In connection with a comparative study of the fatty acid composition of milk fats from various species, a suitable method of esterification prior to gas-liquid chromatography (GLC) was sought. The specimens available from several of the species were very small. Furthermore, certain milk fats contain short-chain acids liable to be lost by volatilization or by incomplete extraction of their esters from reaction mixtures. Thus, a method involving a minimum of transfers and manipulations was desired.

The methods employed by others (1-14) in research on ewe milk fat did not seem suitable for our purposes. The method of Magidman et al. (9), in which the methyl esters are fractionally distilled and each fraction chromatographed, has revealed numerous minor fatty acid components in cow milk fat, but is clearly not suitable for comparative analysis of small specimens for their major acid components. Other workers (13, 14) have reported poor recovery of methyl esters of short-chain acids in methods involving extraction of the esters from the reaction mixture. DeMan, in a recently published note (4), resolved the problem of dealing with the volatile fatty acids by using a procedure which required heating the fat in a sealed tube with sodium methoxide dissolved in absolute methanol.

The method of Mason and Waller (10) appeared promising, inasmuch as it involves transesterification, catalyzed by dimethoxypropane, at room temperature and does not require extraction of methyl esters from the reaction mixture. This method was found to proceed smoothly with cow milk fat, but methyl butyrate could not be separated satisfactorily from the solvent peak. A new procedure was then devised in which ethyl esters are prepared by transesterification with sodium ethoxide and diethyl carbonate. As in the method of Mason and Waller (10), no extraction of the esters is necessary. The method has the additional desirable feature that the reaction is virtually instantaneous at room temperature. Chromatography of the reaction mixtures separated the ethyl butyrate from the ethanol peak, but ethyl caproate was not separated clearly from the diethyl carbonate peak. Separate chromatography of both the methyl and ethyl esters permits a satisfactory analysis for all of the principal acids and also provides a useful check on the analysis. This note describes the methods employed and presents data obtained on a standard mixture and on ewe milk fat.

**Preparation of Ester.** The lipid (0.1 ml) to be transesterified is dissolved in 5.0 ml of a solution consisting of 2 ml diethyl carbonate (Eastman) and 3 ml of absolute ethanol. The addition of 0.2 ml of 2 N sodium ethoxide, prepared by dissolving sodium metal in absolute ethanol, brings about a virtually instantaneous transesterification and the reaction mixture may be chromatographed by GLC immediately. Chromatograms obtained by injection of the reaction mixture into the chromatograph 1 rain. after addition of sodium ethoxide to reaction mixtures gave quantitative recoveries of the ethyl esters.

The alkaline solution of diethyl carbonate decomposes slowly, forming a sodium carbonate gel. If chromatography cannot be carried out within about an hour, it is advisable to neutralize the solution by titration to a phenolphthalein endpoint with 2.5 N ethanoic HCl (prepared by passing anhydrous HCl into absolute ethanol). The fine precipitate of sodium chloride may be filtered off or, as has been our practice, simply allowed to settle and the supernatant drawn directly for chromatography.

If large numbers of samples are to be analyzed it may be convenient to prepare the reagent by making the diethyl carbonate-ethanol mixture 0.08 N in sodium ethoxide and diethyl carbonate. In this manner the aldol condensation precipitate which forms in the sodium ethoxide solution in no way interferes with its catalytic effectiveness. A solution which had been standing for two months and which had turned dark-brown catalyzed complete transesterification.

Methyl esters of fatty acids can be prepared in a completely analogous manner, substituting dimethyl carbonate and sodium methoxide for diethyl carbonate and sodium ethoxide, respectively. Free fatty acids are not esterified under the described conditions, but are esterified at
acid pH, as will be described in a forthcoming publication.

Gas-liquid chromatography. Chromatography of the esterified mixture was carried out, using an F & M Model 609 flame ionization gas chromatograph. The 5-ft aluminum column was packed with 10% diethylene glycol succinate on Diaport W (both from F & M Scientific Corporation, Starr Road and Route 41, Avondale, Pennsylvania). Injection of 10μl samples was made at 75°C and temperature programming at 13°C/min to 190°C was begun 2 min later.

Figure 1 shows two chromatograms obtained from a sample of ewe's milk fat. Part A of Figure 1 shows the methyl esters prepared by the dimethoxypropane-induced transesterification described by Mason and Waller (10). The methyl butyrate peak is obscured by the broad solvent peak obtained using this method. The isopropylidene glycerol peak in this chromatogram appears as a shoulder on the leading edge of the methyl laurate peak, but it is usually satisfactorily separated from the latter. Part B of Figure 1 shows a chromatogram of the ethyl esters of ewe's milk fat obtained by the procedure described here. The peak corresponding to ethyl butyrate is fairly well separated from the ethanol peak. This advantage, however, is somewhat nullified by the partial obscuring of ethyl caproate by the large diethyl carbonate peak. In our work with the milk fats of various species this presented no major problem. A preliminary screening for butyrate and caproate was carried out on the ethyl esters of all samples. If caproate was absent, the chromatogram was used to calculate the composition of the milk fat by triangulation of the peaks. If the presence of caproate was indicated, however, the methyl esters were prepared by the method of Mason and Waller and the percentage of this acid present was determined from a chromatogram of the methyl esters. By the use of a simple proportion comprised of the total peak areas of the fatty acids from C6 to C16 from the chromatograms of the methyl and the ethyl esters, the area of the methyl caproate peak could be transformed to an equivalent ethyl caproate peak and the percentage present of all fatty acids then calculated.

It has been found consistently that within the error to be expected in the measurement of peak areas by triangulation the areas are proportional to the weight percentage of the various fatty acids present. This is at variance with the observations of DeMan (4) and Smith (14) and no explanation is offered here.

Table 1 shows the results calculated from replicate chromatograms of the methyl and ethyl esters of ewe's milk. Included in Table 1 are results obtained from chromatography of the ethyl esters prepared, by the procedure described herein, from a standard mixture of triglycerides. The individual triglycerides were the best grades obtainable commercially. Each had been screened for purity by chromatography of the ethyl esters prepared from them prior to their incorporation into the standard mixture.

It can be seen that the recoveries from the standard mixture indicated in Table 1 are excellent. The values given are means plus or minus their standard errors obtained from individual chromatograms of six aliquots, each of which were transesterified separately.

The milk fat results are the means of six chromatograms obtained from a single transesterification for the ethyl esters prepared by the method described here and for the methyl esters prepared by the method of Mason and Waller (10). The agreement between results obtained by the two procedures is very good.

The procedure described here, because of its simplicity and reproducibility, should find considerable use in operations where the routine
screening of large numbers of samples of fat is desired.

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REFERENCES


PARTIAL ANALYSIS OF MILK TRIGLYCERIDES WITH LIPASE IODINE$^{131}$ RETENTION IN CALVES

The microorganism *Geotrichum candidum* (GC) produces an extracellular lipase highly specific for oleic acid in both natural and synthetic triglycerides (TG) (1, 2). Oleate was removed from the latter, regardless of position. The enzyme also differentiated between geometric isomers releasing about 95 M% oleate (in the free fatty acids) from glyceryl 1-oleate 2,3-dioleate and from margarine (7). The utility of the pancreatic lipase method for studying TG structure encouraged us to determine whether GC lipase could be used for the same purpose with milk fat. The results are reported herein.

Butteroil was obtained by churning cream, melting the butter, and filtering the clear oily layer. To provide a standard substrate for comparisons in several of our investigations, quantities of 15-20 g were dispensed in screw-top tubes and stored at -20 C. As needed, samples were melted and purified on an alumina column, the process monitored by thin-layer chromatography (TLC). The butteroil (0.5 g per 8 ml) was dispersed with a Waring Blender, in phosphate buffer (pH 6.7), containing 1% gum arabic to which were added 0.5 ml of 1% CaCl$_2$ and 25 mg of enzyme preparation in two milliliters of plain phosphate buffer. The enzyme was prepared as described by Alford et al. (2). Two samples, one for column chromatography and one for preparative TLC plus a control containing no enzyme, were incubated with shaking at 37 C for 2 hr. Six runs were made. Synthetic TG's, glyceryl 1-palmitate 2,3 dicaproate (PCC), and glyceryl 1-oleate 2,3 dibutyrate (OBB) were prepared and purified (6). These TG's were mixed (567 mg OBB and 601 mg PCC) and two trials in duplicate run as above. The digestion mixtures from the butteroil and synthetic TG's, respectively, were extracted and the products of lipolysis separated and analyzed as heretofore described, with 4:0 in the FFA determined by column chromatography (3). Net microequivalents of free fatty acid were determined by titration as part of the analysis for 4:0.

The fatty acid composition of the intact butteroil and of the products of lipolysis by GC lipase are listed in Table 1. The specificity for oleate is immediately apparent, as 61.8 M% was present in the FFA as compared to 19.9 in the intact TG's. The low oleate contents of the DG's and MG's reflect this specificity. Very little MG was produced, judging from the relative size of the glyceride bands on the TLC plates. The composition of these MG's does not differ markedly from the MG's resulting from lipolysis of this butteroil by pancreatic lipase (6). However, this comparison is not strictly valid, due to the large difference in activity between GC and pancreatic lipases. For example, 25 mg of GC lipase released 173 microequivalents of FFA from butteroil in 2 hr, whereas 25 mg of pancreatic lipase liberated 300 in 5 min.

From studies of milkfat structure done with pancreatic lipase, most of the 4:0 has been assigned to the 1- or 3-positions, whereas the 2-position has been largely occupied by the saturated (4, 6, 8). If this information is coupled with the data in Table 1, at least one tentative conclusion regarding TG structure can be made. The 4:0 content of the DG's and the 18:1 content of the FFA suggest that TG's of the type; 4:0, S, 18:1 (where S could be 14:0 or 16:0,) exist in milk fat. This tends to confirm the findings of others regarding the presence of this or similar TGs (4, 5).

The presence of butyrate in the FFA prompted us to investigate the action of GC lipase on synthetic glycerides containing 4:0 and 6:0. Results from the lipolysis of a mixture of OBB and PCC are given in Table 2. Data for the MGs are omitted, because these were barely detectable by preparative TLC. Although some saturates were released, most of the FFA was 18:1. More 16:0 than 6:0 was lipolyzed from

$^{1}$ Scientific contribution, Agricultural Experiment Station, University of Connecticut, Storrs. Supported in part by Public Health Service Research grant AM-02605-07 from the Institute of Arthritis and Metabolic Diseases.

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