NEW METHOD FOR EXTRACTION OF MILK PHOSPHOLIPIDS

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ABSTRACT

The whole milk sample was compounded with silicic acid, the mixture placed in a glass chromatographic column, and the phospholipid fraction eluted with 20% (v/v) formic acid in ethyl ether. Lipid phosphorus analysis and thin-layer chromatography were used for the comparison of phospholipid recovery from 10-ml aliquots of milk by each of three methods—the new, the Mohonnier, and the modified Mohonnier (1.5% NaCl added to the milk sample before a Mohonnier extraction). Five trials verified that the modified Mohonnier method and new silicic acid column chromatographic (SACC) method recovered 10.2 and 12.2 mean per cent, respectively, more lipid phosphorus than the conventional Mohonnier procedure. When aliquot samples from the phospholipid extracts recovered by the three methods were compared by thin-layer chromatography, minor differences were evident in the lipid patterns, notably in improved recovery of a component corresponding to phosphatidyl serine by the SACC method and in superior recovery of lysophosphatides by the Mohonnier and modified Mohonnier methods.

Current methods for the extraction of milk fat recover both neutral lipids and phospholipids (P-lipids). An efficient separation method for these two lipid groups is silicic acid chromatography. The concentration of total P-lipids present in a certain milk sample is usually determined by lipid phosphorus analysis of the milk fat extract obtained either by the Roese-Gottlieb or Mohonnier methods. These extraction methods comprise solvent partition systems, with predominantly ethyl and petroleum ethers in the upper phase and basic (ammonia) aqueous-ethanol mixtures in the lower phase. From the structure of P-lipids known to exist in milk, it is apparent that a number of them have acidic groups and would exist as ammonium salts in the basic aqueous phase (particularly phosphatidyl serine and phosphatidic acids), thus making them difficult to extract.

Investigators, studying P-lipids recovered from milk and dairy products, have employed a variety of extraction methods (1, 2, 12, 16, 18, 21). The kinds of solvents used, their concentrations, and variations in the techniques depend upon the product under study and the decision of the investigator. McDowell (12) reports that variations in the milk P-lipid content in the literature may be due to difficulties in the extraction and purification procedures. He suggests that inadequate experimental evidence is available to assume that the Roese-Gottlieb solvents quantitatively extract milk P-lipids. He studied five different methods for extracting butter P-lipids and decided to use a modified Roese-Gottlieb technique (ammonium hydroxide was omitted) because it was rapid, simple, and did not extract inorganic phosphorus.

In studies of whole milk P-lipids, investigators usually have employed the Mohonnier procedure (4) or a Mohonnier modification (12, 21). Since whole milk is not a concentrated source of P-lipids, buttermilk powder which is reported to contain P-lipids representative of whole milk (16) commonly has been used as a concentrated source material. However, given an efficient extraction procedure, the milligram amounts of P-lipid available from relatively small samples of whole milk would be adequate for analysis by the sensitive thin-layer chromatography (TLC) and infrared techniques.

We believed that a type of silicic acid column procedure might enable efficient and direct extraction of milk P-lipids. Numerous experiments were performed to establish suitable conditions for sample preparation and to find the most desirable solvent system for eluting the
EXTRACTION OF PHOSPHOLIPIDS

P-lipids. Harper et al. (8) added dried silicic acid to milk after breaking the lipoprotein complex with 20% sulfuric acid, for recovering the total free fatty acids from milk. Jensen and Morgan (10) modified the method of Harper et al. to recover the milk monoglycerides. Duthie et al. (6) reported that the method of Jensen and Morgan only partially eluted the milk P-lipids. The aforementioned methods were considered, and a chromatographic extraction technique was developed and compared with the Mojonnier method.

While our study was in progress, Walstra and De Graaf (21) reported that by adding 1.5% NaCl to a sample of milk, cream, buttermilk, etc., before Roese-Gottlieb extractions, they recovered (as determined by lipid phosphorus analysis) an additional 10-15% of P-lipids. Their evidence confirmed the postulation that improved recovery of milk P-lipids is possible.

EXPERIMENTAL PROCEDURES

Raw whole milk was obtained from the bulk storage tanks in the University Creamery the morning the sample was to be extracted. The milk fat tests on the samples ranged from 3.7 to 4.1%, as determined by the Gerber method (13). Three methods of recovering the lipids from the milk were employed. These were the Mojonnier modification of the Roese-Gottlieb method, hereafter designated the M method (14), the Mojonnier method as modified by Walstra and De Graaf (21), involving addition of 150 mg of NaCl to the milk sample before extraction, hereafter abbreviated the MM method, and the silicic acid column chromatographic method described below.

Silicic acid column chromatographic method. Reagent-grade methanol, acetone, chloroform, ethyl ether, and formic acid were used. The methanol, ethyl ether, and chloroform (employed during the operation of the column) were redistilled in glass and 0.5% absolute ethanol was added to the chloroform to retard decomposition. Mallinekrodt silicic acid, 100 mesh, labelled suitable for chromatographic analysis by the method of Ramsey and Patterson was employed. The silicic acid used to absorb water from the milk was dried at 100°C for 48 hr. That used to form the plug in the extraction column was washed in a 1,000-ml beaker by suspending approximately 300 g of silicic acid in 700 ml of methanol and decanting that which did not settle in 2 min. The washing was repeated successively with 700 ml of the following solvents: methanol, acetone, and ethyl ether, in that order. The washed silicic acid was dried in a fume hood before use. The purpose of washing was to improve the flow of solvents through the plug by removing the finer particles, and to exclude such particles from the eluted lipid fractions.

The milk sample was warmed to about 40°C to aid liquefaction of the milk fat. Ten milliliters of the milk was added to 1.5 ml of methanol in a porcelain mortar, the mortar rotated gently to mix the ingredients, and the mixture allowed to stand at room temperature.

A silicic acid plug was prepared for the glass chromatographic column (2.8 by 54 cm). A piece of glass wool was inserted up the column tip. A mixture of 8 g of prewashed silicic acid in ethyl ether was poured into the column, using a number of ethyl ether rinsings. When the solvent level was about 2 cm above the plug, the column was ready to receive the sample.

While the ethyl ether was passing through the plug, 30 g of dry silicic acid was added to the milk sample in the mortar. The milk and silicic acid were mixed with a spatula and ground to a fine powder with a pestle. Approximately 70 ml of ethyl ether was added to the mortar, its contents mixed with a spatula and poured through a funnel onto the column plug. Collection of Fraction 1 was started at this point. An additional 80 ml of ethyl ether was employed to transfer the contents remaining in the mortar to the column. When the solvent level approached the surface of the packing, 50 ml of chloroform was added. The eluate collected from the 200 ml of previously mentioned solvents comprised Fraction 1. When the chloroform level neared the top of the packing, 100 ml of a 20% (v/v) solution of formic acid in ethyl ether was poured on the column. As the chloroform passed through the column, the color of the white packing changed to a grayish-brown and the white silicic acid plug became transparent. When the formic acid solution came in contact with the packing and plug, they returned to their original white color and this change marked the progress of the formic acid solution through the column. After this solution moved half-way down the packing, the flask was changed and the solvent remaining in the column was collected and identified as Fraction 2.

Solvents in both fractions were rotary-evaporated at a temperature below 50°C, using reduced pressure from a water aspirator. The two fractions were subsequently analyzed and no P-lipid was found in Fraction 1; however, Fraction 2 contained a mixture of neutral lipids, P-lipids, and nonlipid impurities (5).
This silicic acid column chromatographic method is hereafter designated the SACC method.

**Purification and analysis of P-lipid samples.**

The Borgström method (3), as slightly modified in our laboratory (5), was employed to separate the two major lipid groups extracted by the M and MM methods, and for refining the crude P-lipids obtained as Fraction 2 in the first separation by the SACC method from minute quantities of neutral lipids. Walstra and De Graaf (21) reported that only lipid-bound phosphorus was extracted from milk by the Roese-Gottlieb method. Their procedure and solvents were similar to those employed in this study and thin-layer analysis showed very little evidence of nonlipid impurity recovered by the M and MM methods, so it was believed unnecessary to wash the milk fat samples extracted by those methods. However, it was found that Fraction 2, extracted by the SACC method, contained much nonlipid contaminant, including inorganic phosphorus. The method of Folch et al. (7) was used to remove the nonlipid contaminants and inorganic phosphorus from Fraction 2 before separation of neutral lipids by the modified Borgström procedure. This consists of washing lipid extracts in 2:1 (v/v) chloroform-methanol with 0.2 times their volume of a 0.04% CaCl₂ solution.

Phosphorus was determined by the method of Harris and Popat (9), using the 20-min digestion period recommended by Smith et al. (19).

The basic TLC techniques employed were those described by Mangold (11). The neutral lipid samples were applied to the plate in ethyl ether or chloroform with disposable pipettes. They were separated with solvent mixtures of petroleum ether–ethyl ether–acetic acid, 180:20:2 (v/v). The P-lipid samples were applied to the plate in chloroform-methanol, 2:1 (v/v) with micropipettes. The neutral plate and solvent system of Vogel et al. (20) with chloroform-methanol-water, 160:50:6 (v/v), was used to separate classes of P-lipids. Positions of the neutral and P-lipid components were detected by spraying the plates with a saturated solution of potassium dichromate in 80% (wt) sulfuric acid (15).

Lysolecithin, sphingomyelin, phosphatidyl choline, and phosphatidyl ethanolamine were identified by a combination of two techniques: First, tentative identification of the major P-lipid classes was determined by two TLC techniques using reference compounds; secondly, verification of these P-lipid classes separated on basic TLC chromatograms and eluted from the adsorbent was made by infrared spectral analysis (5). Phosphatidyl serine was detected on the chromatograms of the MM and SACC extracts, but very little was seen in those of the M extract. Presence of cerebrosides and some unidentified phosphatides was also indicated.

**RESULTS AND DISCUSSION**

Lipid phosphorus and TLC analyses were used as criteria of P-lipid recovery by the three extraction procedures. Lipid phosphorus data are presented in Table 1. The values show a consistent trend between the three methods when the different samples are compared. The phosphorus determinations of the MM and SACC fractions are comparable and indicate a mean recovery greater than those obtained by the M method by 10.2 and 12.2%, respectively. This confirms the findings of Walstra and De Graaf (21).

The TLC plate, Figure 1A (no. 4-6), shows that differences do exist between the P-lipid components extracted by the three methods.

**TABLE 1**

<table>
<thead>
<tr>
<th>Methods</th>
<th>(3.7%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(3.9%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(4.0%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(4.1%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(4.1%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean</th>
</tr>
</thead>
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<tr>
<td>Mojonnier</td>
<td>112.0</td>
<td>115.0</td>
<td>119.6</td>
<td>120.2</td>
<td>120.0</td>
<td>119.9</td>
</tr>
<tr>
<td>Modified Mojonnier</td>
<td>122.6</td>
<td>124.0</td>
<td>131.6</td>
<td>129.6</td>
<td>133.0</td>
<td>137.0</td>
</tr>
<tr>
<td>Silicic acid column</td>
<td>125.0</td>
<td>122.0</td>
<td>133.6</td>
<td>135.0</td>
<td>138.0</td>
<td>131.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values indicate micrograms of phosphorus per 10 ml of milk.

<sup>b</sup> Milk fat test of bulk raw milk determined by Gerber method.

<sup>c</sup> Letters a and b following the number indicate a duplicate analysis performed on the same sample.
EXTRACTION OF PHOSPHOLIPIDS


Each P-lipid fraction was dissolved in 0.5 ml of chloroform-methanol, 2:1 (v/v), and 50 μl of each spotted on the plate. A heavy sample application was necessary to show minor differences between the fractions. Presence of the major P-lipid classes in these samples and their behavior on TLC plates had been established. Observation of Figure 1,A (no. 4-6) shows that the three fractions have comparable concentrations of sphingomyelin (first dark spots from origin), phosphatidyl choline (second dark spots from origin), and phosphatidyl ethanolamine (third dark spots from origin). The M and MM methods seemed to recover more lysolecithin (faint grey spots next to origin) than the SACC method. The compound streaking above phosphatidyl choline in the MM and SACC samples, Figure 1,A (no. 5 and 6), corresponds to the manner in which phosphatidyl serine separates on similar chromatograms (17). Therefore, the tentative conclusion is that more phosphatidyl serine is present in the SACC sample than in the MM sample, with least in the M sample. The two acidic and one basic group (unique to phosphatidyl serine) may make it more readily extracted by the acidic SACC procedure than by the basic M or MM procedures. Figure 1,A (no. 4-6) shows two distinct spots directly above phosphatidyl ethanolamine and one at the solvent front in both the M (no. 4) and MM (no. 5) samples; however, three distinct spots directly above phosphatidyl ethanolamine and one at the solvent front are observed in the SACC fraction (no. 6). The identity of these spots is not known. In any event, it is concluded from Figure 1,A and other TLC plates (not shown) that minor differences do exist between components of the P-lipid extracts obtained by the three different methods.

The efficiency of the modified Borgström column in separating the neutral and P-lipid fractions is indicated by the TLC analyses. Figure 1,A (no. 1-3) reveals that this column procedure excluded P-lipids from the neutral lipid fractions. This was further confirmed by negligible lipid phosphorus values obtained on these fractions. Consequently, this evidence indicates that these neutral lipid fractions are apparently free of P-lipids. However, from Figure 1,B (numbers 4-6) it is evident that the P-lipid fractions contain small amounts of neutral lipid. This was observed during the TLC analysis of P-lipid fractions separated by several different silicic acid chromatography techniques. Presence of these neutral lipids suggests that either they are bound in P-lipid micelles or by the column adsorbent.

We do not believe that the new SACC method is an ultimate procedure for recovering milk P-lipids. It is limited from a practical standpoint in being more time-consuming and expensive than the M and MM methods. However, it is considered to be a worthwhile research method simply because it recovers more lipid phosphorus and more phosphatidyl serine.
Also, it recovers P-lipids in a fraction relatively free of neutral lipids which, to the authors' knowledge, is the only extraction technique that does not recover total lipids in one fraction. The fact that the new method employs mild treatment when disrupting the lipoprotein complex, in contrast to the M and MM methods, may be quite significant.

REFERENCES

(13) MILK INDUSTRY FOUNDATION. 1949. Laboratory Manual, Methods of Analysis of Milk and Its Products. Published by the Foundation, Washington, D. C.