Honorable mention is given to T. S. McDonald, Procter and Gamble Company, Dallas, Tex., who was third with a grade of 98.93%.

The grading system used was that adopted in 1950 except for the following:

Sample No. 1. No deduction was made for color where 13, 11B, or 15 were reported.

Sample No. 2. No deduction was made on color where 13, 11A, 11B, or 15 were reported.

Sample No. 3. A tolerance of ±0.5% was used in grading the free fatty acid.

A complete and detailed report was sent to all collaborators.

J. L. TRAUTH, DAN L. HENRY, J. E. MARONEY, C. D. LEAVITT, K. FINK, W. C. AULT, chairman

The Glyceride Structure of Natural Fats. I. A Technique for the Quantitative Determination of Glyceride Types in Natural Fats

A. R. S. KARTHA, Maharaja's College, Ernakulam, India [1,2]

SYSTEMATIC investigation of the natural fats was first undertaken by Hilditch and colleagues. Among their many noteworthy contributions was an oxidative method for the quantitative determination of GS_3[3] (1). A modification of this procedure forms the basis for the present method of determination of the four glyceride types, GS_3, GS_2U, GSU_2, and GU_3.

There have been several attempts to separate and estimate the GS_2U, GSU_2, and GU_3 components of natural fats (3, 4, 5, 6, 7, 8). None of these has been entirely successful.

During attempts at separating the products of fat oxidation according to the Hilditch method, by preferential extraction with bicarbonate (8), Kartha (2a) found that the azelaoglycerides formed in the reaction were partly destroyed by hydrolysis. He then developed the acetic acid-acetone-permanganate method of oxidation and the azelaoglycerides-separation method which will be described in this paper. These procedures are the basis of the method for quantitative estimation of the glyceride types, which will also be presented.

Sources of Error in the Acetone-Permanganate Method for Estimation of GS_3

Oxidation of GS_2U, GSU_2, and GU_3 according to the Hilditch method produces GS_2A GSA_2, and GA_3, respectively. Among the other products of the reaction are carbon dioxide, water, and potassium hydroxide. Some of the carbon dioxide doubtlessly combines with the potassium hydroxide to produce potassium carbonate.

There is evidence for the belief that there is an appreciable loss of azelaoglycerides by hydrolysis during the oxidation process. The hydrolysis is caused by the carbonate solutions produced in the reaction. It is also likely that the azelaoglycerides undergo hydrolysis during the process of separation with aqueous carbonate solutions.

The following experiments are the basis for these conclusions:

1. A sample of GU_3 prepared by synthesis was oxidized according to the Hilditch method, using 12 g. of $KMnO_4$ per gram of fat and a total oxidation time of 10-11 hours. The oxidation products were isolated by extraction with ether, and the volatile acids were removed by steam distillation as suggested by Stainsby (9). The yields of GA_3 were only 0.35-0.45 g. per gram of fat. The theoretical yield is 0.68 g. per gram of fat. The difference is attributed mostly to partial hydrolysis of the GA_3 during oxidation; part of the products were lost because of their comparative solubility in water and insolubility in ether. The great loss can hardly be due entirely to hydrolysis during steam distillation since there is no indication in the work of Stainsby that this would occur.

2. *Garcinia Indica* and *Vateria Indica* fats, which contain about 60% of saturated acids and only traces of GS_3, were oxidized according to the Hilditch method. The products in ethereal solution were washed with an excess of dilute bicarbonate solution. The residues, which varied in quantity and should consist of GS_2A, showed saponification values of 240-260, which are much less than the theoretical value for GS_2A. This fact indicates that GS_2A was lost during the oxidation procedure or during the extraction process, or both, probably by hydrolysis to GS_2OH and by solution in the bicarbonate.

3. A GS_3-GS_2U mixture containing 66.6% of GS_2U was prepared by crystallization of peanut oil, hydrogenated to an iodine value of 45, from acetone at 30-32°C. A sample was oxidized according to the Hilditch method with 9-10 g. of $KMnO_4$ per gram of fat. The reaction period was 10 hours. The oxidation products were collected in ether, and the ether was removed by distillation. The residue was dissolved in cold, dilute carbonate solution. An excess of carbonate was avoided so no hydrolysis should have resulted from this treatment. Evidence of this will be presented in experiments 1 and 2 (see also conclusion 3 following these experiments) under the heading, Procedure for Acid-Acetone-Permanganate Oxidation of Unsaturated Fats.

The GS_2A was precipitated as the magnesium salt by addition of ammonium chloride and finally an excess of magnesium sulfate solution. The GS_3 separated with the magnesium-GS_2A salt, and both were removed by filtration. The filtrate contained no higher acids, showing that the magnesium-GS_2A salt is insoluble in water. The total fatty matter recovered from the magnesium salts was 86.9% of the original fat; the theoretical amount is 92.9%. The quantity of GS_2A found was 80.5% of the GS_2U originally present; the theoretical value is 89.5%. The saturated fatty acids were isolated from the GS_2A-GS_3 fraction and agreed in quantity with the quantity present in the original fat. No hydrolysis should have resulted from the brief, mild carbonate treatment; therefore the loss of azelaoglycerides is most reasonably attributed to hydrolysis during oxidation.

The hydrolysis of azelaoglycerides can affect the GS_3 determination according to the method of Hilditch since hydrolysis of GS_2A and GSA_2 produces

[1] Present address, Indian Agricultural Research Institute, New Delhi, India.

[2] Edited and revised by R. J. Vander Wal, Armour and Company, Chicago, Ill.

[3] The following symbols will be used in the text:
G=Glyceryl radical.
S=Saturated acid group or saturated acids according to context.
U=Unsaturated acid group or unsaturated acids according to context.
A=Azelaic acid group.

neutral products which will separate with the GS_3. Thus positive error in the determination of GS_3 will be introduced.

There is further evidence that the method used by Hilditch for the estimation of GS_3 in fats is not entirely accurate. Only traces of GS_3 could be detected by crystallization procedures in cacao butter (10) and Borneo tallow (11) whereas Hilditch and colleagues reported 2.5% of GS_3 in cacao butter (12) and 5% in Borneo tallow (13). In animal tallows Ault *et al.* (14) and Kartha (2b) found that GS_3 values by crystallization are appreciably below chance values, sometimes by as much as 5-8%, whereas values reported by Hilditch and colleagues (15) are generally 2-5% higher than chance values. Kartha (16) found that it is possible to calculate the GS_3 contents of C_{16}-C_{18} fats directly from the saturated acid contents and the melting points of the fat and its mixed acids. In many cases the GS_3 content so determined is different from that determined by the method of Hilditch whereas they invariably agree with values obtained by crystallization.

If it is true that hydrolysis of the azelaoglycerides occurs during the oxidation process, owing to development of alkalinity, it should be possible to prevent hydrolysis by keeping the mixture acid or neutral. This hypothesis has been found to be correct. By application of the following procedure, fats can be oxidized to azelaoglycerides without loss of the latter through hydrolysis.

Procedure for Acid-Acetone-Permanganate Oxidation of Unsaturated Fats

Five grams of the fat are dissolved in 200 cc. of acetone, and 12 cc. of glacial acetic acid (sufficient to combine with all of the potassium in 31 g. of $KMnO_4$) are added. The oxidation is carried out according to the Hilditch procedure except that after each 16 g. of $KMnO_4$ has been added, 6 cc. of acetic acid are also added. Thus the concentration of acetic acid is always between about 3 and 6%. When the reaction slows, it is continued at the boiling point on a water bath. When the permanganate color disappears very slowly, as when 1 g. is not decolorized on boiling for 45 minutes, the oxidation is stopped. Usually by this time 8-10 g. of $KMnO_4$ per gram of fat will have been added. More would have been required for drying oils.

The acetone is removed by distillation, the last traces being eliminated with reduced pressure, by means of a water pump. About 150 cc. of water are added, and the mixture is heated on a water bath with agitation until the mass breaks up into a suspension. The manganese dioxide is then dissolved by addition of small quantities of sodium bisulfite and sulfuric acid alternately, as usual. The resultant clear layer of fatty matter is separated by extraction with ether. The extract is washed free from mineral acids, and the ether is removed.

The product contains all the azelaoglycerides formed by oxidation, none having been lost by hydrolysis. This is shown by the following experiments:

1. A GS_3-GS_2U mixture containing 66.6% of GS_2U was subjected to acid-acetone-permanganate oxidation. The acid products were separated as the magnesium salts in a manner which will be described later. The amount of fatty material recovered was 93% of the sample, against 92.9%, which is the theoretical amount. The yield of GS_2A was 89.4%; the theoretical quantity is 89.5%. The filtrate contained no higher fatty acids, thus showing that under the conditions of this experiment the magnesium monoazelains were insoluble. These results are to be compared with those described earlier in which the Hilditch procedure was used.

2. Distearolein containing 1% of GS_3 was prepared by crystallization from *Garcinia Indica* fat. It was oxidized by the acetic acid-acetone-permanganate method; the acids were separated as the magnesium salts. The quantity of GS_2A recovered from the precipitate, after correction for the small amount of GS_3 and unoxidized GS_2U present, was 89.6% of the sample against 89.5% present by theory. Higher fatty acids were again absent from the filtrate, and the GS_2A precipitate after hydrolysis and Bertram separation gave 64.5% of saturated acids with an iodine value of 0.0, which is in agreement with the composition of the original fat.

The above experiments establish the following facts:

1. Presence of acetic acid does not interfere with oxidation and acts only to prevent hydrolysis of azelaoglycerides.
2. Magnesium salts of the monoazelaoglycerides are insoluble in water under the specified conditions.
3. The azelaoglycerides are not hydrolyzed during the mild treatment with the alkali carbonate required in their separation as magnesium salts.

Method for Separation of Acid-Acetone-Permanganate Oxidation Products

The products of oxidation cannot be separated satisfactorily by means of their differing solubility in sodium bicarbonate solution because GS_2A is soluble in sodium bicarbonate solution (8). When a dilute solution of bicarbonate is used to minimize this solubility, the tendency for nonanoic acid to remain behind increases.

Investigation has shown that the reported insolubility of the magnesium salts in water (4) is only comparative. Magnesium triazelain is soluble to not less than 3-4% in water at 30°C.; the magnesium salts of GSA_2 show appreciable though more sparing solubility whereas the magnesium salts of GS_2A containing the higher acids are insoluble in water at 30°C.

Based on these findings the following method for separation of the oxidation products has been adopted:

The oxidation product from 5 g. of fat is suspended in about 200 cc. of water (30°C.), and a few drops of phenolphthalein are added, followed by 5% sodium carbonate solution until the solution is just alkaline. The volume is made up to 500 cc. with water (100 cc. per gram of fat oxidized), and 30 cc. of 10% ammonium chloride solution is added and mixed well. Finally 15% magnesium sulphate solution is added until no more precipitate forms. The precipitate is allowed to settle for 5-10 minutes, is filtered on fluted filter paper, and is washed four times with 30-40 cc. portions of water. It is then transferred quantitatively to a flask and heated on a water bath with dilute sulfuric acid until all the magnesium salts are decomposed and a clear oily layer separates. The mixture is then cooled and extracted with ether, and the ether solution is washed free of mineral acids. The ether is removed and the residue heated to constant weight under reduced pressure.

The product is hydrolyzed with alcoholic KOH, and the isolated acids are subjected twice to magnesium salt precipitation as in the Bertram method for determining saturated acids. The weights, iodine values, saponification values, and melting points of the separated acids are determined.

The filtrates are acidified and the acidic fractions are extracted with ether, which is removed by evaporation. The residue is hydrolyzed with alcoholic KOH and the higher fatty acids separated by Bertram's method. Their weights, melting points, and saponification values are determined.

From the accumulated data and the GS_3 content as determined separately, the glyceride-type composition is calculated. The method of calculation will be given later. The GS_3 is determined by a procedure substantially the same as that used by Coffey and Spannuth (18), and Mattil and Norris (19). The sample is dissolved in 3 cc. of dry acetone per gram of fat, and the

solution is held at 25-26°C. for 3 days. The precipitate is filtered and washed with a little acetone at the same temperature. It is then dried under reduced pressure and recrystallized from acetone under the same solvent ratio, time, and temperature conditions. The vacuum-dried residue is weighed, and the iodine and saponification values are determined. From these values the amount of unsaturated acid, as oleic acid, and also the mean molecular weights (M.M.W.) of the S are calculated. From all the data and under the assumptions that the product consists of GS_3 and GS_2U only and that the S in both has the same M.M.W., the proportions of GS_3 and GS_2U are calculated.

The method of separation, as such, is applicable only to fats containing mainly fatty acids of 16 or more carbon atoms, to which the Bertram separation can be applied (19).

Method of Calculation of Glyceride-Type Content of Fat

The fatty matter recovered from the precipitated magnesium salts will contain all the GS_3 originally present in the fat, any unoxidized GS_2U,[4] all the GS_2A, and usually a variable amount of GSA_2. The weight of the GS_3 present in the fat sample, found independently, and that of the unoxidized GS_2U are deducted from the total. The balance consists of GS_2A and GSA_2.

The weight of unoxidized GS_2U (W_2) is calculated from the mean molecular weight (M) of the S found in the precipitated magnesium salts, and the weight of the unsaturated acids (W_1), assumed to be oleic, in the same fraction.

$$W_2 = W_1 \frac{(2M+282+38)}{282}$$

The values of M and W_1 and the weight of the S in the precipitated magnesium salts are calculated from the weight, saponification value, and I.V. of the mixed acids separated from the latter, as described in the previous section.

The combined weights of the GS_2A and GSA_2 and the weight and mean molecular weight of the S in the fatty matter recovered from the precipitated magnesium salts are now known. The weight of the S in the whole mixture minus the weights of the S present as GS_3 and unoxidized GS_2U gives the weight of S present in the mixture of GS_2A and GSA_2. From these data the amounts of GS_2A and GSA_2 can be calculated. It is assumed that the S in both has the same M.M.W., which has been found to be true in a large number of fats (2c).

From the weights of GS_2A and GSA_2 found in the precipitated magnesium salts the weights of the GS_2U and GSU_2 from which they were derived may be calculated. It is assumed that the U is oleic acid. If the M.M.W. of the U in the fat is known, this value may be substituted.

The weight of GS_2U thus obtained is added to the weight of the unoxidized GS_2U to give total weight of the GS_2U in the original fat sample.

The filtrates from the precipitated magnesium salts contain part of the GSA_2, the remainder having been precipitated with the insoluble fraction. The weight of the S in the fatty material recovered from the filtrates is determined by isolation of the fatty acids by the procedure described in the previous section. It is present in the filtrate entirely as GSA_2 and from its weight the weight of the GSA_2 can be calculated. The weight of GSU_2 from which the GSA_2 was derived can be calculated from the result. The total GSU_2 in the fat is the sum of that found in the soluble and insoluble fractions of the magnesium salts. The percentage of GU_3 is calculated by difference.

The total S in the fat is the sum of that found in the GS_3, GS_2U, and GSU_2 fractions. It can also be determined by Bertram separation of the acids produced by hydrolysis of the product of acetic acid-permanganate-acetone oxidation. It is also the sum of that found in the fatty matter recovered from the soluble and insoluble magnesium salt fractions.

The amount of fat required for glyceride-type analysis by this method is small. The time required is also relatively short. Duplicate determinations rarely show more than 0.5% difference in the GS_2U content.

The results of application of the method to the glyceride-type estimation of 27 fats of saturated acid content, varying from 8 to 99 moles per cent, and the significance of the results will be presented in the next paper of this series.

[4] This fraction should contain any GSUA present in the oxidation product. It is assumed that none is present. The close agreement between experimental and calculated results (2) (and part II of this series, which is expected to follow) indicate that the assumption is correct.

Summary

The acetone-permanganate oxidation method for determination of GS_3 has been shown to be unreliable because some hydrolysis of the azelaoglycerides occurs, the products of which are determined as GS_3. The presence of excess acetic acid throughout the oxidation corrects this defect. GS_2A can be precipitated quantitatively as the magnesium salt. Although it is contaminated with GSA_2, its proportion can be calculated and from this the proportion of GS_2U in the sample can be found.

The remainder of the precipitate is GSA_2; its weight, when added to that found in the filtrates, gives the total GSA_2. From this, the percentage of GSU_2 in the sample can be derived. The GS_3 is determined separately, and the content of GU_3 can be estimated by difference.

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