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QUANTITATIVE ESTIMATION OF GLYCEROL IN LIPIDS AS ITS TRIMETHYLSILYL ETHER BY GAS-LIQUID CHROMATOGRAPHY

A. RAJIAH, M. R. SUBBARAM AND K. T. ACHAYA

Regional Research Laboratory, Hyderabad 9 (India)

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SUMMARY

Glycerol has been quantitatively estimated by gas-liquid chromatography of its trimethylsilyl ether, using hexadecane as an inert internal standard. The glycerol contents of some vegetable oils are reported. Kamala seed oil and kusum oil have a low glycerol content. Triglyceride groups separated by argentation chromatography could be estimated from glycerol determination by gas-liquid chromatography.

INTRODUCTION

Since glycerol plays an essential role in the metabolism of triglycerides, phospholipids, and other materials in both animal and plant tissues, a quick and accurate method for its quantitative estimation is of great importance. Earlier methods used to estimate glycerol quantitatively include, among others, periodic acid oxidation¹⁻⁷, copper complex formation⁸, an enzymatic procedure (ref. 9 and references cited therein), microbiological determination¹⁰ and paper chromatography¹¹. Methods involving oxidation with periodic acid are not very accurate because of interference by biological compounds such as glucose, glycerophosphate and ethanolamine¹². Microbiological, enzymatic and paper chromatographic methods are rather tedious. More recent methods are based on gas-liquid chromatography (GLC)¹³⁻¹⁷. A procedure for the simultaneous quantitative determination of glycerol and fatty acid contents of fats and oils involved interesterification of fat with methanol and analysis of the methyl esters and of glycerol as isopropylidene-glycerol by GLC¹³. In other methods¹⁴⁻¹⁷, the acetyl derivative of glycerol was analysed by GLC against an internal standard such as butane-1,4-diol or hexadecanyl acetate. The different rate of acetylation of a hydroxyl-containing standard and glycerol could perhaps influence the quantitation procedure, and, therefore, use of an inert internal standard seemed preferable. In this paper we report a rapid and quantitative method for the estimation of glycerol as its trimethylsilyl ether by GLC, using hexadecane as an inert internal standard. The method has been applied to determine the glycerol content of vegetable oils and of fractions separated from them on silver nitrate-impregnated silica gel thin-layer plates.

MATERIALS AND METHODS

Apparatus

This consists of an F & M Model 1609 gas chromatographic unit with a hydrogen flame ionization detector system. A 2-ft. \times $\frac{3}{16}$ in. I.D. coiled stainless steel column packed with 2% SE 30 (silicone rubber gum) on Chromosorb W 60-80 mesh was used.

Materials

Petroleum ether AR (b.p. 40-60°) was not purified further.

Pyridine AR (BDH) was refluxed over barium oxide, distilled and kept over solid potassium hydroxide.

Glycerol AR (BDH) was rendered anhydrous by heating it to 120° before use with continuous stirring, and kept under anhydrous condition. Glycerol standards were prepared in dry pyridine to give concentrations from 1.0-3.0 mg/ml.

Methanol GR (Merck) grade was used.

n-Hexadecane was purified by percolation through a column of activated silica gel (BDH quality). A stock solution of 0.5 g hexadecane in 50 ml dry pyridine was prepared (1 ml = 10 mg).

Anhydrous methanolic HCl (5%) was prepared by passing dry hydrogen chloride into methanol till the requisite concentration was obtained.

Hexamethyldisilazane (Peninsular Chemresearch, Gainesville, Fla., U.S.A.) and *trimethylchlorosilane* (K.K. Labs, Plainview, N.Y., U.S.A.) were used without further treatment.

Potassium hydroxide (Merck) pellets.

Tripalmitin was synthesised by refluxing a solution of palmitoyl chloride and glycerol (4:1) in a pyridine and chloroform medium for 4 h¹⁸, followed by column chromatographic purification using the procedure of QUINLIN AND WEISER¹⁹. The product melted at 65.5°.

Vegetable oils. Triglycerides were obtained from the oils by chromatography on a silica gel column according to QUINLIN AND WEISER¹⁹. Kamala seed oil and kusum oil (*Schleichera trijuga*) were used as such after removing moisture.

Methods

Silylation procedure. Silylation was carried out by the method of SWEELEY *et al.*²⁰ incorporating the improvements of WOOD and co-workers²¹ as follows: Aliquots of a standard solution of glycerol equivalent to 1.0, 2.0 and 3.0 mg were taken in duplicate in 15-ml glass-stoppered test tubes. To each tube was added successively 30 μ l of hexadecane solution, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. The reaction mixture was shaken vigorously for 1 min and set aside for 10 min. At the end of this period 5 ml distilled water and 5 ml petroleum ether were added to the contents of the test tube. After shaking thoroughly, the layers were allowed to separate whereby the silyl ether of glycerol passed into the petroleum layer. Extraction of the aqueous phase was repeated twice with 5 ml aliquots of petroleum ether. The petroleum extracts were combined and, after drying over anhydrous sodium sulphate, the solvent was removed on a water bath until free from the odour of pyridine. The final volume was *ca.* 0.2 ml. Until GLC analysis, these silylated products were stored in a deep-freezer.

Liberation of glycerol from triglycerides. Tripalmitin (12.5 and 25 mg samples, in duplicate) was refluxed for 2 h with 2 ml of 6% methanolic potassium hydroxide. The solution was cooled, neutralised with a solution of 5% methanolic HCl (to Congo red) and the solvent removed on a water bath. The liberated glycerol was taken up in 2 ml pyridine, and the contents mixed thoroughly and silylated by the procedure described above, again incorporating hexadecane (30 μ l) as the internal standard.

The vegetable oils (10 mg samples) or glyceride fractions were similarly hydrolysed and silylated.

Analysis by gas-liquid chromatography. Injection port temperature, 170°; detector block temperature, 160°; flow rates of hydrogen, nitrogen and compressed air were respectively, 40, 100 and 400 ml/min; attenuation, 1600; chart speed, 30 in./h. The column was run isothermally at 100° till the glycerol derivative emerged, and thereafter the temperature was increased to 110° by programming at the rate of 5°/min, after which it was again run isothermally till hexadecane emerged. Peak areas were measured by triangulation.

RESULTS AND DISCUSSION

Standardisation experiments with glycerol and with tripalmitin

Fig. 1 shows that silylated glycerol and hexadecane were well separated. Peak areas of silylated glycerol were measured with respect to hexadecane (Table I) and the ratios were found to be close to simple multiples. Standardisation with tripalmitin likewise gave ratios proportional to the amounts taken. These values (ratio of the

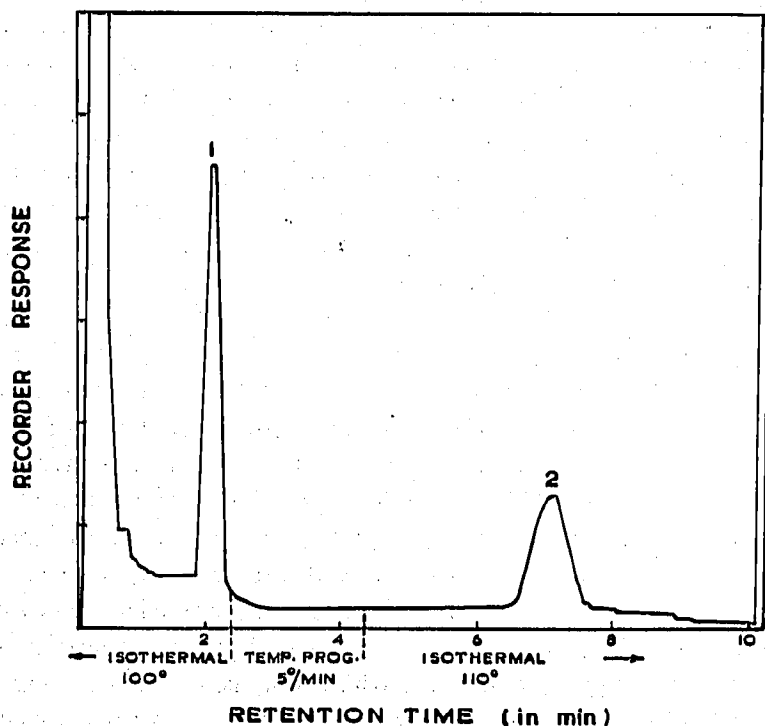


Fig. 1. GLC separation of silylated glycerol (peak 1) and hexadecane (peak 2); conditions are given in the text.

TABLE I

ANALYSIS BY GAS-LIQUID CHROMATOGRAPHY OF GLYCEROL AS ITS TRIMETHYLSILYL ETHER IN STANDARD GLYCEROL SOLUTION AND TRIPALMITIN USING HEXADECANE AS INTERNAL STANDARD

Material added (mg)	Pyridine solution of hexadecane (μ l)	Area of glycerol-derived peak (α)	Area of hexadecane peak (β)	α/β
Glycerol				
1.0	30	5.60	4.77	1.17
2.0	30	3.06	1.50	2.04
3.0	30	9.35	3.15	2.97
Tripalmitin				
12.5	30	2.14	1.28	1.66
25.0	30	3.90	1.21	3.22

peak area of silylated glycerol to that of hexadecane) were later used when determining glycerol in glyceride groups isolated from cocoa butter by argentation chromatography.

Glycerol estimation in vegetable oils

Most of the vegetable oils were purified by QUINLIN AND WEISER's procedure¹⁹. Kamala seed oil is unusual in that it contains triglycerides of exceptionally high molecular weight²², and such chromatographic purification was not feasible. Kusum oil contains nonglyceridic compounds and, therefore, it also was not purified by column chromatography. All the vegetable oils (10 mg samples) were treated for glycerol recovery, and subsequently silylated with addition of the internal standard as outlined in the experimental section. From the relative peak areas of known quantities of silylated pure glycerol and hexadecane, and the relative areas of the silylated glycerol derived from the vegetable oil and hexadecane, the percentage glycerol in the oil can be calculated. For example, using 1 mg glycerol and 0.35 mg hexadecane, the ratio of the peak areas for silylated glycerol and hexadecane was $5.60/4.77 = 1.17$. When 10 mg groundnut oil was taken, and 0.35 mg hexadecane added during silylation of the glycerol released, the ratio of the peak areas obtained was $5.21/4.20 = 1.24$. Hence the glycerol present in 10 mg groundnut oil is $1.24/1.17 = 1.06$ mg, *viz.* 10.6%.

The amounts of glycerol obtained from vegetable oils are given in Table II. Present values for the normal vegetable oils are in good agreement with those reported in the literature²²⁻²⁴. This seems to indicate that the method is of general applicability. The higher value for refined cottonseed oil, which was consistently obtained cannot be explained. Kamala seed oil consists of glycerides in which kamolenic acid (18-hydroxy-9-*cis*, 11-*trans*, 13-*trans*-octadecatrienoic acid) is believed to occur in linked chains terminated by a normal fatty acid. As a result, the mean molecular weight of the oil is around 1800 (ref. 22), and a low glycerol content, as now obtained, was also earlier observed by chemical analysis²⁵. Kusum seed oil (from *Schleichera trijuga*) is unique among vegetable oils in that it contains only 37% triglycerides, the rest being nonglyceridic components²³. The reported glycerol content of this oil is 3.7%, which is in fair agreement with the value of 3.8% now obtained.

TABLE II

GLYCEROL CONTENT OF VEGETABLE OILS

<i>Oil</i>	<i>Glycerol (%) by other methods^a</i>	<i>Glycerol (%) by the present method</i>
Safflower oil	10.7	10.9
Groundnut oil	10.8	10.6
Cottonseed oil	10.8	11.9
Cocoa butter	10.8	10.3
Mustard oil	9.9	9.7
Kamala seed oil	3.6	3.4
Kusum oil	3.7	3.8

^a Refs. 22-24.

Quantitation of glyceride groups in oils by determination of glycerol

The amounts of glycerides present in fractions, into which the parent fats have been resolved by argentation chromatography, have been indirectly estimated by calculation based on the glycerol content estimated in each of these fractions using the periodate oxidation procedure^{20,27}. This has now been done using the present method of glycerol estimation for cocoa butter, which was separated into glyceride groups by preparative TLC on silver nitrate-coated silica gel plates using an ether-petroleum ether solvent system (25:75). The separated zones, consisting of glycerides containing 0, 1, 2 and 3 double bonds, were visualised with a dichlorofluorescein spray and viewed by ultraviolet light. Bands were marked lightly. Each fraction was separately scraped off and the fatty material extracted in a continuous solvent extractor using ether²⁸. The extracts were dried and suitable aliquots refluxed with 2 ml of 6% methanolic potassium hydroxide solution. The glycerol was liberated, hexadecane added and GLC carried out after silylation. In a separate run, a standard glycerol solution representing 1 ml glycerol was also silylated and analysed by GLC.

The amounts of the various glyceride groups in cocoa butter calculated from

TABLE III

EXAMINATION OF GLYCERIDE GROUPS IN COCOA BUTTER BASED ON GLYCEROL ESTIMATION

<i>Glyceride group, no. of double bonds</i>	<i>Glyceride present based on glycerol estimation (%)</i>	<i>Glyceride present based on weight eluted by column chromatography^a (%)</i>
0	traces	1.4
1	76.2	73.8
2	16.1	18.1
3	7.7	6.7

^a Ref. 29.

their glycerol contents determined in this way are shown in Table III. Also given are the proportions of glyceride groups reported by SUBBARAM AND YOUNGS²⁰ for this fat using the argentation column chromatographic method of DE VRIES³⁰ for the separation and estimation by weight of glyceride groups. Fully-saturated glycerides, which occur in trace amounts, could not be definitely estimated. The results for other groups agree to within $\pm 2.5\%$ with those obtained by column chromatography on a different sample.

Although only analysis of vegetable oils is now reported, the method should also be suitable for the quantitative estimation of glycerol in animal fats, mono- and diglycerides, and other derivatives of glycerol.

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