Chromatographic Procedure for Determination of Fat Content of Small Specimens of Milk

Abstract

Milk, 30-60 mg, is extracted with a mixture consisting of methanol–dimethyl carbonate–benzene (3:2:3 v/v) containing an internal standard of methyl tridecanoate. After centrifugation, the clear upper layer is made alkaline with sodium methoxide and then acidified with methanolic-HCl. Gas-liquid chromatography of the resulting methyl esters gives a chromatogram from which the fat content and fatty acid composition may be calculated.

Materials

Extractant, methanol–dimethyl carbonate–benzene (3:2:3 v/v), containing a standard amount of methyl tridecanoate (about 5 mg/gram). The extractant is routinely prepared in 50-ml lots. Since the gas chromatography is gravimetric and since it is sometimes desirable to measure the extractant, the methyl tridecanoate is weighed accurately and the density of the extractant is determined by weighing a measured volume.

Sodium methoxide approximately 2 N, prepared by dissolving the appropriate amount of metallic sodium in absolute methanol.

Methanolic-HCl approximately 10%, prepared by passing dry HCl into absolute methanol.

Gas-liquid chromatography (GLC) was performed on an F & M Model 810 dual-column hydrogen flame chromatograph equipped with a disc chart integrator. Aluminum columns (1.5 M by 4 mm id) were packed with 80–90-mesh Anakrom F6 containing 10% by weight of diethylene glycol succinate. Samples (2 μliters) were injected at a column temperature of 90 C. After 3 min this was raised at 6 degrees/minute to 210 C, which was held for 10 minutes. Injection and detector port temperatures were maintained at 250 C and the helium flow rate was approximately 120 ml/minute.

Experimental Procedure

Samples of 30–60 mg of milk are weighed into tared 6- by 50-mm test tubes. To it is added approximately 3 vol of extractant which is accurately measured or weighed. Thorougly mixing with an external mixing device is followed by centrifugation in a clinical-type centrifuge.

The top layer of the centrifugate consists of benzene, essentially all of the fat (including methyl tridecanoate), and sufficient methanol and dimethyl carbonate for the transesterification. To a 40-μliter aliquot is added 15 μliters of sodium methoxide, followed after 5 min by 30 μliters of the methanolic HCl. The purpose of the HCl is twofold. First, it disperses the gel of sodium carbonate which forms and makes sampling for GLC difficult. Secondly, although transesterification of glycerides is complete in 5 min at room temperature, any free acids which may be present are not esterified in the alkaline medium. These are, however, esterified within 30 min at room temperature in the acidified mixture. Free fatty acids are not normally found in fresh milk, in which case the acidification step is not necessary. Only sufficient HCl is then added to disperse the gel. If, however, free fatty acids are present, as revealed by thin-layer chromatography (TLC) of the extract, the acidification step must be carried out.

Fig. 1 shows a thin-layer chromatogram of the successive steps of the procedure when applied to a sample of human milk which, although apparently fresh, contained free fatty acid. A sample of the original extract was applied at Spot 1 and showed the principal components to be triglycerides (B) and fatty acids (C). Following the alkaline transesterification reaction and acidification, the material was applied at Spot 2. Although transesterification has occurred, as shown by the presence of methyl esters at A, unchanged fatty acids are still present at C. Spot 3, applied 30 min after acidification, shows the material to be ready for GLC. We find that nothing more drastic than acidification at room temperature is required to esterify free fatty acids in the presence of excess methanol. The dimethyl carbonate, necessary for the transesterification reaction, is not required for esterification of free acids. The material remaining at the origin of the chromatogram consists of sodium chloride and glycerol.

The precipitate of sodium chloride that forms upon acidification of the reaction mixture presents no problem but may, if desired, be removed by centrifugation. A procedure to which no valid objection has been found is to inject

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FIG. 1. Thin-layer chromotogram of human milk fat and reaction mixtures.

1. Unesterified fat.
   A. Methyl esters
   B. Triglycerides
   C. Fatty acids

2. Reaction mixtures after transesterification.

3. Reaction mixture after transesterification and acidification. Chromatography on silica gel G (0.5-mm layer) in petroleum ether-ethyl ether-acetic acid (90:10:1 v/v).

the entire reaction mixture into the chromatograph without further treatment. No impairment of injection port function or column efficiency or life has been observed after such treatment. During periods of continuous daily use the oven is left at 210°C overnight, to clear it of high boiling material such as cholesterol or glycerol. Columns are usually in use for several months and replacement is made only when poor resolution of the 18 carbon fatty acid esters indicates that the liquid phase has become exhausted. It is felt that direct chromatography of the reaction mixture is one of the principal advantages of the dimethyl carbonate procedure and that any purification steps applied to the ester mixture are unnecessary as well as undesirable.

Calculations. Following GLC the areas under the methyl tridecanoate peak and under the total tracing are determined by reference to the integrator. It is imperative that good baseline compensation be obtained. Subtraction of the area of the standard peak from the total area gives the milk fatty acid esters.

The area of the standard peak when divided by the weight, in milligrams, of standard added to the sample gives units of area per milligram of standard ester. The area of the sample esters when divided by this value is converted directly into milligrams of fat present in the original sample. It is recognized that recovery will be greater than 100% because the fatty acids are measured as methyl esters rather than as triglycerides. We consider that this error, which varies from 1.32% for tributylin to 0.45% for tristearin, may be ignored as negligible.

Methyl butyrate is obscured by the solvent peak and was not determined directly in our study. The butyrate content of the milks used was estimated to be 3%, using the ethyl ester procedure described earlier (3), and this value was added to the areas of all chromatograms. Since the procedure described here was developed for measuring the fat content of rodent milk which does not contain butyrate and only trace amounts of caproate, its absence from the chromatogram is of no importance. It is unlikely that this procedure would be used for routine determination of fat in cow milk. The latter was used to evaluate the method because sufficient sample of homogenized material was required to eliminate sampling as a source of error in the several determinations made. Milk specimens from small animals are drawn from the nipple directly into small tared tubes and no subdivisions of the sample are subsequently made.

Results

Table 1 summarizes the results obtained from several different samples when analyzed by the method described and by the Roese-Gottlieb method (1). Samples 1 and 2 were commercial homogenized milk and coffee-cream (Half and

<table>
<thead>
<tr>
<th>Samples</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Homogenized milk</td>
<td>Half and Half</td>
<td>Milk fat</td>
<td></td>
</tr>
<tr>
<td>Gravimetric*</td>
<td>3.3 ± 0.1 (4)</td>
<td>10.9 ± 0.1 (4)</td>
<td>107.8</td>
<td>70.0</td>
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<td>Chromatographic</td>
<td>3.6 ± 0.1 (10)</td>
<td>10.9 ± 0.3 (10)</td>
<td>105.6 ± 0.3 [4]</td>
<td>69.6 ± 1.9 [4]</td>
</tr>
</tbody>
</table>

* Measured by Roese-Gottlieb Method (1).
* Mean and standard deviation of single determination.
* Numbers in parentheses are number of individual specimens analyzed.
* Numbers in brackets indicate replicate chromatograms of individual esterified samples.

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Half), respectively. In both cases the indicated number of samples were subjected to the procedures and the results given are the means of individual results from individual samples. The average sample size was 51.0 mg (range 44.1–55.8 mg) for Sample 1 and 65.6 mg (range 50.1–83.4 mg) for Sample 2.

Samples 3 and 4 were prepared by suspending a weighed amount of melted milk fat in 1 ml of water and treating with 3 vol of extractant weighed as described. The results given were obtained from four successive chromatograms of a single esterified aliquot of each of these two samples.

We believe these results substantiate the validity of the chromatographic procedure for the determination of milk fat and that it should be of practical importance for the analysis of small samples whose fat content cannot easily be measured by existing methods. An additional advantage of the method is, of course, that the fatty acid composition can be obtained from the same chromatogram used for estimating fat content.

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References

Simultaneous Phenotyping Procedure for Milk Proteins—Improved Resolution of the β-Lactoglobulins

The procedure of starch gel electrophoresis for the simultaneous phenotyping of the principal proteins of cow's milk described by Aschaffenburg and Thymann (1) suffers from the drawback that the β-lactoglobulin bands are not so well separated from the leading β-casein band as to permit recognition of the rare, slow-moving β-lactoglobulin Variant C. Nor has it been possible to resolve the recently discovered Variant D (2) which migrates even slower. One of us (3) has published an improved method which provides clear resolution of these slow-moving β-lactoglobulins. Michalak’s (3) gels were longer than, and twice as thick (3 mm) as those used by Aschaffenburg and Thymann. Attempts to adapt his method to gels of the dimensions recommended by these authors met with an unexpected difficulty. Though improved resolution was obtained, the gels were disfigured by an irregular band (salt boundary?) forming across the region of the κ-caseins. The difficulty was overcome by changing the buffer systems, eliminating the borate ion which appeared to be implicated, and the following modifications are recommended when it is desired to use gels of the original dimensions.

Procedures

Apparatus buffer. Dissolve 6.0 g tris and 28.8 g glycine in water to 1 liter; pH about 8.5.

Gel buffer. Dissolve 9.0 g tris and 1.8 g citric acid to 100 ml. Adjust the pH to 8.6 by stirring in small amounts of solid citric acid.

Starch gels. To prepare two gels boil approximately 10 g of hydrolyzed starch (Connaught Medical Laboratories, Toronto) in 8 ml of gel buffer plus 40 ml water until the fluid loses its high viscosity (at least 1 min), then stir in 26 g urea and proceed as described before. Add four instead of three drops of 2-mercaptoethanol, but do not add this reagent to the milk samples. Allow the gels to set for 30 min, then transfer to the cold room for at least 5 hr before use.

Electrophoresis. Apply a constant 210 v, corresponding initially to about 10 mamp per gel, for approximately 16 hr.

Staining. Use the Amido Black 10B-nigrosine stain (3), which brings out the protein bands much faster than the black dye on its own.

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