
Butterfat	1.4	1.3	-
Castor Oil	125.	-	-
Coconut Oil	0.4	0.6	-
Cod Liver Oil	-	2.3	-
Corn Oil	2.0	3.2	2.3
Cottonseed Oil	2.9	4.0	-
Lard	0.7	0.8	-
Linseed Oil Raw	3.4	3.8	-
Linseed Oil Boiled	3.9	4.0	-
Neatsfoot Oil	3.8	5.6	-
Olive Oil	0.4	-	-
Peanut Oil	2.4	2.9	2.4
Salmon Oil	2.2	-	-

A. The entire preparation of fatty acids carried out in the presence of air.

B. The saponification was done in an atmosphere of nitrogen; the rest of the procedure in carbon dioxide.

C. The entire preparation of fatty acids carried out in an atmosphere of nitrogen.

Table XIII

Neutral Equivalents of Free Fatty Acids

Source of Acids	Neutral Equivalents Calculated on basis of acidity of acids after heating with pyridine			Neutral Equivalents Calculated on basis of saponification values of acids		
	A_1	B_1	C_1	A_2	B_2	C_2
Butterfat	253	253	-	253	252	-
Castor Oil	305	-	-	301	-	-
Coconut Oil	210	207	-	210	206	-
Cod Liver Oil	-	291	-	-	287	-
Corn Oil	282	281	282	282	278	279
Cottonseed Oil	278	275	-	277	273	-
Lard	277	276	-	276	274	-
Linseed Oil Raw	285	283	-	283	278	-
Linseed Oil Boiled	288	283	-	284	280	-
Neatsfoot Oil	288	286	-	285	282	-
Olive Oil	280	-	-	278	-	-
Peanut Oil	284	284	284	284	282	280
Salmon Oil	284	-	-	280	-	-

A, B, C have the same designation as in Table XII.

Discussion

Regarding the acetyl values of fatty acids as determined by Benedikt and Ulzer's method, Lewkowitsch said that

> "----most of the numbers contained in the older literature and stated to indicate the presence of hydroxylated fatty acids in natural oils and fats (with the exception of castor oil and, perhaps, grape seed oil and quince oil) must be rejected as fictitious values."

We have evidence, however, from the analyses of a considerable number of free fatty acids by an improved method for determining acetyl values, that the presence of small amounts of hydroxylated fatty acids in fats and oils of plant and animal origin is quite general. This fact becomes of some significance in consideration of the possible role of hydroxylated fatty acids in fatty acid metabolism. The demonstration of increased amounts of hydroxy-acids in the tissues of animals in a state of increased fat metabolism would lend direct proof to Dakin's theory as to the mechanism of fatty acid oxidation. The improved method is well suited for this purpose, and research along such lines is contemplated.

Summary

1. Methods of determining the acetyl values of fats and fatty acids have been discussed.

2. An improved procedure for the determination of the acetyl values of fats and fatty acids has been described and compared with other methods.

3. A study was made of the acetyl values of a number of fats and fatty acids and the following points were demonstrated:

a. The concurrent rise in acetyl values of fats with an increase in free acidity;

b. The auto-oxidation of free fatty acids on long exposure to air, with the formation of hydroxylated acids;

c. The general presence of hydroxylated fatty acids in fats and oils of both plant and animal origin.

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