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A technique for the determination of serum glycerides*

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» The present communication describes a method for the determination of serum glycerides that combines the features of several already reported. It enables a single technician to complete 20 determinations, in duplicate, of sera varying from about 30 to at least 12,000 mg of glyceride per 100 ml in one day with an accuracy of nearly 4%. The color reaction used is a

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modification of the technique of Randrup (1). This provides significant advantages over procedures employing the chromotropic acid technique of Lambert and Neish (2), by permitting the use of a highly standardized color reagent easily prepared daily, and eliminating prolonged heating to develop color.

Lipids were extracted by a modification of Bragdon's procedure (3). One-half milliliter of serum or plasma was added to 12 ml of chloroform–methanol 2:1 (v/v) in a 20 x 125-mm culture tube fitted with a Teflon-lined screw cap. The tube was then shaken horizontally for 10 min in a 280-stroke-per-min Kahn shaking machine, and 2.5 ml of distilled water was added. The tubes were inverted 10 times and then centrifuged at 3000 rpm ($2000 \times g$) for 15 min at 4°. Three layers were formed: the lower layer, 9.0 ml of chloroform containing essentially all the lipid; the middle layer, a disk of tightly packed protein; and an upper aqueous alcohol layer. The upper layer was removed by aspiration and discarded. The lower chloroform phase can also be obtained by centrifugation at room temperature, but then must be clarified by placing the tube in an ice bath for 5 min followed by recentrifugation for 3–4 min.

To 5 ml of the chloroform layer, placed in a 16 x 125-mm culture tube with a Teflon-lined screw cap, were added 5 ml of chloroform and 0.5 g of activated silicic acid.[1] This was shaken as above for 30 min and the silicic acid was then packed by centrifugation.

One milliliter of the supernatant chloroform solution was transferred to a 15-ml graduated centrifuge tube and evaporated at 60–63° under a current of air or nitrogen. One milliliter of 4% potassium hydroxide in 95% ethanol was added, and the solution was then mixed and heated in a 60–63° water bath for 30 min. One milliliter of 8% HCl (v/v) was added, followed by distilled water to a total volume of 10 ml; *N*-hexane was then added to the 15-ml mark. The glass-stoppered tube was shaken vigorously by hand for 45 sec and then centrifuged. The upper hexane phase along with about 1 ml of the lower aqueous alcohol phase was then discarded.

Commercial reagent grade glycerol was used as standard (4). The water content of the glycerol was determined from the specific gravity according to Lawrie (5). Approximately 1 g of glycerol was diluted to 100 ml with distilled water. From this solution, stable for at least 9 months at 4°, a working standard containing 4–5 μg glycerol/ml was prepared daily. For each set of determinations, 1, 3, and 5 ml of working standard were employed.

[1] Silicic acid, minus 325 mesh, activated 4 hr in a 110–125° oven. Bio-Rad Laboratories, Richmond, California.

Fifty milliliters of "blank" were prepared, using the above saponification technique with a 5-fold quantity of reagents. The final hexane extraction was not performed with blank or standards.

The following reagents were prepared for colorimetry: periodic acid, 0.067 M, prepared daily as 153 mg H_5IO_6 in 10 ml of distilled water; phenylhydrazine hydrochloride, 0.125 M, prepared daily by dissolving 338 mg of phenylhydrazine base in about 20 ml of distilled water, followed by addition of 2.5 ml of concentrated HCl, and distilled water to a total volume of 25 ml ($C_6H_5NHNH_2$, rather than $C_6H_5NHNH_2 \cdot HCl$, was used because of the relatively lower blank it provided); potassium ferricyanide, 0.137 M, as 4.5 g of $K_3Fe(CN)_6$ dissolved in distilled water to a total volume of 100 ml; concentrated HCl.

Five-milliliter aliquots of the aqueous alcohol phase of blank, standards, and samples were transferred to 19 x 105-mm colorimetric cuvettes. In a semi-darkened room with no direct light, 0.05 ml of periodic acid solution was added to each from a calibrated dropper, taking care to have the drops strike the liquid surface. The solutions were mixed.[2] After 10 min, 0.5 ml of phenylhydrazine hydrochloride solution was added and, following a 10-min interval in the dark, 0.2 ml of potassium ferricyanide solution. The cuvettes were then placed in an ice bath for 4–5 min before the addition of 2.5 ml of concentrated HCl.

Twelve minutes later, the optical density was read at 530 mμ. Earlier than 12 min, the optical density varied irregularly; subsequently, it diminished steadily at a rate of about 0.25% per minute. If the optical density exceeded 0.8, appropriate dilutions of hydrolysate were prepared using the blank solution previously prepared.

When the original Bragdon procedure for extraction of lipids (3) was compared with the one adapted here, there was no significant difference in amount of lipid recovered.

No significant quantity of phosphorus was recovered in the eluate from silicic acid even when phospholipid was added to the chloroform extract of serum to a final concentration of 40 mg of lipid phosphorus per 100 ml. To test the recovery of glycerides in this eluate, pure mono-, di-, and triglycerides[3] were added separately to chloro-

[2] It is highly desirable to use a mechanical tube mixer, such as the Vortex Jr. Mixer (Scientific Industries, Inc., Queens Village, N.Y.) in mixing each added reagent with the solution in the colorimetric procedure.

[3] The author wishes to express his appreciation to Dr. A. I. Holtz and Dr. J. E. Berger, National Heart Institute, Bethesda, Maryland, the first for supplying a mixture of the total phospholipid extract of beef spinal cord and the second for providing pure monostearin and dilaurin; and to Dr. F. H. Mattson, Procter and Gamble Co., who supplied all tripalmitin employed in this technique.

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TABLE 1. Recovery of Cotton Seed Oil Emulsion over a Wide Range of Concentrations

Sample No.	Glyceride Concentration		Difference
	Actual*	Found	
	mg/100 ml		%
1	40	36	−10.0
2	65	68	+ 4.6
3	100	105	+ 5.0
4	120	130	+ 8.3
5	150	152	+ 1.3
6	190	188	− 1.1
7	260	248	− 4.6
8	325	317	− 2.5
9	600	640	+ 6.7
10	1100	1130	+ 2.7
11	1350	1408	+ 4.3

* Coded dilutions were kindly supplied by Mr. O. Young and Mr. T. Shiratori, Laboratory of Metabolism, National Heart Institute, Bethesda, Maryland.

form extracts of normal serum. The recoveries were 54%, 91%, and 99% respectively. The occurrence of mono- and diglycerides in human plasma is controversial. Carlson and Wadström (6) reported that approximately 1.5% and 4.8% of total glycerides were mono- and diglycerides, respectively. Hirsch and Ahrens (7) reported the absence of partial glycerides in plasma from normal and hyperlipemic patients, except during heparin-induced clearing. If the quantity of partial glycerides in plasma samples is, indeed, negligible, then the error in glyceride determination due to selective retention of partial glycerides should not exceed 1%.

The results of a single-blind determination of known amounts of a triglyceride emulsion ranging in concentration from 40 to 1350 mg are shown in Table 1. The addition of a known triglyceride emulsion to nine serum samples also resulted in a mean recovery of 99% of the added fat. Identical recovery was obtained with standards of pure tripalmitin, a mixture of oils (C_{10} 0.55%, C_{14} 1.46%, $C_{14:1}$ 0.36%, C_{16} 1.97%, $C_{16:1}$ 3.28%, $C_{18:1}$ 88.01%, and $C_{18:2}$ 4.34%), freshly distilled glycerol,[4] glycerol stored for nine months at 4°, and freshly prepared glycerol.

As shown in Table 2, the mean difference between duplicate determinations on 22 different serum samples was ± 4.1%.

To test the adaptability of the present technique to sera differing widely in glyceride concentration, an aliquot of the chloroform extract of serum #22, Table 2, was diluted 1:40 with chloroform and this sample was again carried through the entire procedure. The value for initial concentration obtained on this sample differed less than 3% from that obtained in the original extract.

[4] Glycerol was distilled in an all-glass apparatus at low pressure and only the middle third of the distillate was used; glycerol samples used in this technique ("Certified Reagent," Fisher Scientific Co., Fair Lawn, N.J.) were found to contain an average of 5.0% of water.

TABLE 2. Duplicate Glyceride Determinations on Serum Samples Made on Two Different Days

Sample	Glyceride Concentration A	Glyceride Concentration B	Difference
	mg/100 ml		%
1	48	45	6.2
2	50	53	6.0
3	54	57	5.6
4	56	55	1.8
5	61	58	4.9
6	61	58	5.0
7	69	68	1.5
8	78	81	3.9
9	79	81	2.5
10	83	85	2.4
11	88	89	1.1
12	92	87	5.4
13	115	105	8.7
14	153	147	3.9
15	158	161	1.9
16	459	442	3.7
17	769	754	2.0
18	1108	1063	4.1
19	2092	1999	4.5
20	2138	1945	9.0
21	5004	4933	1.4
22	12126	11674	3.7
		Mean	4.1 ± 2.2*

* Standard error.

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