Limitations of Periodate-Permanganate Oxidation in Determination of Glyceride Composition: Nonquantitative Oxidation and Hydrolysis of Azelaoglycerides

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Abstract

Oleic acid, methyl oleate, mono- and diunsaturated triglyceride concentrates, triolein, *Garcinia indica* fat and safflower oil were oxidized with periodate-permanganate reagent in aqueous 60% *tert*-butanol at 80 C (reflux temperature), 65 C and 28 to 30 C under different conditions. Significant proportions of unoxidized and partially oxidized compounds, mainly ketohydroxy compounds, were found in oxidation products. Hydrolysis of azelaoglycerides, particularly triazelain also occurred. The di- and trisaturated glyceride contents are therefore likely to be higher than the actual for all fats when determined by Youngs and Subbaram's method and for fats containing high proportions of triunsaturated glycerides by Youngs' method.

Introduction

The oxidative scission of unsaturated fatty acids, esters and oils with sodium metaperiodate in the presence of potassium permanganate at pH 8–9 is claimed to be quantitative (1–8). Based on this oxidation, Youngs (4) proposed a method for the determination of six glyceride types in natural fats. The oxidized fat was fractionated on a silicic acid partition column. The fractions, viz., GS_3 + GS_2A and GSA_2 + GA_3 (G, glyceryl; S, saturated acid; A, azelaic acid radicals) were subjected to pancreatic lipase hydrolysis after esterification and the liberated fatty acids were analyzed by gas liquid chromatography (GLC). In a later method by Youngs and Subbaram (5), total azelaoglycerides were esterified and analyzed by GLC. The probabilities which may affect the accuracy of these methods have been scrutinized in the present investigation. These are nonquantitative oxidation leading to the presence of residual unsaturated glycerides and intermediate oxidation products, e.g., ketohydroxy compounds and hydrolysis of azelaoglycerides.

Experimental Procedures

Materials

Garcinia indica fat (9a) and safflower oil (9b) were genuine. GS_2U and GSU_2 (U, unsaturated acid) concentrates were isolated from *G. indica* fat by intensive crystallization from acetone (10). Their compositions are given in Table I. These were calculated from the fatty acid compositions, determined by GLC on a diethylene glycol succinate (DEGS) column at 205 C, assuming that the GS_2U concentrate contained only GS_2O (O, oleic acid) and GS_3 and the GSU_2 concentrate contained only GS_2O, GS_2L (L, linoleic acid) and GSO_2.

Fatty acids of the GS_2U concentrate were subjected to urea adduction thrice and then to ester-fractionation to give methyl oleate. GLC on a DEGS column at 205 C showed it to contain 2.7% (wt.) of stearate and no other impurity.

Triolein was synthesized from oleic acid, which was obtained from methyl oleate, by esterification with glycerol using *p*-toluene-sulfonic acid. After purification by chromatography on alumina (10) and subsequently silicic acid columns (11), triolein was found to be a pure triglyceride by thin layer chromatography (TLC) on Silica Gel G (E. Merck) using the solvent system petroleum ether (60–80 C)-ether-acetic acid (90:10:1 v/v). It had an iodine value (IV) 78.7. It would have 92.3% of triolein, if esterification had taken place at random as expected. Tristearin was similarly prepared from stearic acid, obtained from GLC-pure methyl stearate and purified.

9(10),10(9)-Ketohydroxystearic acid (mp 64.5 C) was prepared from reagent grade elaidic acid according to King (12). TLC of the methyl ester on Silica Gel G impregnated with silicone oil (Dow Corning 200) using the solvent system acetonitrile-acetic acid-water (70:10:20 v/v) and GLC on a silicone gum rubber methyl-GE SE-30 column at 210 C, showed the ester to be at least 95% pure. Pure 9,10-dihydroxystearic acid was obtained as a by-product. Azelaic and sebacic acids were purified by recrystallization from water and ethyl acetate. GLC of the methyl esters on a SE-30 column at 150 C showed that they were pure.

Methods of Analysis

The official and tentative method of the AOCS (13) for the determination of IV was carried out on a semimicro scale. About 100 mg of oxidized material was dissolved in 5 ml of alcohol-free chloroform in a 100 ml round-bottomed flask with a ground glass stopper. Wijs reagent (5 ml) was pipetted. After 30 min in the dark, 5 ml of 15% KI solution was pipetted and the contents were titrated with 0.02 N thiosulfate solution.

Neutral products were isolated from oxidation products by alumina column chromatography (10). Using a 2% solution, the sample was analyzed in CCl_4 by IR spectrophotometry in 1 mm NaCl cell. A F&M

TABLE I

Composition of Glyceride Concentrates

Glyceride concentrate	Fatty acid mole %				Glycerides mole %			
	Palmitic	Stearic	Oleic	Linoleic	GS_3	GS_2O	GS_2L	GSO_2
GS_2U	0.0	67.4	32.6	0.0	1.7	98.3	0.0	0.0
GSU_2	5.8	36.1	54.7	3.4	0.0	8.0	10.2	81.8

Model 720 dual column programmed temperature gas chromatograph equipped with a flame ionization detector was used. A 2 ft × $^3/_{16}$ in. bronze column packed with 2% SE-30 on Chromosorb W (60–80 mesh) was used. The rates of flow of hydrogen, nitrogen and air were 40, 100 and 400 ml/min, respectively.

Oxidation Procedures

The conditions of oxidation described by Youngs (4), Youngs and Subbaram (5) and Tulloch and Craig (6) were strictly followed including the size of the sample. Where necessary, the oxidation products were pooled from a number of experiments. Sodium bisulfite was used to reduce the excess oxidant in place of ethylene, which was not available. The oxidation products were extracted with chloroform after saturation with NaCl (5).

Determination of Residual Unsaturation

The percentage of residual unsaturation was calculated from the weight of fatty material taken for oxidation and the weight of unoxidized glycerides present in the oxidation products. The latter value was obtained from the weight of thiosulfate equivalent to Wijs reagent consumed by the unoxidized material, assuming that a portion of the least unsaturated glyceride was left unoxidized. The residual unsaturated acid was identified. The methyl esters of oxidized *G. indica* fat and safflower oil were shown by GLC to contain small amounts of oleate and linoleate respectively, in addition to the saturated esters, probably because these are the predominant unsaturated components in the respective fats.

Determination and Characterization of Partially Oxidized Products

The percentage of partially oxidized products was calculated from the weight and IV of the neutral products obtained from the oxidation products by alumina column chromatography and the weight and the composition of the material subjected to oxidation.

The neutral products isolated from oxidized methyl oleate were subjected to reversed phase TLC along with pure ketohydroxystearate under the conditions described earlier. The IR spectrum was recorded before and after acetylation. The acetylated products were analyzed by GLC at 210 C. The original neutral products were saponified by refluxing with excess 0.5 N alcoholic potash. The acids including azelaic and pelargonic acids, the split products of ketohydroxystearate if present (14), were liberated by cold acidification. These were extracted with ether, esterified with diazomethane and analyzed by GLC at 150 C.

The neutral products isolated from glyceride concentrates were analyzed similarly. TLC on Silica Gel G using petroleum ether (60–80 C)-ether-acetic acid (90:10:1 v/v) was employed instead of the reversed-phase TLC used for products from methyl oleate.

Extent of Hydrolysis of Azelaoglycerides

A known amount of glyceride concentrate or oil was oxidized. The oxidation products were recovered as usual by extraction with $CHCl_3$ after saturation with NaCl (5), esterified with diazomethane and weighed. To the esters a known amount (about 2%) of dimethyl sebacate was added as an internal standard for estimating dimethyl azelate, if present. A suitable amount of the ester mixture was injected into the gas chromatographic column to give a measurable peak for sebacate. At the column temperature of 150 C, only the methyl esters of short chain mono- and dicarboxylic acids were eluted; esters of azelaoglycerides and neutral glycerides were retained. From the area percentages of sebacate and azelate, the percentage of dimethyl azelate present in the ester mixture was calculated. From this, the percentage of azelaic acid derived from glyceride concentrate or fat by hydrolysis of azelaoglycerides during oxidation was calculated. The percentage of hydrolysis was calculated assuming that all azelaic acid radicals in the azelaoglyceride were hydrolyzed and that GS_2O, GSO_2, GO_3 and GLLO were hydrolyzed in the GS_2U, GSU_2 and triolein concentrates and safflower oil, respectively. These percentages were, however, lower than the actual because of the poor recovery of azelaic acid during isolation of oxidation products as demonstrated in the blank experiments which were carried out as follows. To a mixture of tristearin (ca. 100 mg) and varying quantities of azelaic acid (0.2 to 2.0 mg) all the reagents were added as in the regular oxidation, but the oxidant was reduced immediately. Thereafter the material was processed and extracted as usual (5). The amount of azelaic acid incorporated into the synthetic mixture was approximately the same as that found experimentally by GLC in each of the oxidized glyceride concentrates and oil. The recovery percentage of azelaic acid in the blank experiment was 70.2 for GS_2U concentrate, 60.5 for GSU_2 concentrate and 58.5 for triolein and safflower oil. The percentages of hydrolysis calculated from the azelaic acid contents determined by GLC were accordingly corrected.

The oxidized GS_2U and GSU_2 concentrates were examined for the presence of di- and monoglycerides by TLC on alumina according to Lederkremer and Johnson (15).

Results and Discussion

Residual Unsaturation

The periodate-permanganate oxidation of unsaturated fatty materials has been considered as a quantitative reaction (1–8), on the basis of oxygen consumption, azelaic acid recovery and absence of unsaturated fatty acids in the oxidation products. However, Kuemmel (7) reported low recoveries of short chain mono- and dicarboxylic acids and also some unidentified material. Extraction of dibasic acids by trituration with petroleum ether (1,3) is alleged to be nonquantitative (16) because of intersolubility effects. It is probable that residual unoxidized oleate could have been masked by stearate (4) or could have been interpreted as C_{11}-dicarboxylic acid (6) in the earlier standardization experiments using GLC. None of the earlier investigators has estimated residual unsaturation by determining the iodine value as in the present investigation. The percentages of unoxidized material left over after oxidation of various types of unsaturated compounds are given in Table II. For oleic acid and methyl oleate these ranged from 1.0% to 1.6%. No improvement was observed when methyl oleate was oxidized for 70 hr with 600% excess oxidant at room temperature. The percentage of unoxidized oleic acid was almost the same when the oxidation was carried out either in aqueous or in 60% *tert*-butanol medium. Glycerides oxidized at 28–30 C contained maximum amounts of unoxidized material.

TABLE II

Extent of Residual Unsaturation after Periodate-Permanganate Oxidation of Unsaturated Fatty Materials

Material	Unoxidized material, %[a]		
	At 80 C (reflux) (4)	At 65 C (5)	At 28–30 C (6)
Oleic acid	.....	.....	1.0[b]; 1.5[c]
Methyl oleate	1.6	1.2	1.2
GS_2U concentrate	2.3	1.7	8.5
GSU_2 concentrate	.....	1.8	7.6
Triolein concentrate	10.1	10.0	10.6
G. indica fat	9.3	8.9	12.3
Safflower oil	2.3	3.7	5.6

[a] Calculated on the basis of material taken for oxidation, assuming that the unoxidized glycerides were GS_2O in GS_2U and GSU_2 concentrates and *G. indica* fat, stearodiolein in triolein concentrate and palmitodilinolein in safflower oil.
[b] Aqueous medium.
[c] 60% Aqueous *tert*-butanol medium.

Partially Oxidized Products

The presence or absence of partially oxidized products in the oxidation products was not scrutinized carefully in the earlier investigations. These were isolated in the present study, together with unoxidized material, by chromatography on an alumina column. Partially oxidized neutral products were found to the extent of 4.6%, 4.5% and 7.4% respectively when methyl oleate, GSU_2 concentrate and triolein were oxidized at 65 C (5). When methyl oleate was oxidized at 28–30 C (6) these products were found to the extent of 6.4%. In the case of methyl oleate oxidized according to Youngs (4) and Youngs and Subbaram (5) these were shown by reversed-phase TLC to consist of mainly ketohydroxystearate, small amounts of dihydroxystearate and unoxidized oleate. IR spectrum of neutral products isolated from methyl oleate after oxidation according to Youngs and Subbaram (5) showed a peak at 1710 cm^{-1} characteristic of free C = O, apart from the peak at 1730 cm^{-1} due to ester group. The peak at 1710 cm^{-1} became more prominent after acetylation. A comparison of these spectra with those of standard ketohydroxystearate before and after acetylation gave further proof for the presence of a keto group. GLC gave a peak corresponding to ketoacetoxystearate, after acetylation of the neutral products. Vicinal ketohydroxy fatty esters are known to split on saponification (14) giving in the present case azelaic and pelargonic acid soaps. Confirmatory evidence was thus obtained for the presence of such compounds by saponification of the neutral products, acidification in the cold and GLC of the esterified acids.

Neutral products isolated from oxidation at 65 C (5) of GS_2U and GSU_2 concentrates and triolein were found to contain products of higher polarity than that of triglycerides, by TLC on Silica Gel G. IR spectrophotometry, however, did not show the presence of keto or hydroxy groups, probably due to the low percentages of such compounds and the high molecular weight of the glyceride. Pelargonic and azelaic acids were again found to be obtained by alkali-scission of the neutral products derived from GS_2U concentrate after oxidation at 65 C (5) indicating the formation of ketohydroxy compounds during oxidation of these glycerides.

Hydrolysis of Azelaoglycerides

Hydrolysis of glycerides was deduced to be absent by Youngs (4) by GLC of the oxidized fat after esterification under conditions wherein only the methyl esters of free fatty acids were eluted. The name of the fat used in this standardization experiment was not mentioned. If it was a comparatively saturated fat like lard, giving on oxidation predominantly GS_2A, any of the hydrolysis products, namely, azelaic acid, saturated acids and diglycerides, would not be detected, since the present investigation shows that hydrolysis of GS_2U is negligible. For the same reason, diglycerides could not be detected in oxidized fats by Youngs and Subbaram (5) using GLC and by us in the present investigation using alumina TLC as described by Lederkremer and Johnson (15). Again monoglycerides and glycerol, the products of hydrolysis of GSA_2 and GA_3 respectively, were not detected in the oxidation products, since, as expected, they were further oxidized by periodate. Therefore, the absence of azelaic acid in the oxidation products is the only sure means of proving the absence of hydrolysis of azelaoglycerides. Only 60–70% of the azelaic acid formed by hydrolysis could be recovered by the procedure used for the isolation of oxidation products. Even so, peaks for dimethyl azelate were seen in our gas liquid chromatograms. The percentages of hydrolysis of unsaturated glycerides calculated from azelaic acid contents of oxidation products were corrected on the basis of recovery percentages of azelaic acid obtained in blank experiments and are given in Table III.

TABLE III

Extent of Hydrolysis of Unsaturated Glycerides in Periodate-Permanganate Oxidation

Material	Hydrolysis, %[a]		
	At 80 C (reflux) (4)	At 65 C (5)	At 28–30 C (6)
GS_2U concentrate	1.0	0.5	0.0
GSU_2 concentrate	1.6	1.1	0.7
Triolein concentrate	7.1	6.4	1.7
Safflower oil	8.1	5.3	1.2

[a] Calculated on the basis of material taken for oxidation, assuming that GS_2O, GSO_2, GO_3 and GLLO were hydrolyzed in GS_2U, GSU_2 and triolein concentrates and safflower oil, respectively.

Effects of Nonquantitative Oxidation and Hydrolysis on Glyceride Analysis

In the present investigation only the oxidation step was examined. As to how the accuracy of the methods proposed by Youngs (4) and Youngs and Subbaram (5) is affected by nonquantitative oxidation and hydrolysis of azelaoglycerides could only be speculated. The unoxidized unsaturated glycerides may be expected to emerge with the GS_3 + GS_2A fraction on the silicic acid partition column of Youngs giving rise to higher values for GS_3 and GS_2U. On the other hand, the partially oxidized products, being more polar, may emerge with the GSA_2 + GA_3 fraction giving higher values for GSU_2 and GU_3 at the expense of GS_3 and GS_2U. These two effects may therefore counteract each other to some extent. Hydrolysis of azelaoglycerides may tend to increase apparent GS_3 and GS_2U, since hydrolysis of both GSU_2 and GU_3 is greater than that of GS_2U. Youngs' method may, therefore, give serious errors with oils containing a high percentage of GSU_2 or GU_3. On the GLC column of Youngs and Subbaram the unoxidized unsaturated glycerides may emerge with GS_3 and inflate its percentage. Since Youngs and Subbaram found that even tristearin was not eluted completely from the GLC column there is reason to believe that partially oxidized products, being more polar and having a higher molecular weight, may not be eluted. Since more partially oxidized products arise when glycerides of higher unsaturation are oxidized, and since the final composition in GLC charts adds up to 100, higher values for GS_3 and GS_2U may be obtained.

As with Youngs' method hydrolysis of azelaoglycerides will give higher values for GS_3 and GS_2U. Since all these effects are additive, higher values for GS_3 and GS_2U may be obtained by Youngs and Subbaram's method. As an example of the possible occurrence of these effects, the results on cocoa butter may be cited. Youngs obtained 73% for GS_2U, whereas Youngs and Subbaram 82%, while Vander Wal's theoretical value is 71% (4).

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