A Modification of the Periodic Acid Method for the Determination of Monoglycerides and Free Glycerol in Fats and Oils

W. D. POHLE and V. C. MEHLENBACHER, Swift and Company, Chicago, Illinois

THE usefulness of the periodic acid method for the determination of monoglycerides in fats and oils (5) has been confirmed by its continued usage. It has been applied to research investigations and routine testing in several laboratories (1, 2, 3, 4, and 6). However Hanschumaker and Linteris (4) found the original method insufficiently rapid and convenient for routine purposes, and the present authors confirm their opinion.

The most critical and time-consuming steps in the original method (5) were the removal of free glycerol by salt-water extraction, maintenance of a satisfactory yet safe temperature during the reaction when testing solid fats, and the necessity of vigorous shaking to obtain complete reaction between the sample and reagent which are not completely miscible. Hanschumaker and Linteris (4) suggested an improved modification in which the sample was dissolved in chloroform-acetic acid solution. This method was carried out at room temperature, thus eliminating the danger of secondary reactions that may occur when solid samples are melted for testing, and is applicable to most samples except certain high melting fats.

The most logical approach to the problem of extending the range of applicability and at the same time eliminating all of the aforementioned shortcomings appeared to be solvent separation for removal of glycerol from fat and a single phase solvent medium for the reaction of periodic acid and monoglyceride. This then resolved itself into a search for suitable solvents. The high degree of solubility in water immediately suggested that glycerol might be removed by water washing. The requirements of a monoglyceride solvent are more demanding since it is desirable to carry out the reaction in a solvent that will give a homogeneous solution when mixed with the periodic acid reagent.

To summarize the results of tests with several solvents, chloroform and carbon tetrachloride were found to be the best, and of these chloroform eventually proved to be superior. High melting triglycerides and monoglycerides are sufficiently soluble in chloroform so that glycerol can be removed by repeated aqueous extractions. This step involving removal of free glycerol is frequently necessary because it is often present in significant amounts, even in natural fats. Because of difference in molecular equivalents, 0.1% glycerol is equivalent to about 0.75% monoglyceride; thus the need for the treatment to remove glycerol is obvious unless glycerol is known to be absent.

TABLE I

Rate of Reaction Between Monoglyceride and Periodic Acid at Room Temperature, 28°C.

Minutes allowed to stand after adding sample to reagent	Monoglyceride, %
0	45.4
5	90.9, 91.5, 91.0
10	91.2
30	91.7
60	91.2, 91.0
120	91.6, 91.2
240	91.4, 91.0

In order to obtain a homogeneous solution when the periodic acid was added to the sample in chloroform, it was necessary to modify the periodic acid reagent. The original reagent (5 g. periodic acid in 200 ml. water and 800 ml. of acetic acid) is not satisfactory because it yields a 2-phase system. Periodic acid in glacial acetic acid is not satisfactory either because periodic acid precipitates from solution when added to chloroform, but a reagent intermediate between the two yields a clear homogeneous solution. Actual analyses revealed that it is better to add the sample-in-chloroform to the periodic acid reagent than in the reverse order. When performed in this order, both the sample and periodic acid remain in solution. The subsequent cloudiness that appears is due to the formation of insoluble reaction products.

Briefly, as finally evolved, the method follows. The sample is dissolved in chloroform and the free glycerol removed by four extractions with water. The glycerides remain in the chloroform solution. An aliquot of the aqueous solution is tested for glycerol, and an aliquot of the chloroform solution is tested for monoglycerides.

TABLE II

Analyses of Pure Monoglycerides and Known Mixtures

Sample	Free Glycerol, %		Monoglyceride, %	
	Present	Found	Present	Found
Monostearin	......	......	99.2	99.3
Monopalmitin	......	......	99.2	99.4
Monostearin and tristearin	......	......	49.6	49.7
Monostearin and glycerol and hydrogenated fat	5.00	4.97	9.92	9.97
Monostearin and glycerol and hydrogenated fat	5.00	4.98	0.99	0.98

The rate of reaction between monoglyceride and periodic acid under the conditions of the proposed method was determined by titration of a series of samples at selected intervals after addition of the sample to the periodic acid reagent. The results appear in Table I.

These data indicate the reaction is complete in five to 10 minutes. A reaction time of 30 minutes was adopted thereafter to insure a sufficient safety factor for complete reaction.

A group of samples of known monoglyceride and glycerol content was prepared, using known amounts of pure synthetically prepared mono- and triglycerides and C. P. glycerol. The purity of the monoglycerides and triglycerides was established from melting points and acetylation of hydroxy groups. The purity of the glycerol was determined from its specific gravity, moisture content, and analysis by the periodic acid method (7). These results appear in Table II.

Completeness of the removal of free glycerol was further checked by determining the glycerol in the combined first three extractions and the fourth extraction separately. A series of six samples of commercial monoglycerides, which ranged in glycerol

TABLE III

Determination of Monoglyceride and Free Glycerol in Monoglyceride Products

Sample	Monoglyceride, found by proposed method, %	Free Glycerol, %	
		In comb. extract	In 4th extract
1	36.9	4.60	0.01
2	38.6	5.31	0.01
3	38.4	5.36	0.01
4	30.5	2.06	0.01
5	41.4	4.23	0.01
6	37.2	2.21	0.01

content from 2 to 5%, were analyzed in this manner for free glycerol and for monoglyceride by the proposed method. These data appear in Table III.

The small amount of periodic acid reducing material in the fourth extraction calculated as glycerol indicates that the first three extractions remove essentially all the glycerol and also that there is no appreciable solubility of the monoglyceride in the water used to remove the glycerol.

The precision of the methods under ideal conditions was determined by analyzing 10 portions of the same well-mixed sample. All of these analyses were made by one individual. The results appear in Table IV.

Method

A. APPARATUS:

1. Buret, 50-ml. accurately calibrated.
2. Meniscus magnifier suitable to permit reading the buret to 0.01 ml.
3. Flasks, volumetric, 1000-ml.
4. Flasks, volumetric, 100-ml., glass-stoppered are preferred, but regular volumetric flasks and rubber stoppers may be used.
5. Pipets, volumetric, 10-ml. 25-ml., and 50-ml. The 25- and 50-ml. pipets must conform to Bureau of Standards tolerances and be accurately calibrated to deliver 25 and 50 ml. respectively.
6. Glass siphon with blow-tube and stopper similar to that shown in A.O.C.S. Official Method Da 8a-42.
7. Beakers, 400-ml. and watch glasses to serve as covers.
8. Variable speed electric stirrer with glass stirrer.
9. Graduated cylinders, 100-ml. and 1,000-ml.

B. REAGENTS:

1. Periodic acid (H_5IO_6) reagent grade. Manufactured by G. Frederick Smith Chemical Company, Columbus, Ohio. *Test for Quality:* To 0.5 to 0.6 g. of C. P. glycerine in 50 ml. of distilled water add 50 ml. of periodic acid reagent with a pipet. Prepare a blank, using only 50 ml. of distilled water. Allow to stand 30 minutes and titrate as described in E, 10 and 11. Titration of soln. containing glycerol divided by titration of the blank will be between 0.75 and 0.76 when the periodic acid is satisfactory.
2. Sodium thiosulfate, A.C.S. grade.
3. Potassium iodide, A.C.S. grade.
4. Glacial acetic acid, A.C.S. grade, 99.5%.
5. Soluble starch.
   Test for Sensitivity: Place 2 ml. of starch soln. (C, 4) in 100 ml. of distilled water and add 0.05 ml. of 0.1 *N* iodine soln. The deep blue color produced must be discharged by 0.05 ml. of 0.1 *N* sodium thiosulfate.
6. Chloroform, U.S.P. or reagent grade. Blank tests run on periodic acid with and without 50 ml. of chloroform must check within 0.5 ml. If they do not, get a new supply of chloroform.
7. Potassium dichromate, A.C.S. grade. The potassium dichromate is finely ground and dried to constant weight at ca 110°C. before using.

   Note: A standard sample of potassium dichromate with a certificate of analysis may be obtained from the National Bureau of Standards at Washington, D. C. This sample is strongly recommended as the primary standard for this method. Treat as directed in the certificate of analysis accompanying the sample.
8. Hydrochloric acid, A.C.S. grade, sp. gr. 1.19.

C. SOLUTIONS:

1. Periodic acid soln.; dissolve 5.4 g. of periodic acid in 100 ml. distilled water and then add 1,900 ml. of glacial acetic acid and mix thoroughly. Store the soln. in a dark glass-stoppered bottle or store in the dark in a clear, glass-stoppered bottle (see Note 2).
2. Sodium thiosulfate soln., 0.1 *N*; dissolve 24.8 g. of sodium thiosulfate in distilled water and dilute to 1 liter. *Standardization:* Pipet 25 ml. of the standard dichromate soln. into a 400-ml. beaker. Add 5 ml. of hydrochloric acid, 10 ml. of potassium iodide soln. and rotate to mix. Allow to stand for 5 minutes and then add 100 ml. of distilled water. Titrate with sodium thiosulfate soln., stirring continuously until the yellow color has almost disappeared. Add 1 to 2 ml. of starch indicator soln. and continue titration, adding the thiosulfate soln. slowly, until the blue color has just disappeared. The strength of the thiosulfate is expressed in terms of its normality.

$$\text{Normality of } Na_2S_2O_3 \text{ soln.} = \frac{2.5}{\text{Ml. sodium thiosulfate soln. required}}$$

3. Potassium iodide soln.; dissolve 150 g. of potassium iodide in distilled water and dilute to 1 liter.
4. Starch indicator soln.; make a homogeneous paste of 10 g. of soluble starch in cold distilled water. Add to this 1 liter of boiling distilled water, stir rapidly, and cool. Salicylic acid (1.25 g. per liter) may be added to preserve the indicator. If long storage is required, the soln. must be kept in a refrigerator at 4° to 10°C.(40° to 50°F.). Fresh indicator must be prepared when endpoint of the titration from blue to colorless fails to be sharp.
5. Standard potassium dichromate soln., 0.1 *N*; dissolve 4.9035 g. of finely ground and dried potassium dichromate in distilled water in a 1,000-ml. volumetric flask and make up to volume at 25°C.

D. PREPARATION OF SAMPLES:

1. *Solid Samples in Flake Form.* Mix without melting and take portion for test.

TABLE IV

Example of Precision of Method

Test	Monoglyceride, %	Free Glycerol, %
1	34.9	2.55
2	35.1	2.57
3	35.0	2.51
4	35.0	2.58
5	35.1	2.59
6	34.6	2.53
7	35.1	2.53
8	34.9	2.53
9	35.1	2.56
10	34.8	2.54
Average	34.96	2.55
Standard deviation	±0.15	±0.025

2. *Solid Samples Not in Flake Form.* Melt at not more than 10°C. above melting point, mix thoroughly, and take portion for analysis. Do not attempt to test samples which contain so much free glycerol that it separates when the sample solidifies.
3. *Semi-Solid and Liquid Samples.* Liquefy by heating at not more than 10°C. above melting point, mix thoroughly, and take portion for analysis. Do not attempt to test sample which contains so much glycerol that it separates from the sample when cooled to room temperature.

   Caution: The sample must not be subjected to temperatures in excess of that required to melt them since the monoglyceride content may be reduced if any soap is present.

E. PROCEDURE FOR MONOGLYCERIDE:

1. Weigh duplicate samples accurately into 100-ml. glass-stoppered volumetric flasks. The proper size of sample is indicated in the following table:

Monoglyceride, %	Approximate size of sample, g.	Weighing accuracy, g.
100	0.30	±0.0002
75	0.40	±0.0002
50	0.60	±0.0003
40	0.70	±0.0005
30	1.00	±0.001
20	1.50	±0.001
10	3.00	±0.002
5	6.00	±0.004
3.0 or less	10.00	±0.010

2. Add 50 ml. of chloroform with a graduated cylinder. Dissolve the sample in the chloroform and mix thoroughly by shaking. Warm flask on a steam bath if necessary to effect complete soln. and then cool to room temperature.
3. Add 25 ml. of distilled water, stopper flask tightly, and shake vigorously for 30 to 60 seconds so that good contact occurs between aqueous and chloroform phases. The flask must be tightly stoppered and adequate precaution taken so that none of the aqueous extraction soln. or chloroform soln. is lost.
4. Set the flask aside until the aqueous and chloroform layers separate. Transfer the aqueous layer to a glass-stoppered 100-ml. volumetric flask, using a glass siphon. The aqueous layer must be transferred as completely as possible without including any of the chloroform layer. *Note:* When an emulsion forms due to the presence of soap in the sample, add 3 or 4 ml. of glacial acetic acid to break the emulsion.
5. Extract 3 more times using 25, 25, and 20 ml. of distilled water as described in D, 3 and 4.
6. Add chloroform to the flask until the level of the chloroform coincides with the 100-ml. mark on the flask. Using the glass siphon, transfer as much as possible of the aqueous layer above the chloroform layer to the flask containing the aqueous extracts. The aqueous extracts in the volumetric flask are saved for the determination of free glycerol.
7. Stopper the flask tightly and mix thoroughly by inverting.
8. Pipet 50 ml. of periodic acid reagent into a series of 400-ml. beakers. Prepare 3 for blanks, adding 50 ml. of chloroform to two and 50 ml. of water to the third. The titrations of the water and chloroform blanks are used as a check on the chloroform, see B, 6.
9. Pipet 50 ml. of chloroform-sample soln. into a 400-ml. beaker containing 50 ml. of periodic acid reagent, and shake gently to effect thorough mixing. Cover with a watch glass and allow to stand for 30 minutes (see Note 1).
10. Add 20 ml. of KI soln. mix by gentle shaking, allow to stand at least 1 minute, but never more than 5 minutes before titrating. Do not allow to stand in strong sunlight.
11. Add 100 ml. of distilled water and titrate with 0.1 N $Na_2S_2O_3$ soln. Use the variable speed electric stirrer to keep the soln. thoroughly mixed. Continue the titration to the disappearance of the brown iodine color from the aqueous layer. Add 2 ml. of starch indicator soln. and continue the titration to the disappearance of iodine from the chloroform layer and the disappearance of the blue iodo-starch color from the aqueous layer. Vigorous agitation is essential for complete removal of iodine from the chloroform layer.
12. Read the buret to the hundredth of a ml.
13. The blanks are handled exactly like the samples, E, 10 and 11.
14. If titration of sample E, 12 is less than 0.8 of the titration of the blank E, 13, there is not a sufficient excess of periodic acid to assure complete reaction. When this occurs, repeat the analysis using smaller portions (25, 10, or 5 ml. in E, 9) until the titration of the sample is more than 0.8 of that of the blank. When 10 ml. (or less) is required, the analysis should be repeated by starting at the beginning with a smaller sample, referring to E, 1 to find proper amount of sample to weigh.
15. For the best results the difference between the titration of the blank D, 13 and the titration of the sample should be more than 4 ml. When less than 4 ml., it is advisable to repeat the analysis beginning with E, 1, and using twice as much sample. If doubling the sample size exceeds 10 g., use only 10 g.

F. PROCEDURE FOR FREE GLYCEROL:

1. Add distilled water to combine aqueous extracts from monoglyceride test E, 6 until volume is 100 ml. and mix thoroughly.
2. Analyze in same manner as prescribed for monoglycerides, E, 8 to E, 15 inclusive.

G. CALCULATIONS FOR MONOGLYCERIDES:

1. The following calculations assume the monoglyceride to be monostearin.

$$\text{Monoglyceride, \%} = \frac{(B - S) \times N \times 17.927}{W}$$

B = Titration of blank containing 50 ml. chloroform.
S = Titration of sample.
N = Normality of $Na_2S_2O_3$ soln.
W = Weight of sample, represented by aliquot pipeted for test, E, 9.
17.927 = Mol. wt. of monostearin divided by 20.

The monoglyceride may be calculated to some monoester other than monostearin by dividing the molecular weight of the monoglyceride by 20 and substituting this value for 17.927 in the formula above.

If the molecular weight of the monoglyceride is not known, proceed as follows:

a) Separate the fat acids as described in A.O.C.S. Method Cd 6-38.
b) Determine the acid number of the fat acids as directed in A.O.C.S. Method 14-42.
c) Average mol. wt. of fat acids $= \frac{56104}{\text{acid no.}} = M.$
d) Mol. wt. of monoglyceride $= (M + 92.09) - 18.02.$

H. CALCULATIONS FOR GLYCEROL:

$$\text{Free glycerol, \%} = \frac{(B - S) \times N \times 2.30}{W}$$

B = Titration of blank containing 50 ml. of water.
S = Titration of sample.
N = Normality of $Na_2S_2O_3$ soln.
W = Weight of sample represented by aliquot taken for test.
2.302 = Mol. wt. of glycerol divided by 40.

I. NOTES:

1. Samples may be allowed to stand 1½ hours at room temperature before titrating, but never longer.
2. *Cork stoppers must never be used where periodic acid can come in contact with them.*

Summary

A modified and improved method for the determination of monoglyceride by the periodic acid method has been presented as well as a method for free glycerol in fatty products. The advantages of the method for monoglyceride are 1. eliminates danger of secondary reactions due to elevated temperatures, 2. eliminates the need of shaking to effect good contact and complete reaction between sample and reagent, and 3. makes removal of free glycerol from the sample easier.

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