corresponding *cis* bonds. In those cases where contamination occurred, which could be seen by the presence of unsaturated fatty methyl esters, the percentage of the sum of saturated fatty esters was corrected by substracting twice the percentage of the unsaturated esters from the above mentioned sum with the assumption that the contaminating glycerides were mono-unsaturated. The empirically corrected values were in agreement with those values obtained without any observed contamination. The general validity of the correction has, however, not been investigated.

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Estimation of trisaturated glycerides in fats by argentation thin-layer chromatography

The classical methods for the estimation of trisaturated glycerides(GS_3) in fats originally developed by HILDITCH[1] and later modified by KARTHA[2] and VON RUDLOFF[3] were essentially based on the oxidation of the unsaturated portion(GU) of the mixed glycerides. In recent years these methods have largely been replaced by modern chromatographic techniques such as column[4], thin-layer[5] and gas–liquid[6] chromatography, by which rapid and accurate determination of triglyceride composition has been achieved.

For the evaluation of mixed glycerides the application of argentation thin-layer chromatography, initially developed by DE VRIES[5], has been well established. This method is based on the separation of mixed glycerides into components according to the degree of unsaturation, for example GS_3, GS_2U, GSU_2 and GU_3, and also according to the isomeric configuration of unsaturated glyceride molecules. Taking advantage of this principle, quantitative methods for the analyses of both natural and synthetic triglycerides have been developed. Thus BARRETT *et al.*[7] have separated mixed glycerides of a number of natural fats on a silver nitrate-impregnated silica gel plate using chloroform–acetic acid–ethanol as solvents. They subsequently estimated the

individual glyceride components by first charring the spots with phosphoric acid and then employing densitometric measurement. Other workers[8–10] have utilised the same principle in separating various natural and synthetic triglycerides on a preparative scale (80–100 mg) on a thick-layer of adsorbent using benzene–diethyl ether, etc. as solvent. The triglyceride components thus separated in band form were detected under ultraviolet light after the application of a dichlorofluorescein spray and subsequently extracted from the adsorbent with dry ether. Each fraction was then analysed either by a titrimetric[8] or colorimetric[9,10] determination of the glycerol liberated from the triglyceride moiety. Among the recently developed micro techniques[11,12] for the quantitative estimation of triglycerides, the method of VAN HANDEL[13] is found to be widely used.

The present work was undertaken to develop a micro argentation thin-layer chromatographic method for estimating in a single spot the trisaturated glycerides in fats of biological importance.

Experimental

The materials under investigation were standard tripalmitin (Hormel Institute, U.S.A.), refined and bleached groundnut oil fortified with known amounts of pure tripalmitin, coconut oil, groundnut oil randomised with 15% pure trimyristin, commercially available butter, butter fat(ghee) and hydrogenated groundnut oil. For the present study, these samples were prepared as 1% solutions in dry benzene, except for coconut oil which was 0.5%.

The solvents and other reagents used were all of analytical grade. Diethyl ether was made peroxide-free by washing repeatedly with saturated solutions of $FeSO_4$, followed by distilled water, and then being dried over a mixture of anhydrous Na_2SO_4 and fused $CaCl_2$, and finally being distilled at a constant boiling temperature.

All-glass apparatus including scratchless and uniform glass plates (10 × 20 × 0.2 cm) were thoroughly cleaned and washed with chromic acid and distilled water and finally dried.

The preparation of a silver nitrate–Silica Gel G layer of 325 μ on plates has been reported elsewhere[14].

Definite volumes in μl (Lamda pipette, Fisher, U.S.A.) of standard tripalmitin solution were taken in glass-stoppered test tubes (16 × 2 cm) and after the removal of the solvent on a water bath, the glycerol content was estimated according to VAN HANDEL *et al.*[13] as follows: μg amounts of tripalmitin left in the test tubes were saponified with 0.5 ml of 0.4% ethanolic (aldehyde-free absolute ethanol) KOH at 60–70° for 30 min, and acidified with 0.5 ml of 0.25 *N* H_2SO_4. After the complete removal of ethyl alcohol on a boiling-water bath, 0.1 ml of 0.05 *M* $NaIO_4$ was added and mixed. Ten minutes later 0.1 ml of 0.5 *M* Na_2AsO_3 was added and allowed to stand for 10 min. Five ml of chromotropic acid, prepared by mixing in the cold an aqueous solution of sodium salt of chromotropic acid (0.448 g sodium salt of chromotropic acid in 40 ml distilled water) with 180 ml of sulphuric acid (120 ml conc. H_2SO_4 mixed with 60 ml distilled water), was then added and allowed to develop colour for 30 min on a boiling-water bath. After the solution had cooled, the optical density was measured at 570 mμ against the "reagent blank" with the help of a Zeiss or Unicam spectrophotometer.

Artificial mixtures containing groundnut oil and graded amounts of standard tripalmitin were prepared. Known volumes of the solution were spotted quantitatively

on a silver nitrate–Silica Gel G plate with a Lamda pipette. The plate was developed in a chloroform–acetic acid (99.5 :0.5, v/v) solvent system. When the solvent front reached 14 cm (45 min), the plate was taken out of the chamber, heated at 110° in an oven for 15 min to remove the solvent, cooled and carefully sprayed with ammonia solution (40 ml of liquor ammonia diluted with 100 ml distilled water) until the layer was uniformly wetted. When GS_3 was identified as a white visible spot near the solvent front, the spot area was marked immediately with a needle. The plate was then air dried for 30 min, heated at 110° in an oven for 15 min and finally cooled at room temperature until the ammonia was removed. The contents in the marked area were then scraped off by means of a scalpel or sharp-edged stainless-steel spatula and put directly into a wide-mouthed miniature column (8 × 0.8 cm) fitted with a grease-free stopcock having a small filter bed (1.5–2 cm length) of acid-washed Celite 545 over a cotton plug. The column had been previously flushed with ether and the filter bed was always kept under ether. Thus tripalmitin was extracted quantitatively with 15 ml ether and the ether eluate was collected in test tubes. After careful removal of the solvent on a warm-water bath, the glycerol content was estimated from the residue as described above. In this case the colour was measured against an "experimental blank" obtained by scraping the portion covering an approximately equal blank area as that of GS_3 from the plate being subjected to similar treatment. A "reagent blank" was also simultaneously made for comparison with the "experimental blank" value.

In all subsequent experiments, the GS_3 content of all the samples was determined accordingly. For each plate and every determination an "experimental blank" and a "reagent blank" were performed.

Results and discussions

By drawing a standard curve with increasing amounts of tripalmitin (μg) against the corresponding values of the optical densities (O.D.), a straight line is obtained as shown in Fig. 1. Deviation was observed, however, only at an appreciably high concentration of tripalmitin.

In all the investigated samples the trisaturated glyceride content determined

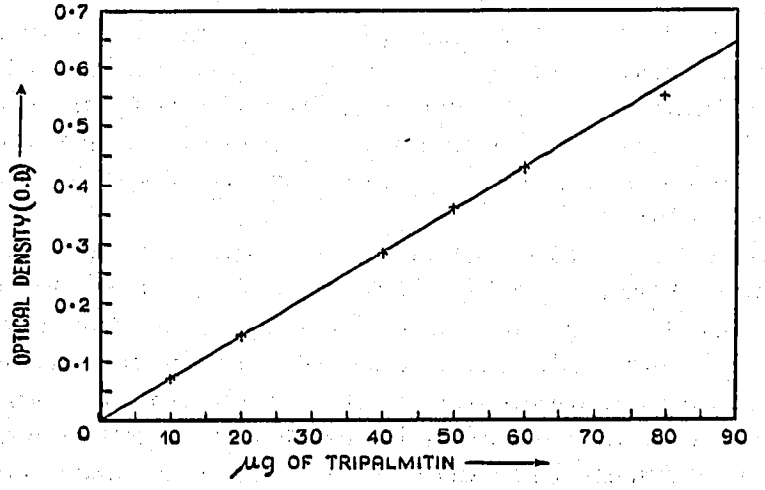

Fig. 1. Standard curve of μg amounts of tripalmitin against the corresponding experimentally determined optical densities (O.D.).

by the present method was derived from the standard curve (Fig. 1) by comparing the corresponding O.D. values measured in each case. The percentage composition was calculated from the known amount of material spotted.

Table I represents known and experimentally determined GS_3 content of groundnut oil mixed with pure tripalmitin. The experimental values are the means of three determinations; two were spotted on a silver nitrate–Silica Gel G plate at equal concentrations (100 μg each of 1% solution) and the other one was spotted at a higher concentration (150 μg of 1% solution). For the experimental value of (a), however, larger amounts (200–250 μg) of a 2% solution were subjected to investigation.

Table II indicates the GS_3 content of other fats. Here also, three determinations for each sample were made according to Table I and the mean values are given. In these cases, however, a sample of groundnut oil containing a known amount of pure

TABLE I

ESTIMATION OF FORTIFIED TRIPALMITIN IN GROUNDNUT OIL MEDIUM BY ARGENTATION THIN-LAYER CHROMATOGRAPHY

Sample	*% Tripalmitin (GS_3)*	
	Known	*Found*
(a)	1	0.98
(b)	3	2.91
(c)	5	4.88
(d)	10	9.92
(e)	30	29.4
(f)	50	49.2
(g)	60	58.2

tripalmitin was also spotted on the plate as "reference sample" and the tripalmitin content was estimated along with each material to study the efficacy of the procedure and its reproducibility. It should be noted that in the case of coconut oil, the amount spotted on the plate for two determinations was 25 μg each and that for one determination was 50 μg.

For the detection of GS_3 on a silver nitrate–Silica Gel G plate, the present method requires aqueous ammonia as a specific spray reagent, although other triglyceride components can also be visualised as faint spots. By using dichlorofluorescein as a spray reagent in the present case, high values with inconsistent results were obtained due to contamination of trace amounts of dye that remained even after extraction of GS_3 through an activated silica gel column.

Further, the use of a small filter bed column of celite was sufficient for the quantitative recovery of GS_3 with ether. A single-packed bed permits about five such successive extractions including the blank from each plate, provided the flow rate (which should be moderately adjusted) is not sufficiently disturbed by the formation of large air bubbles. It may, however, be mentioned that during the removal of ammonia from the plate at 110°, some blackening of spots in the lower region of the chromatogram, consisting mainly of unsaturated glyceride molecules, was observed, whereas the trisaturated glyceride spots remained unchanged.

By comparing the optical densities of a number of "experimental blanks" from

TABLE II

ESTIMATION OF GS_3 IN FATS BY ARGENTATION THIN-LAYER CGROMATOGRAPHY

Sample	*% GS_3*
Groundnut oil randomised with 15% trimyristin	3.0 (2.7)*
Coconut oil	85.0
Commercial butter	13.5
Commercial butter fat (ghee)	18.5
Hydrogenated groundnut oil	10.1

* Denotes the calculated value.

a number of plates measured against "reagent blanks", a positive deviation of O.D. values between 0.02–0.03 was always observed. If the deviation exceeds 0.05, erroneous results may be expected.

A preliminary qualitative study of fat samples by argentation thin-layer chromatography is recommended to adjust the amounts to be employed in the present quantitative micro method in order to obtain well defined spots.

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