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A New Oxidation Method for the Determination of Saturated Fatty Acids

J. FITELSON, U. S. Food and Drug Administration, Federal Security Agency, New York City

METHODS available for the estimation of saturated fatty acids in natural fats are not entirely satisfactory. Fats containing appreciable quantities of highly unsaturated acids, solid unsaturated acids, or lower saturated acids can be analyzed only by special, elaborate methods unsuitable for routine work. The widely used Twitchell and Bertram methods are time-consuming, restriced to certain fats, and may yield inaccurate results (1, 2). Recent collaborative studies by several participating laboratories on three analytical methods produced widely divergent results (3). However the average results obtained by a modified Bertram oxidation method (4) and a low-temperature crystallization procedure (5) were in agreement and were higher than those obtained by the A.O.C.S. lead salt-alcohol method (6).

In a search for a rapid method, procedures based upon the solubility of the fatty acids or their salts in various solvents were rejected as too limited in application. Oxidation without disruption of the unsaturated acids, which would preclude formation of interfering lower saturated acids, offered a promising method of approach to the problem. These oxidative properties have been claimed for peracetic acid in its use as a quantitative reagent to determine the unsaturation of fats with isolated ethylenic linkages (7). Peracetic acid has also been used in investigations of the structure of oleic acid, elaidic acid, and various natural fats (8). More recently, performic acid has been proposed as an efficient and rapid hydroxylating agent for certain mono-unsaturated compounds (9), and a modified performic acid solution has been used in the work described here.

Preliminary experiments on the oxidation of unsaturated acids with performic acid indicated the formation of only small quantities of decomposition products. The main oxidation products are saturated hydroxy and hydroxy-formoxy acids, together with variable quantities of the ill-defined, oily acids first described by Hilditch and Lea (8c). Partial purification of the saturated acids can be obtained by extraction with petroleum ether since most of the oxidized acids are insoluble in this solvent. The esterified hydroxy acids are soluble in petroleum ether, and it is necessary to hydrolyze these compounds to the insoluble hydroxy acids to effect a further concentration. The saturated acids at this stage contain small quantities of the oily, oxidized acids, together with traces of hydroxy acids. These contaminants can be removed by a chromatographic procedure, using activated silica as the selective adsorbent. The final residue is essentially free from unsaturated acids but contains a small quantity of unsaponifiable matter formed by disruptive oxidation of the unsaturated acids.

Experimental

Most of the reagents used in this work are described in the method. Saturated fatty acids, C_6 to C_{18}, were obtained from Eastman Kodak Company and used without further purification. Corrections for iodine values of 0.12, 0.42, and 0.67, found respectively in the myristic, palmitic, and stearic acids, were applied when required. Oleic, linoleic, and linolenic acids were prepared under non-oxidizing conditions from the methyl esters furnished by the Hormel Foundation, Austin, Minn. The following pertinent analytical information was supplied with these samples: methyl oleate, iodine value 85.88, saturated acids 0.12%; methyl linoleate, iodine value 172.4, saturated acids—none; methyl linolenate, iodine value 258.6, saturated acids—none. Dihydroxystearic acid, m.p. 94.5°-95°C., was prepared by performic oxidation of oleic acid (9) and recrystallized from ethyl acetate and dilute alcohol.

a) Oxidation of Unsaturated Acids

1. *Preparation of Oxidizing Reagent:* Mixtures of hydrogen peroxide (30%) and formic acid (98-100%) yield maximum concentrations of performic acid within one to two hours and then slowly decompose (10, 11). At higher temperatures decomposition increases and becomes quite rapid at 80°C. (12). To avoid the long reaction period necessitated by the insolubility of the fatty acids in the aqueous performic acid, a glacial acetic acid solution of this oxidant was made by adding acetic anhydride. Ready solution of the fatty acids in the non-aqueous reagent results in rapid oxidation. The reagent contains no acetic anhydride, thereby preventing excessive acylation of the hydroxyl groups formed in the oxidation. Maximum performic acid concentration of about 5% by weight is usually reached one hour after addition of the anhydride and can be maintained for about one week with only slight loss by storage in the refrigerator. Performic acid content was determined in most of the preparations by the permanganate-thiosulfate titration of D'Ans and Frey (13). The recently published ceric sulfate-thiosulfate titration proposed by Greenspan (14) is more satisfactory and was used in some of the later work. In most of the experiments peracid titrations of 18 to 19 ml. of 0.1 N thiosulfate per ml. of oxidizing reagent were found.

2. *Optimum Oxidation Conditions:* Separation of the saturated acids from the oily, oxidized substances produced in the performic acid oxidation proved to be the most difficult operation in the method. Since

the largest quantities of these contaminants are produced by oleic acid, olive oil fatty acids were used to obtain oxidation data. Controlled experiments on variations in the temperature, weight of sample, time of reaction, and quantity and concentration of oxidizing reagent were conducted. Optimum conditions were found at 70°-75°C., using 25 ml. of reagent, 2.5 gm. sample, and a 30-minute oxidation period. Lower temperatures resulted in formation of excessive undesirable products (8c) while higher temperatures were precluded by thermal decomposition of the reagent before completion of the oxidation. The conditions selected for the reaction are not critical so that slight deviations from the procedure are permissible.

Although other investigators have reported only negligible oxidation of saturated fatty acids by peracids in the absence of catalysts (15), the effect of the performic acid reagent on C_{10} to C_{18} saturated acids at 70°C. was studied. All of the saturated acids could be quantitatively recovered after this oxidation treatment. Absence of oxidized acids was demonstrated by the chromatographic purification method described elsewhere.

b) PRELIMINARY SEPARATION OF THE SATURATED ACIDS

1. *Solvent Purification:* Saturated acids, co-precipitated with hydroxy acids, cannot be quantitatively separated by direct extraction with petroleum ether (2). However separations can be effected by agitation with a mixture of petroleum ether and dilute alcohol. A 55% alcohol solution is an effective solvent for dihydroxystearic acid and dissolves only minor quantities of higher saturated acids. These small losses can be avoided by diluting the alcoholic solution with water in a second separatory funnel and extracting these precipitated acids with petroleum ether. In one experiment palmitic and dihydroxystearic acids were precipitated together from an alcohol solution. Direct extraction of these mixed acids with petroleum ether recovered only 90% of the palmitic acid while treatment by the immiscible solvent procedure gave a 99.5% recovery. Corrections for the solubility of dihydroxystearic acid in the petroleum ether (3 mg. per 100 ml.) were applied.

When oxidized natural fatty acids are extracted, the petroleum ether dissolves some interfering material, mainly partially esterified hydroxy acids. Conversion to the insoluble hydroxy acids is readily accomplished by cold hydrolysis with alcoholic potash so that the final petroleum ether residue consists mainly of saturated acids, together with a small proportion of oxidized acids. When the fatty acids from rancid oils were analyzed, intractable emulsions frequently formed in the second separatory funnel. These could be broken by adding a small quantity of saturated sodium chloride solution, acidified with hydrochloric acid, and permitting the layers to separate for 10 minutes after agitation. This modification permitted sharper separations and resulted in better recoveries of saturated acids.

Since the lower saturated acids are appreciably soluble in dilute alcohol, it was necessary to ascertain the limitations of this extraction procedure. Weighed quantities of saturated acids were dissolved in 150 ml. of redistilled petroleum ether, washed successively with four 50-ml. portions of 55% alcohol, 350 ml. of 7% alcohol, and finally with two 50-ml. portions of water. Each washing was transferred to a second separatory funnel, where the 55% alcohol portions were each diluted with 300 ml. of water. After agitation and separation, each of the washings was successively extracted in a third separatory funnel. The petroleum ether solutions from each funnel were evaporated separately and the residues weighed. Table I shows that the C_{10} and higher saturated acids can be satisfactorily treated by this technique while losses with C_8 become appreciable only when this acid is present in large quantities. Lower saturated acids are too soluble in the aqueous solutions and cannot be recovered by this process. For fats containing only minor quantities of C_{12} or lower acids the use of two separatory funnels for the extraction procedure should be satisfactory.

TABLE I

Application of Extraction Procedure to Pure Saturated Acids

Saturated Acids	Per Cent Saturated Acids Found in the			Total Loss of Saturated Acids Using	
	First Separatory Funnel	Second Separatory Funnel	Third Separatory Funnel	Two Separatory Funnels	Three Separatory Funnels
C_{16}	96.5	2.7	0.3	0.8	0.5
C_{14}	94.9	4.2	0.3	0.9	0.6
C_{12}	86.7	11.8	0.9	1.5	0.6
C_{10}	74.8	21.7	1.6	3.5	1.9
C_8	42.8	43.2	8.0	14.0	6.0
C_6	4.5	1.2	4.0	94.3	90.3

2. *Drying Temperatures for Saturated Acids:* Lewkowitsch (16) records losses on heating saturated acids on the steam bath. In some of the preceding experiments considerable volatilization of the lower acids was also noted so that it became necessary to determine appropriate drying temperatures. It was found that losses at 100°C. were significant for the C_{12} or lower acids so that fats containing appreciable quantities of these acids must be dried at 80° or lower temperatures. At 80°C. the volatilization of C_6 and higher acids becomes unimportant if a non-volatile fixative (palmitic acid) is present. To determine potential maximum heating losses of natural products, mixtures were made to resemble coconut oil and milk fat saturated acids. The former mixture showed an average loss of 2.5 mg. per hour at 80°C. while the latter lost 9.0 mg. per hour. In some of these heating experiments, it was observed that the last traces of petroleum ether (b.p. 60°-70°C.) were removed with difficulty when heated at 80°C. and therefore the lower boiling redistilled A.O.C.S. solvent (35°-60°C.) was used in all experimental work.

c) FINAL PURIFICATION OF THE SATURATED ACIDS

1. *Preliminary Chromatographic Investigations:* The contaminants in the petroleum ether residue, ranging from 0.04 to 0.12 grams for the majority of the fats examined, exhibited most of the properties of saturated acids so that the usual methods of removal cannot be applied. Williams (17) separated air-oxidized acids from normal acids by adsorption on a column of calcium carbonate, but this adsorbent exhibited no selectivity for the residues obtained here. Cassidy (18) and Kaufmann (19) used activated silica in some of their work on the separation of saturated acids. Preliminary experiments showed that silica, activated by heating at 100°C., permitted ready elution of the saturated acids while retaining the oxidized acids. However extreme variations in activity were found among the several batches examined. These differences in the efficiency of the adsorbent depend largely upon the particle size, the

coarser lots producing poor separations. Finer silica, with 80% passing through a 200-mesh screen, is quite efficient but packs down too tightly in the adsorption column. Good flow characteristics can be obtained by mixing the silica with half its weight of Hyflo Supercel, a siliceous filter aid.

Many liquids were examined to find an efficient solvent for the selective elution of the saturated acids. Non-polar solvents are inactive while oxygenated liquids remove all adsorbed acids without separation. Benzol, washed and dried chloroform, and dichloromethane are satisfactory developing solvents. The dichloromethane permits a sharp separation, and the saturated acids are quantitatively extracted in one hour by passage of about 400 ml. through a column 7 cm. high and 2.6 cm. diameter. Wide variations in rate of flow disclosed no effects on the extraction efficiency. Some acid-base indicators in the adsorbent can be used to follow the course of the elution process. Although all of the indicators tested were relatively insensitive under the non-aqueous conditions of use, methyl orange exhibited sufficient color change to locate the acids as pink colored bands.

2. *Standardization of the Adsorbent:* Although control of particle size and method of activation eliminated the extreme variability in activity previously encountered, the differences in adsorptive capacities of various batches of adsorbents were still sufficient to prevent adoption of a uniform elution procedure. Standardization of adsorbents to some arbitrary activity value seemed preferable to adjustment of the elution volumes with each new lot. Separation of dyes (20) and threshold volume (21) have been proposed for the characterization of adsorbents. Le Rosen (22) used R, the ratio of the rate of movement of an adsorbed zone to the rate of flow of the developing solvent, as well as several other values, to measure adsorption activity. However this activity is best indicated by the adsorption isotherm. Cassidy and Wood (23) found that the adsorption of lauric acid by carbon could be described by a Freundlich type of isotherm. Papps and Othmer (24) obtained Langmuir isotherms for stearic acid and carbon with some solvents while other solvents gave Freundlich equations.

The activity of several batches of the silica-filter aid mixture was investigated over a wide range of solute concentrations by placing 2 gm. of the adsorbent in contact with 100 ml. of a Skellysolve B solution of palmitic acid at 25°C. (±2°C.) for one hour. Equilibrium was rapidly attained; longer periods of contact showed no increase in adsorption. All of the adsorbents examined were found to follow a Freundlich type of isotherm. The curves obtained by plotting c, the equilibrium concentration of palmitic acid, expressed as mg. per 100 ml. of solution, against Q, mg. of palmitic acid adsorbed by 1 gm. of adsorbent, were similar for all batches and exhibited parallel linearity over the 300-500 mg. per 100 ml. concentration range. These linear portions of the curves can be described by the equation $Q=k+0.055c$, where k, denoting the Q axis intercept, is a constant characterizing the activity of the adsorbent. Values of k can thus be readily obtained by a single determination, using an initial concentration of about 500 mg. of palmitic acid per 100 ml. solution to insure the desired equilibrium concentration.

The chromatographic qualities of several batches of adsorbents with k values ranging from 40 to 80 were tested. Since the lower saturated acids are eluted less readily than the higher acids, weighed quantities of both palmitic and capric acids were passed through the columns. The highly active mixtures (k greater than 60) retained appreciable quantities of the saturated acids after passage of the arbitrarily chosen 400 ml. of dichloromethane through the column. Since adsorbents with low activity may permit leakage of oxidized acids into the percolate, a k of 50 (±3) was taken as optimum for standardization purposes.

The standardized silica adsorbent can be prepared in several ways. Activation by heating or deactivation by exposure to a moist atmosphere can be controlled so that the final adsorbent has the required activity. Changes in efficiency can also be obtained through variation in the proportion of silica in the adsorbent. Since the 2:1 ratio of silica and filter aid has satisfactory packing characteristics, this mixture was kept constant and the standardized adsorbent prepared by mixing an inactive batch with a highly active lot in the proper proportions.

The precision obtained by this method of standardization and calculation is illustrated in Table II.

TABLE II

Activity Constants of Silica Adsorbent Mixtures

Inactivated Adsorbent	Activated Adsorbent	Final Adsorbent Mixture			
Activity Constant k_x	Activity Constant k_y	% Inactive Adsorbent x	% Active Adsorbent y	Activity Constant, Calculated k_z	Activity Constant, Found k_z
28.0	65.3	50	50	46.7	45.8 45.9 Av. 45.9
28.0	58.9	28.6	71.4	50.1	48.6
28.0	64.9	30	70	53.8	52.8
38.9 37.7 Av. 38.3	75.1 76.6 Av. 75.9	67	33	50.8	50.8 50.2 Av. 50.5
25.3 24.8 Av. 25.1	78.8 81.3 Av. 80.1	53	47	51.0	51.4

3. *Chromatographic Removal of Oxidized Acids:* The insolubility of dihydroxy acids in petroleum ether restricts their importance as interfering impurities in the extracted residues. Removal of these traces of hydroxy acids by the chromatographic procedure is readily accomplished since they remain at the top of the adsorption column during the elution of the saturated acids. Various mixtures of palmitic and dihydroxystearic acid (m.p. 95°) were tested and easily separated by passage through a 7-cm. column, using 400 ml. of dichloromethane as developing solvent.

Most of the foreign acid material in the saturated acid residue is a yellow oil which usually remains in the upper half of the adsorption tube and separates into two bands during the elution process. The lower band, which is barely perceptible with the methyl orange indicator, can be washed through the column with U.S.P. chloroform and consists of a yellow, viscous oily acid. The more highly adsorbed band can be extracted as a yellow oil with ethyl ether. The chromatographic separation of these oxidized acids from saturated acids becomes difficult only with oils high in oleic acid, such as olive oil. In the examination of such fats larger quantities of these acids appear in the petroleum ether residues and are observed to move farther down the column during elu-

TABLE III

Analysis of Pure Fatty Acid Mixtures by Proposed Method

Experiment No.	Weight Unsaturated Acids (Grams)			Weight Saturated Acids (Grams)							Total Mixed Acid Basis				
	Oleic (d)	Linoleic	Linolenic	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	(a) % Saturated Acids	% Saturated Acids	% Unsaponifiable Matter in Sat. Acids	Iodine Number of Sat. Acids	Corrected % Sat. Acids
											Calcu'ted	Found			Found
1	1.5756											0.64(b)	0.46		0.18
2		0.9102										0.64(b)	0.64		None
3			1.4477									0.41(b)	0.41		None
4	2.0103	0.1420						0.0143	0.2694	0.0468	13.34	14.03(b)	0.54	0.70	13.4
5-1	1.0315	1.2652							0.1210	0.1383	10.13	10.09(b)	0.24	0.91	9.8
5-2	1.0578	1.2380							0.1202	0.1539	10.67	10.91(c)	0.12	0.90	10.7
6-1	0.2363	0.9920	1.0606					0.0084	0.1460	0.1057	10.14	9.87(b)	0.29	0.93	9.5
6-2	0.2497	0.9810	1.1601					0.0135	0.1436	0.1399	10.99	10.99(c)	0.11	0.40	10.8
6-3	0.2250	0.7647	1.2580					0.0177	0.1340	0.1369	11.32	10.90(c)	0.15	0.41	10.7
7	0.9560	0.0762							0.6253	0.9055	59.40	60.22(b)	0.65	0.00	59.6
8-1	0.9405	0.3987					0.9390		0.2312		43.68	44.43(b)			
8-2	0.9656	0.4624					0.9378		0.2577		45.50	45.28(c)	0.19	0.14	45.0
9-1	0.2227	0.0523		0.0030	0.1953	0.2532	1.1068	0.4320	0.2350	0.0508	89.31	83.27(b)			
9-2	0.2099	0.0547		0.0205	0.1796	0.2773	1.1938	0.4616	0.2525	0.0707	90.00	87.82(c)	0.30	0.00	87.5

(a) Corrected for 0.12% saturated acids in oleic acid and for unsaturation of saturated acids.
(b) Two separatory funnels used in extraction procedure.
(c) Three separatory funnels used in extraction procedure.
(d) Oleic acid, prepared from methyl oleate, contained 0.12% saturated acids.

tion of the saturated acids. Since the methyl orange indicator used is not sensitive to very small quantities of acids, an additional 100 ml. of dichloromethane was passed through the column and collected separately as a precautionary measure to detect leakage of these oxidized acids. In most cases this residue was less than 2 mg. This indicated a satisfactory chromatographic separation since a second passage of the main residue through the column gave no appreciable change in the weight of the saturated acids. In a few analyses of olive oil fatty acids, where the methyl orange indicated that the oxidized acids had reached the bottom of the column, check residues of 2 to 6 mg. were obtained. When the corresponding saturated acids were rechromatographed, the results were lowered by 0.1 to 0.3%.

d) Application of the Proposed Method to Mixtures of Pure Acids

Pure saturated and unsaturated acids were examined by the proposed method. The unsaturated acids with isolated double bands gave 0.4-0.6% of final residues. The residue from oleic acid contained 0.18% saturated acid (original sample basis) together with some unsaponifiable matter while only non-acidic material was present in the residues from the linoleic and linolenic acids. Since pure saturated acids are recovered quantitatively with no unsaponifiable matter when examined by this method, these non-acid residues must originate from decomposition of the oxidized unsaturated acids. The residues obtained from mixtures of pure saturated and unsaturated acids were also examined for unsaponifiable matter by the S.P.A. (25), and the fatty acids were extracted with petroleum ether after acidification of the unsaponifiable free soaps. The iodine values (Hanus) of these saturated acids were not significant, resulting in corrections of less than 0.1% as oleic acid.

Analytical data for these synthetic mixtures, made to resemble the acid composition of various natural fats, are shown in Table III. In the mixtures containing only higher saturated acids the maximum loss of 0.6% is shown by the linseed type acids (Experiment 6-1). The use of three separatory funnels in the extraction procedure results in a slight increase in recovery when only higher saturated acids are present. However this additional extraction becomes significant when large quantities of C_{12} or lower acids are present, as in laurel oil acids (Experiment 8). Fats containing significant amounts of C_8 or lower acids, such as coconut oil, cannot be satisfactorily analyzed by this method (Experiment 9).

e) Recovery Experiments on Saturated Acids Added to Natural Fatty Acids

As an added check on the adequacy of the method, saturated acids were added to the olive oil acids used in the oxidation experiments and the mixed acids examined carefully. These experiments (Table IV) showed no additional difficulties, and the recoveries of the added acids were quite good.

TABLE IV

Recovery of Saturated Acids Added to Olive Oil Acids

Weight Mixed Olive Oil Acids	Weight Saturated Acid Added	Total Weight Saturated Acids Found	Weight Added Saturated Acids Recovered	% Added Saturated Acids Recovered
gm.	*gm.*	*gm.*(a)	*gm.*	
2.1692		0.3373 (15.55%)		
1.7476	0.3992 Stearic	0.6657	0.3939	98.7
2.0958	0.3404 Lauric	0.6634	0.3375	99.2
1.8496	0.4660 Capric	0.7529	0.4653	99.9

(a) Three-separatory funnel procedure used.

Method

A. Apparatus

Glass adsorption tube—2.6-cm. internal diameter; ca 30 cm. long; joined at the bottom to a narrow glass tube serving as outlet.

Cotton pad (milk-sediment disc)—ca 2.8-cm. diameter.

Glass Buchner funnel with fritted disc—medium porosity; 40-mm. disc diameter; 50 mm. high; 60-ml. capacity. (For use in an all-glass suction filter device, the exit tube of the funnel passes through a short section of a wider tube to which it is fused at the upper end. This wide tube is provided with a side arm and an inner 29/42 standard taper joint.)

Erlenmeyer flask, 1-liter. (A flask with outer 29/42 standard taper joint is used with the modified Buchner funnel in the glass filtering device.)

Extraction flask, 100-ml.—wide mouth; flat bottom.

B. Reagents and Solutions

Hydrogen Peroxide, 30%, C.P.

Formic Acid, 98-100%. (Eastman Kodak Co., Rochester, N. Y.)

Acetic Anhydride, A.C.S.

Ethyl Alcohol, 95%. (U.S.S.D. Formula 30 and 3A are satisfactory.)

Ethyl Alcohol, 55%. Dilute 580 ml. of 95% alcohol with water to one liter.

Petroleum Ether, 35°-60°C., A.O.C.S. grade. (Specification H2-41.) Redistill before use.

Petroleum Ether, 60°-70°C.

Silica. Precipitated silica, C.P., 80% to pass through a 200-mesh screen; or activated silica gel, "Thru 200, grade 22," Davison Chemical Co., Baltimore, Md.

Dichloromethane, C.P. Redistill before use.

Palmitic Acid, C.P.

Methyl Orange.

Filter aid (Hyflo Super-cel, Johns-Manville Corp., N. Y.)

Sodium Sulfate, anhydrous, powder, A.C.S.

Concentrated Potassium Hydroxide solution (50%). Dissolve 60 gm. potassium hydroxide, A.C.S. grade, in 40 ml. of water and cool.

Acid-Salt solution. Dilute 5 ml. of conc. hydrochloric acid with water to 100 ml. and saturate with sodium chloride.

Ceric Sulfate solution (ammonium tetrasulfatocerate), 0.1N in 0.05N sulfuric acid.

Ferroin Indicator solution (o-phenanthroline-ferrous ion).

Potassium Permanganate solution, 0.1N.

Manganese Sulfate solution, saturated.

Sodium Thiosulfate solution, 0.1N.

Potassium Iodide solution, 10%.

Starch solution. Mix ca 1 gm. of soluble starch with enough cold water to make a thin paste, add 100 ml. of boiling water and boil while stirring for one minute.

Performic Acid Oxidizing solution. Mix 10 ml. of 30% hydrogen peroxide with 10 ml. of 98-100% formic acid and cautiously add 40 ml. of acetic anhydride in several small portions. Allow sufficient time after each addition of the acetic anhydride for initiation of the reaction, shown by a rise in temperature, and do not add the next portion until the temperature begins to fall. Keep the temperature of the solution below 40°C., cooling with water, if necessary. The reagent is ready for use one hour after addition of the last acetic anhydride portion and may be stored in the refrigerator for about one week without significant deterioration. The peracid concentration should be equivalent to at least 15 ml. of 0.1N thiosulfate for each ml. of oxidizing solution, determined immediately before use by one of the two following methods: (a) Transfer 2 ml. of the reagent to a 500-ml. Erlenmeyer flask containing 150 ml. of 5% sulfuric acid and sufficient cracked ice to maintain a 0°-10°C. temperature. Add three drops of ferroin indicator and titrate with the 0.1N ceric sulfate to disappearance of the salmon color of the indicator. Add 10 ml. of the 10% potassium iodide solution and complete the titration with 0.1N thiosulfate solution, using starch indicator near the end of the titration. Or (b) Add three drops of saturated manganese sulfate solution to the cold 5% sulfuric acid containing the 2 ml. of reagent. Titrate rapidly with 0.1N potassium permanganate to a pink color lasting 5-10 seconds. Immediately on the appearance of the end point, add 10 ml. of the 10% potassium iodide solution and titrate the liberated iodine with the 0.1N thiosulfate.

C. Standardization of the Adsorbent

Mix the silica with half its weight of Hyflo Super-cel and add sufficient indicator powder to provide 300 mg. of methyl orange for each 1,000 gm. of adsorbent mixture. (To prepare this indicator, dissolve sufficient methyl orange in ca 10 ml. of water and add enough Hyflo Super-cel to make a thick paste. Evaporate on the steam bath, complete the drying in a 100°C. oven and then powder the cooled residue.) Mix the adsorbent thoroughly, avoiding prolonged exposure to the atmosphere. Vigorous agitation in closed containers on a shaking machine is recommended since it will insure uniform distribution of the indicator. Divide the mixed adsorbent into approximately two equal parts. If precipitated silica was used, activate one portion by heating overnight at 100°C. If activated silica gel was used, inactivate one portion by exposure overnight in shallow pans to a moist atmosphere.

Determine the activity constant of each portion as follows: Prepare a solution of palmitic acid in petroleum ether (60°-70°C.), containing 0.49-0.51 gm. of palmitic acid in 100 ml. Weigh 2 gm. (±2 mg.) of mixed adsorbent, transfer to a dry, glass-stoppered flask and pipette 100 ml. of the palmitic acid solution into the flask. Stopper the flask, mix and allow to remain at 25°C.(±2°) for one hour, with occasional swirling. Permit the adsorbent to settle and pipette 50 ml. of the clear, supernatant solution into a weighed flask. Evaporate the solvent, dry at 100°C. and weigh. Obtain the weight of palmitic acid in 50 ml. of the original solution in a similar manner. Make all determinations in duplicate. Calculate the activity constant, k, for each of the two adsorbent portions by the equation $k = Q - 0.055c$, where Q is the weight of palmitic acid in milligrams adsorbed by one gram of the adsorbent and c is the corresponding equilibrium concentration of palmitic acid, in milligrams per 100 ml. of solution, at which the Q was determined. Calculate the proportions of inactive and active adsorbents to make the final standardized mixture with an activity constant of 50.

$$y = 100\left(\frac{50 - k_x}{k_y - k_x}\right)$$

$$x = 100 - y$$

where k_x is the activity constant for the inactivated portion and k_y is the activity constant for the activated portion; x is the per cent of inactive adsorbent in the final mixture and y is the per cent of active adsorbent in the final mixture. Weigh the calculated quantities of the two portions, mix thoroughly in closed containers, avoiding exposure to the atmosphere and store in tightly closed bottles.

D. Preparation of the Adsorption Column

Place a wad of absorbent cotton in the bottom of the tube and add a one-cm. layer of anhydrous sodium sulfate powder. Tamp firmly with a smooth-surfaced cork attached to a rod and apply suction to the tube. Add the adsorbent, while applying suction, until a 7-cm. column is obtained and tamp firmly. Place a cotton disc on top of the column and fill the tube with dichloromethane. When the solvent reaches the bottom of the column, remove the suction and apply sufficient air pressure at the top of the tube to cause a rapid stream of solvent to emerge at the exit tube. (The air pressure can be controlled by a T-tube with a rubber tube and screw clamp inserted in the pressure line as a "bleeder." A gage serves as a useful adjunct in controlling the rate of flow.) Continue passage of the solvent until the column becomes smooth and adheres uniformly to the glass walls without any visible air pockets. Release the pressure and maintain a small volume of solvent over the top of the column until ready for use.

E. Procedure

Saponify ca 10 gm. of fat by refluxing for one hour with 30 ml. of 95% alcohol and 6 ml. of concentrated potassium hydroxide solution. Transfer the soap solution into a separatory funnel with ca 300 ml. of water, acidify with dilute sulfuric acid and extract with 100 ml. of ethyl ether. Repeat this extraction if lower saturated acids are present. Discard the aqueous solution and wash the ether free from mineral acid by shaking with several portions of water. Dry the ether with anhydrous sodium sulfate, filter, evaporate, and finally dry the mixed acids at 100°C. Dry at 80°C. if lauric or lower saturated acids are present. Highly unsaturated fats should be dried under an inert gas. (A.O.C.S. method Cd 6-38 can also be used to prepare the mixed acids.)

Weigh accurately ca 2.5 gm. of the mixed fatty acids into a 125-ml. conical beaker with pouring spout, add 25 ml. of the peracid oxidizing solution (use 40 ml. for fatty acids with iodine values above 150) and warm cautiously, using a thermometer in the liquid to observe the temperatures. Keep at 40°-50°C. until a clear solution is obtained, cooling, if necessary, to control the rise in temperature from the heat of reaction. When the initial oxidative reaction has subsided, immerse the beaker in a water bath held at ca 70°C. and permit the oxidation to continue for 30 minutes at 70°-75°C.

Cool to ca 35°C. and transfer the solution to a 500-ml. separatory funnel containing 300 ml. of water, rinsing the beaker with ca 15 ml. of 95% alcohol and then with 150 ml. of redistilled petroleum ether (35°-60°C.), adding the rinsings to the separatory funnel. Shake vigorously and allow the liquids to separate for ca ten minutes. Transfer the lower aqueous layer, which is usually slightly turbid, to a second separatory funnel containing 100 ml. of the petroleum ether. Retain any intermediate layer of oily or semi-solid material in the first funnel. Shake the second funnel vigorously, allow the layers to separate ca ten minutes and transfer the lower layer to a third separatory funnel containing 100 ml. of petroleum ether. Repeat the shaking operation and finally discard the separated lower aqueous layer.

Add 50 ml. of 55% alcohol to the first separatory funnel and shake gently but thoroughly, in order to extract the insoluble material without formation of persistent emulsions. After separation, transfer as much of the clear lower layer as possible to the second funnel, from which the aqueous layer had previously been removed. Shake the second funnel vigorously, add 300 ml.

TABLE V

Saturated Acid Content of Some Natural Fats by Proposed Method

Total Mixed Acids							Calculated to Unsaponifiable-Free Total Mixed Acid Basis	
Fat	% Unsaponifiable Matter	% Saturated Acids Found	% Unsaponifiable Matter in Sat. Acids (original sample basis)	Iodine Number of Saturated Acids	Mean Molecular Weight of Saturated Acids	Corrected % Saturated Acids (b)	Corrected % Saturated Acids	% Saturated Acids Found
							Proposed Method	Bertram Method
Spanish Olive Oil	0.89	15.69(c)	0.49	0.93			15.0	
		15.44(c)	0.28	0.50			15.1	
		15.09	0.28	0.51	262	14.73	14.9	16.6
		15.24	0.16	0.58	264	14.98	15.1	
		15.04*		0.29(a)				
		15.27		0.42(a)				
		15.55*		0.30(a)				
Sunflower Oil	0.75	12.06	0.39	0.74	271	11.57	11.7	11.9
		12.11(c)	0.28	0.49			11.8	
		11.99(c)						
		11.96(c)						
Peanut Oil	0.65	22.54*	0.39	0.47	292	22.04	22.2	20.1
		22.75*	0.35	0.54	296	22.27	22.4	21.4
Linseed Oil	1.15	9.58	0.28	0.50	271	9.25	9.4	
		9.64		0.65(a)				10.3
		9.61(c)	0.46	0.67	265		9.1	
		9.55*		0.48(a)				
		9.20*(c)		0.36(a)				
Cocoa Butter A	0.77	58.78	0.39	0.19		58.27	58.7	59.0
		58.70 (c)	0.18	0.24	277		58.4	58.5
		58.14		0.13(a)				
Cocoa Butter B	0.73	61.33*(c)		0.12(a)				
		61.20(c)		0.29(a)				
		60.53(c)	0.45	0.12			60.1	
		60.42(c)	0.10	0.19			60.2	58.0
		60.95(c)	0.32	0.15			60.5	
		60.52	0.11	0.13		60.33	60.8	
Whale Oil	1.78	25.65*	0.20	0.19	252		25.4	
		24.95(c)	0.20	0.20	250		24.7	23.9
		25.34	0.33	0.20		24.95	25.4	
		25.10*	0.31	0.19	254	24.74	25.2	

(a) Determined directly on original saturated acid residue; other iodine numbers determined on unsaponifiable free saturated acids.
(b) Corrected for unsaponifiable matter and unsaturated acid, calculated as oleic acid.
(c) Unsaponifiable matter removed from total mixed acids prior to analysis for saturated acids.
* Three separatory funnels used in extraction procedure; all other determinations made by the 2-separatory funnel method.

of water and 50 ml. of the acid-salt solution and again shake vigorously. Allow the layers to separate for ca 10 minutes and transfer the lower, turbid aqueous layer to the third funnel. Retain in the second funnel any insoluble material precipitated by the addition of the water to the alcoholic solution. Shake the third funnel vigorously and discard the separated aqueous layer after 10 minutes.

Repeat this extraction procedure with successive 50-ml. portions of 55% alcohol until all insoluble material has been removed from the first separatory funnel and transferred to the second funnel. A minimum of two of the 55% alcohol extractions should be made with all samples and a 10-minute separation period allowed before transfer of the aqueous layers.

Add 25 ml. of 95% alcohol and 1.5 ml. of the concentrated potassium hydroxide solution to the first separatory funnel and mix thoroughly. Allow to remain at room temperature for 15 minutes, add 300 ml. of water and acidify with 5 ml. of concentrated hydrochloric acid in 20 ml. of water. Shake vigorously and, after 10 minutes, pass the lower turbid layer successively through the other two funnels, using vigorous agitation and permitting separation for 10 minutes each time. Extract any insoluble material retained in the first funnel by the 55% alcohol treatment described above and transfer it to the second separatory funnel. After agitation, dilute each alcoholic extract with water and acid-salt solution, shake thoroughly and allow to separate for 10 minutes. Transfer the aqueous layers to the third funnel for a final extraction with petroleum ether. Finally wash with two 50-ml. portions of water, passed successively through each of the separatory funnels.

Draw off as much water as possible from the first separatory funnel, retaining any precipitate formed in the last water washing of the petroleum ether. Dry the exit tube of the funnel with a small piece of absorbent cotton. Add ca 0.5 gm. of Hyflo Super-cel to this separatory funnel, shake and then filter the contents through the Buchner funnel with fritted disc, containing ca 4 gm. of Hyflo Super-cel as a filter bed. Use gentle suction and catch the filtrate in a 1-liter flask. (The Buchner funnel and a tube for suction can be fitted to an Erlenmeyer flask with a 2-hole rubber stopper or the more convenient equivalent glass attachment described under Apparatus can be employed.) Rinse the separatory funnel and tip with five 20-ml. portions of petroleum ether, passing each rinsing through the filter.

Add the contents of the third separatory funnel to the second separatory funnel, using two 20-ml. portions of petroleum ether to complete the transfer. Draw off as much water as possible from the second funnel, dry the exit tube, add ca 0.5 gm. of Hyflo Super-cel and shake. Filter the contents through the same Buchner filter and finally rinse the separatory funnel with three 20-ml. portions of petroleum ether and pass each rinsing thru the filter. Wash the tip of the Buchner funnel exit tube with petroleum ether, add a few grains of 20-mesh carborundum or a few pieces of broken porcelain to the Erlenmeyer flask and remove the petroleum ether on the steam bath, using a current of air to speed the evaporation. (If appreciable quantities of lauric or lower saturated acids are present, remove the flask from the steam bath when ca 10 ml. of solvent remain and complete the evaporation by passing a current of air through the flask without using any external heat.)

Dissolve the residue in the flask in ca 10 ml. of redistilled dichloromethane and transfer quantitatively to the previously prepared adsorption tube, rinsing the flask with several small portions of this solvent. Apply gentle pressure, catching the effluent in a 1-liter Erlenmeyer flask (preferably with a standard taper connection). Release the pressure when the solvent barely covers the column, wash the tube above the column with ca 5 ml. of dichloromethane and allow this washing to flow through the column under pressure. Repeat the washing with two more 5-ml. portions of solvent, forcing each portion into the column but keeping the top of the column covered with liquid. Finally, fill the tube with dichloromethane and wash the column with a total of 400 ml. of this solvent, using sufficient air pressure to collect this volume at a rapid, dropping rate of ca 5 to 8 ml. per minute. Rinse the exit tube with dichloromethane, distill the 400 ml. of solvent and transfer the residue quantitatively to a weighed 100-ml. flask with the aid of petroleum ether. Remove the solvent on the steam bath and finally dry to constant weight at 100°C. (If lauric acid or lower saturated acids are present in appreciable quantities, the last portions of solvent must be removed by a current of air without the aid of heat. Final drying is made at 80°C., weighing at hourly intervals until the loss in weight is less than 3 mg.)

Although the methyl orange indicator permits visual observation of the chromatographic development on the adsorption column, a more precise check should be made after the 400-ml. washing by passing an additional 100 ml. of dichloromethane through the column and weighing the residue in this filtrate. Satisfactory separation is shown by a residue weight of not more than 2 mg. A greater residue indicates possible leakage of undesired oxidized acids into the main effluent and it is necessary to rechromatograph the saturated acids.

The saturated acids obtained by this method contain ca 0.3% of unsaponifiable matter and 0.1% of unsaturated acids, calculated on the original sample basis. For exact corrections, unsaponifiable content and iodine value must be obtained. The latter constant can be determined directly on the total saturated acids or on the acids extracted with petroleum ether after removal of the unsaponifiable matter. Unsaturated acids are usually calculated as oleic acid from this iodine value.

The petroleum ether-55% alcohol extraction procedure can be shortened by elimination of the third separatory funnel. In the absence of material quantities of lauric or lower saturated acids, fats can be examined by this shorter method without any significant loss of accuracy.

Results and Discussion

Several mixed fatty acids have been examined by this method (Table V). Multiple analyses of the same fat gave results within a range of one unit per 100 parts of total mixed acid. These values compare favorably with the results obtained on these fats by the Bertram method and show a smaller experimental error than the ± one unit per cent reported for this latter method (2).

The saturated acids obtained by the proposed method are usually white to lemon yellow in color. Their mean molecular weights agreed well with the values calculated from the composition of the fats (26). Small quantities of unsaponifiable matter were present in all of the saturated acid residues. Removal of the natural unsaponifiable matter in the total mixed acids before analysis did not influence the unsaponifiable content of the separated saturated acids, indicating that the small non-acid portion of this final residue originated in the oxidative disruption of unsaturated acids. The analyses of the whale oil fatty acids demonstrate that the presence of relatively large quantities of natural unsaponifiable matter have little effect on the composition of the isolated saturated acids. Iodine values of the saturated acids show only negligible unsaturation, on the order of 0.1 unit per cent, calculated as oleic acid. In all of the complete analyses listed in Table V the unsaponifiable matter was removed from the saturated acids by the S.P.A. method (25) and the saturated acids then extracted with petroleum ether after acidification of the soap solution. The iodine values of these treated residues did not differ materially from the iodine values found by direct examination of the untreated saturated acids. Table V also shows that the two-separatory funnel extraction method, applied to natural fats containing only the higher saturated acids, gave results comparable to those obtained by the more elaborate three-separatory funnel procedure. Experiments with these fats showed that the third separatory funnel contained from 0.1 to 0.3% of saturated acids, the latter figure appearing in the examination of fats with greater saturated acid contents. However the increased yields contributed by this third separatory funnel are largely hidden by the experimental errors inherent in the proposed method, and for most purposes, the shorter separation procedure should be adequate for the examination of normal fats.

Collaborative work on saturated acid methods has been published recently (3). The fats used in these studies have now been examined by the proposed method, and the results are listed in Table VI. With the exception of the higher values for the beef fat the corrected results are in good agreement with the averages reported for the Bertram and crystallization methods. The isolated saturated acids again show only negligible unsaturation but contain appreciable unsaponifiable matter.

Many fats containing certain distinctive or less common unsaturated acids cannot be analyzed for saturated acids by available methods. Several of these fats have been examined by the proposed method (Table VII) and, with the exception of rape seed and celery seed oils, exhibited no analytical difficulties. The dihydroxybehenic acid, formed by the peracid oxidation of the erucic acid present in rape seed oil, is only slightly soluble on the 55% alcohol solution so that removal of this oxidized acid re-

TABLE VI

Application of Proposed Method to A.O.C.S. Collaborative Fat Samples

Fat	Total Mixed Acid Basis						
	% Saturated Acids	% Unsaponifiable Matter in Saturated Acids	Iodine Number of Saturated Acids (b)	% Saturated Acids	Average A.O.C.S. Collaborative Results (d)		
	Found (a)			Corrected (c)	Twitchell %	Bertram %	Crystallization %
Beef	52.82 52.79 53.01	0.27 0.39	0.12 0.16	52.4 52.5	45.3	50.0	49.2
Lard	37.61 37.03 36.59 37.06	0.73 0.43	0.25 0.30	36.2 36.0	33.2	36.5	35.7
Cottonseed	26.94 27.16 27.15	0.41 0.41 0.26	0.36 0.60 0.34	26.4 26.5 26.8	23.3	26.6	25.3
Soy	14.38 14.20 15.12	0.27 0.20 0.22	0.40 0.30 0.44	14.1 14.0 14.8	11.7	14.2	14.1
Linseed	9.21 8.91 9.20 9.24	0.20 0.26 0.20	0.50 0.42 0.58	9.0 8.6 8.9	7.0	9.4	8.9

(a) Three-separatory funnel extraction procedure used.
(b) Determined after removal of unsaponifiable matter in saturated acids.
(c) Corrected for unsaponifiable matter and unsaturated acid, calculated as oleic acid.
(d) Jour. Amer. Oil Chem. Soc., *25*, 145 (1948).

quires a large number of washings. This tedious procedure could probably be shortened by use of a more concentrated alcoholic wash solution, but since normal values for saturated acids were obtained by the method, it was not considered necessary to devise a modification for this special case.

TABLE VII

Application of Proposed Method to Fats Containing Distinctive Unsaturated Acids

Total Mixed Fatty Acids	Distinctive Unsaturated Acid	% Saturated Acids Found (uncorrected)	Iodine Number of Saturated Acids (b)	% Saturated Acids Reported in Literature (c)
Castor Oil	Ricinoleic	1.4	5.0	0.3-2.4
Celery Seed Oil	Petroselinic	6.1(d) 6.9(d)	1.2	3
Chaulmoogra Oil	Chaulmoogric	5.3	1.5	4.0
Chinawood Oil	Elaeostearic	4.8	1.9	3.4-6
Hydrogenated Cottonseed Oil	Iso-oleic	29.1	0.2	.....
Rapeseed Oil	Erucic	6.9(a) 6.3(a) 6.8(a)	0.8 0.6 0.9	1-6

(a) Contains 0.20-0.31% unsaponifiable matter, calculated on original total mixed acid basis.
(b) Corrections for unsaturated acid are less than 0.1%, calculated as oleic acid.
(c) See Reference 26.
(d) Special purification of saturated acid residue necessary to remove interfering lactones. See text.

With the exception of celery seed oil the saturated acid content found in these oils agrees well with published data. No difficulty was noted in the oxidation and removal of the characteristic petroselinic acid. However celery seed oil contains non-fatty material which appears in the final residues. These contaminants were identified as saturated lactones, which were readily hydrolyzed by alkali but reformed immediately upon acidification. This material probably originated in the various unsaturated anhydrides and lactones which are known to be present in celery seed oil. Christian and Hilditch (27) also found that the analysis of celery seed oil, as well as other fats of the Umbelliferae, was complicated by the presence of non-fatty material, particularly lactonic compounds, which distilled with the methyl esters of the fatty acids. Attempts to remove these substances by steam distillation prior to analysis was unsuccessful. A more satisfactory solution to this problem was devised when it was found that the lactones present in the residues obtained by the proposed method could be separated from the fatty acids by a simple lead-salt procedure. Addition of neutral lead acetate to the alcoholic solution of the residue caused precipitation of the lead salt, leaving the lactone in solution. After filtration and washing, the saturated acids can be regenerated by treatment with acid and extraction with petroleum ether. In two experiments total residues of 10 to 11% were obtained. The lead salt treatment on these two residues yielded 6.1 and 6.9% of fatty acids, with mean molecular weights of 265 and 269 respectively. Careful examination failed to disclose any evidence of the presence of lactones. The corresponding lactone fractions of 3.9 and 3.5% showed no free fatty acid.

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Summary

A method for the quantitative determination of the saturated acids in fats has been developed. This method is based upon the non-disruptive oxidation of the unsaturated acids by performic acid, extraction of the oxidized mixture with petroleum ether and, finally, chromatographic purification of the saturated acids. The final residue contains a small quantity of non-acid material, usually 0.1 to 0.7%, based on the total mixed fatty acids. The unsaturation of these saturated acids is equivalent to about 0.1%, as oleic acid, so that a total absolute correction of about 0.4% must be applied. The method is fairly simple and rapid, requiring about six hours for one determination and can be applied to a wide variety of fats.

The method has been tested with known mixtures of pure saturated and unsaturated acids, with results varying up to 0.6% from the calculated values. Recovery of added saturated acids to olive oil fatty acids has also been satisfactory. Application of the method to the common fats shows good reproducibility, with results that compare favorably with the best accepted values. Fats containing distinctive acids, such as iso-oleic, eleaostearic, chaulmoogric, and ricinoleic acid, presented no analytical difficulties. However celery seed oil (petroselinic acid) contains interfering non-fatty material so that the final residue must be further purified. The method can also be used with fats containing lauric, capric, and small quantities of caprylic acids but is not satisfactory when appreciable quantities of water-soluble saturated acids are present.

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