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DETECTION OF BUTTERMILK SOLIDS IN MEAT BINDERS BY ELECTRON MICROSCOPY

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Abstract

Nonfat dry milk and buttermilk solids used as ingredients in meat binders can be differentiated by transmission electron microscopy. The meat binders are suspended in water and coarser ingredients such as wheat and mustard flours are separated from the milk solids by low-speed centrifugation (415 g for 30 min). The milk solids thus purified are concentrated by ultracentrifugation (8×10^4 g for 90 min) and the resulting pellets are embedded in a resin, thin-sectioned, stained, and examined by transmission electron microscopy. Buttermilk solids are revealed by the presence of fat globule membrane fragments. In the absence of buttermilk solids only casein micelles are found in the pellets. Sensitivity of this technique is 1 part of buttermilk solids in 20 parts of milk solids, *i.e.* 5% of buttermilk (w/w).

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Introduction

Binders used in Canadian comminuted meat products are principally blends of cereal products (wheat flour, baked crumb, and starches), milk products (nonfat dry milk and buttermilk), salt, and spices (which include mustard flour). Meat binders save time and labour to meat processors as they are handled as a single ingredient which already contains salt and spices as well as the binding substances. The meat binders must not introduce any undesirable properties into the finished meat products. Nonfat dry milk (NDM) is one of the ingredients important for the quality of the meat binders and, consequently, for the quality of the meat products. Yet, there is a possibility that NDM may contain buttermilk solids as was mentioned earlier[3]. Mixtures of NDM and buttermilk solids may occur unintentionally if sweet uncultured buttermilk, which is a by-product in the manufacture of butter, is added to whole milk destined for cream separation in order to retrieve the residual fat from the buttermilk. After the skimmed milk is spray-dried, the buttermilk solids become part of the NDM. Another kind of unintentional mixing occurs when skim milk is pumped into a storage tank which had been previously used to store buttermilk but had not been completely emptied before the introduction of skim milk. However, buttermilk may also be added intentionally to skim milk because comparable functionality[5] might be achieved at lower price. Apart from being illegal without a proper declaration, such practice may introduce some unknown factors into the products in which NDM is an ingredient. A higher susceptibility to oxidation of lipids present in buttermilk is one of such factors. Lipids in fat globule membrane fragments, particularly the cephalin (phosphatidyl ethanolamine) fraction of phospholipids, contain relatively large amounts of polyunsaturated fatty acid residues susceptible to oxidation[6]. This was demonstrated by a considerable decrease in the rate of oxidation of butter following the removal of the membrane substances from it[1] although membrane lipids comprised only a minute fraction of total lipids. Membrane substances have been assumed to play an important role by initiating the oxidation reactions. Although consequences of the presence of milk fat globule membrane fragments on the development of a rancid flavour in finished meat

products have not yet been established, preference has been given by meat processors to NDM free from such substances, *i.e.* to NDM free from buttermilk solids.

Chemical detection of buttermilk solids in NDM is difficult if not impossible because the composition of both products is similar except that spray-dried buttermilk contains approximately 5% lipids whereas NDM generally contains less than 1% lipids. To be useful, a test for differentiating NDM and buttermilk solids must be able to detect the latter at ratios lower than 15% or more probably at 5 to 10%.

Description of an attempt to detect buttermilk solids in NDM by electron microscopy was published earlier[3] . The test was based on the presence of large amounts of fat globule membrane fragments in buttermilk. As churning disrupts fat globules in the cream allowing the fat to aggregate into butter, most membrane fragments are released in the buttermilk[4]. Being distinctly different from other milk constituents, the fragments are detectable under electron microscope. The same principle has been used in this study to detect buttermilk solids in meat binders in the presence of other ingredients after such ingredients had been removed by low-speed centrifugation.

Materials and Methods

Materials

Meat binders were prepared on laboratory and pilot plant scales from the following ingredients: hard wheat flour, yellow mustard flour, milk solids, and salt. Milk solids were represented by spray-dried NDM and buttermilk, and by mixtures of buttermilk and NDM solids at ratios of 1:29, 1:24, 1:19, 1:14, 1:4, and 1:2 as shown in Table 1. All components were of Canadian origin.

Table 1.
Composition of milk solids (% by weight) in typical meat binders containing 60% wheat flour, 15% mustard flour, and 10% salt, analyzed by electron microscopy.

Sample number:	NDM:	Buttermilk:
1	15.0*	0
2	0	15.0**
3	14.5†	0.5†
4	14.4†	0.6†
5	14.25†	0.75†
6	14.0††	1.0††
7	12.0††	3.0††
8	10.0††	5.0††

* Four brands tested; fat content 0.1 to 1.5%.
** Five brands tested; fat content 4.1 to 4.5%.
† Two brands of NDM and two brands of buttermilk solids tested in mixtures.
†† One brand of NDM and five brands of buttermilk tested in mixtures.

Methods

The meat binders (2 g) were dispersed in 15 mL of distilled water in graduated centrifuge tubes. The suspensions were allowed to stand undisturbed at 22°C for 30 min and were then centrifuged at 415 g for 30 min. Centrifugation separated each suspension into a thin top layer of residual fat, a turbid supernatant containing milk solids and salt, and a pellet consisting of wheat and mustard flours. Central portions of the supernatants (9 mL) were withdrawn and were subjected to ultracentrifugation at 8×10^4 g for 90 min. All the corpuscular solids present in the supernatant from low-speed centrifugation were sedimented by ultracentrifugation in the form of a pellet (maximum thickness of 2 mm) leaving a clear supernatant.

Each pellet was divided into the top, middle, and bottom portions. Small particles, approximately 0.5 mm in diameter, were excised from the portions and were fixed in a 1.4% glutaraldehyde solution for 30 min and postfixed in a 2% buffered (0.05 M veronal-acetate buffer, pH 6.75) OsO_4 solution for 2 h. The particles were then dehydrated in a graded alcohol series, embedded in Spurr's low-viscosity medium[7], sectioned (<100 nm), placed on 200-mesh hexagonal copper grids, and stained with uranyl acetate and lead citrate solutions as reported earlier[3]. The stained sections were examined in a Philips EM 300 electron microscope operated at 60 kV and micrographs were taken on a 35-mm film.

Composition of the specimens was not known to the electron microscopist and was revealed only after conclusions from electron microscopical analysis had been drawn. All experiments were made in 4 repetitions.

Results and Discussion

In preliminary experiments, meat binders were wetted with distilled water to a dough-like consistency, fixed, and embedded for electron microscopy. Of all the corpuscular ingredients present, starch granules in the wheat flour were most prominent (s in Fig. 1). Milk solids were characterized by the presence of casein micelles, *i.e.* globular protein bodies approximately 100 nm in

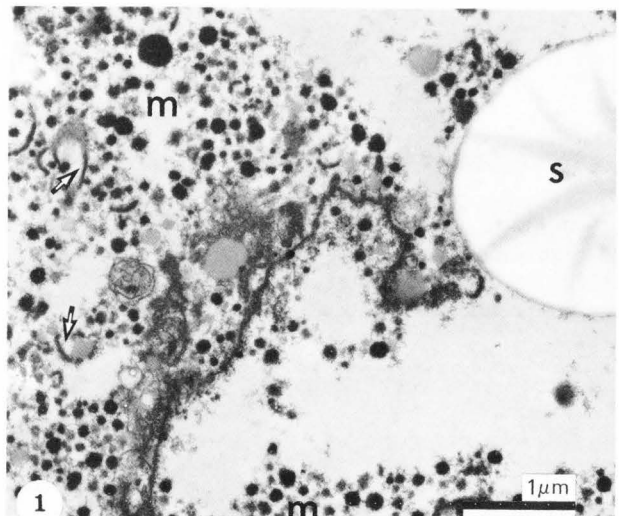

Fig. 1. Whole meat binder. Electron microscopy reveals the presence of starch (s) from wheat flour and casein micelles (m) representing milk solids. Fat globule membrane fragments (arrows) are difficult to identify in the presence of other materials.

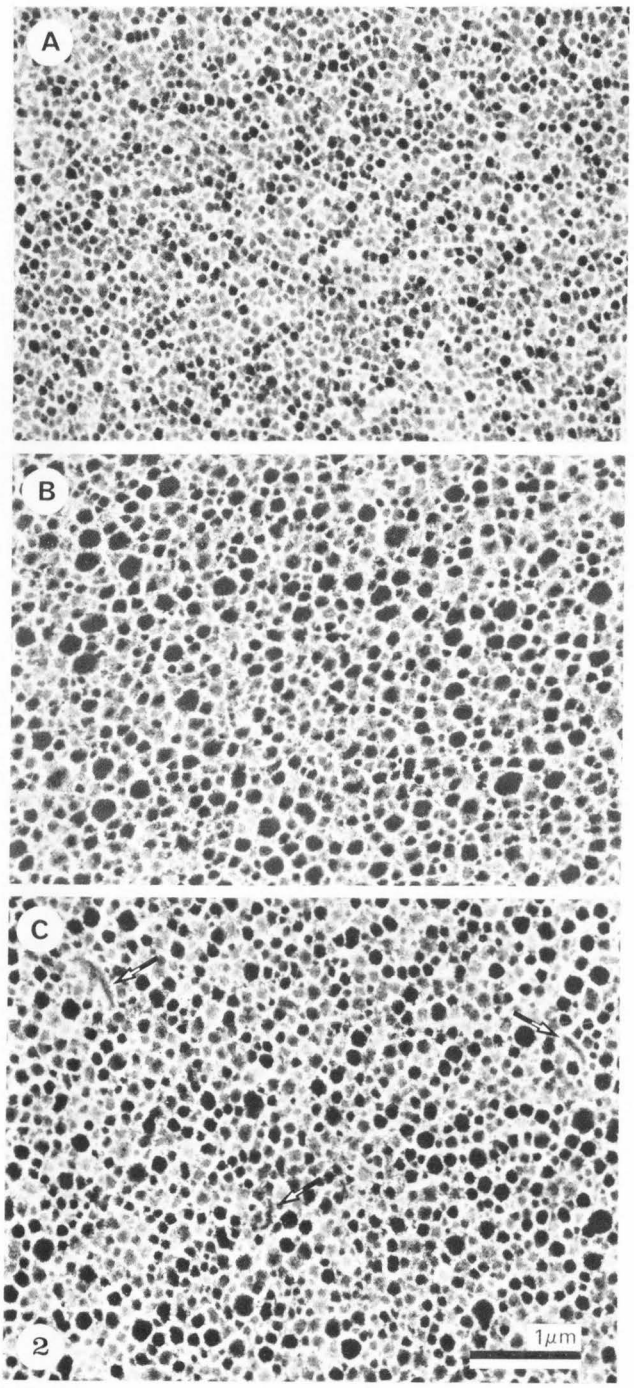

Fig. 2. Milk solids isolated by ultracentrifugation (8 x 10^4 g for 90 min) from a purified aqueous suspension of a meat binder made with pure nonfat dry milk. The pellet was divided into top (A), middle (B), and bottom (C) portions; large casein micelles (dark discs) sedimented first (C) and the smallest micelles sedimented last (A). Individual fat globule membrane fragments (arrows) were found in the bottom portion (C).

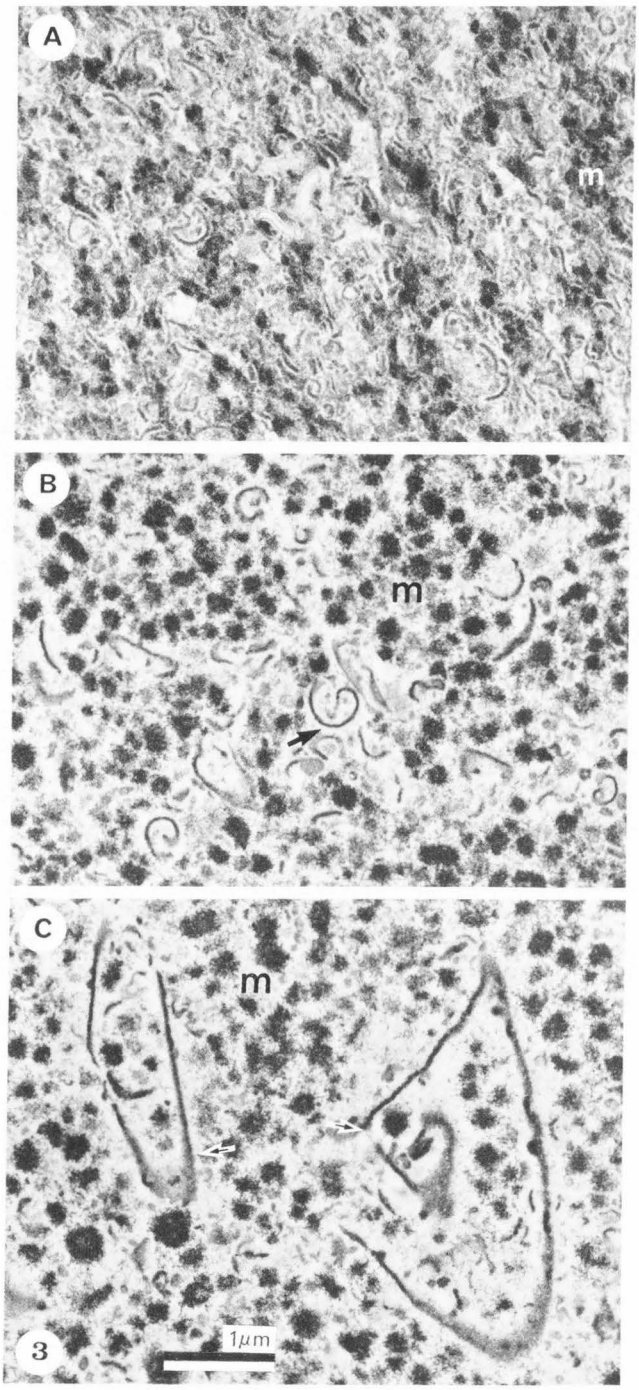

Fig. 3. Milk solids isolated by ultracentrifugation (8 x 10^4 g for 90 min) from a purified aqueous suspension of a meat binder made with buttermilk solids as the only source of milk solids. Fat globule membrane fragments (arrows) were abundant in all portions of the pellet (A = top; B = middle; C = bottom) with the largest particles sedimented in the bottom (C) and the finest particles sedimented in the top (A) portions. Casein micelles (m) appear in the form of dark discs.

diameter (m in Fig. 1). It was difficult, however, to distinguish fat globule membrane fragments in such heterogeneous mixtures. A preliminary purification of the milk solids appeared to be necessary. It was accomplished by the removal of coarse components of the meat binders such as wheat and mustard flours by low-speed centrifugation; resulting pellets were discarded. Corpuscular milk solids in the supernatants were subsequently concentrated by ultracentrifugation and the pellets were examined by electron microscopy. Electron micrographs of pellets obtained from meat binders made with pure NDM (Fig. 2) and with buttermilk solids (Fig. 3) differed considerably.

As it is known that heavier particles sediment first and the finest particles sediment last, each pellet was analyzed from top to bottom. In the case of pure NDM there were only casein micelles present in all the three portions (Fig. 2). Fat globule membrane fragments were found only seldom at a rate of less than one fragment per an area of 58 μm^2, which means that a great number of such areas within a single opening in the hexagonal grid mesh, as visualized on the microscope screen, contained not a single fat globule membrane fragment. In a small number of areas 1 to 3 fragments could be found (Fig. 2 C).

In contrast, pellets obtained with meat binders which contained buttermilk solids as the only source of milk solids showed a different composition. Fat globule membrane fragments were present in all the 3 portions of the pellets (Fig. 3) and ranged in sizes from small in the top portion (Fig. 3 A) to large in the bottom portion (Fig. 3 C). The fragments were abundant within a single area of vision (58 μm^2) under the electron microscope at the suitable magnification (10,000 X on the viewing screen) at which the detection of the membrane fragments was possible. Presence of fat globule membrane fragments in all the portions of the pellet is interesting and indicates that the sedimentation rate of the fragments is similar to that of casein micelles. Although the original fat globule membranes in cream contain lipoproteins[2] and, thus, would be anticipated to be of a density lower than the casein micelles, the lipid component may be gradually lost from the membranes during processing (churning, spray-drying). Wooding[9] found that the original membrane started to disintegrate immediately after the secretion of milk and Brunner[2] showed that the lipoproteins were partially removed from the membranes by washing with water. The fat globule membrane fragments found in the pellets are different from lipoprotein particles and membranes which in the ultracentrifuge sediment in a layer just above the casein micelles[8] and which are present in both NDM and buttermilk[3]. Such particles are believed to mainly consist of detached microvilli and remnants of Golgi apparatus vacuoles[8]. Noticeable differences in the dimensions of casein micelles were found only between the top and the middle portions of the pellet; casein micelles in the bottom and middle portions were of similar dimensions (Fig. 2 A to C). A more marked separation of fat globule membrane fragments by their dimensions is evident in Fig. 3 A to C.

There were some slight differences in the dimensions and shapes of the fat globule membrane fragments found in meat binders made with various brands of buttermilk solids but in general the micrographs were similar to those obtained with pure spray-dried buttermilk which had been reconstituted and ultracentrifuged[3]. Differences between the micrographs of milk solids isolated from meat binders made with NDM or with buttermilk solids were distinct and indicated that this technique may be suitable to detect the presence of buttermilk solids in NDM used as an ingredient in meat binders.

To establish sensitivity of the detection, meat binders containing buttermilk and NDM solids at ratios varying between 1:29 and 1:2 were analyzed. In such cases it was necessary to examine larger areas of the sections to establish the most probable distribution of the fat globule membrane fragments. If "clean" areas, *i.e.* areas completely free of fat globule membrane fragments were impossible to find, it was concluded that buttermilk solids were present; these conclusions were correct for mixtures at ratios of 1:2 to 1:19 as compared with the composition of the meat binders not known to the electron microscopist. A single micrograph, such as in Fig. 4, however, would not sufficiently reflect the mean composition of the milk solids in the meat binder and is presented only as an example to show a local accumulation of fat globule membrane fragments in a 1:14 mixture; many areas of the same dimensions contained a somewhat lower number of the membrane fragments.

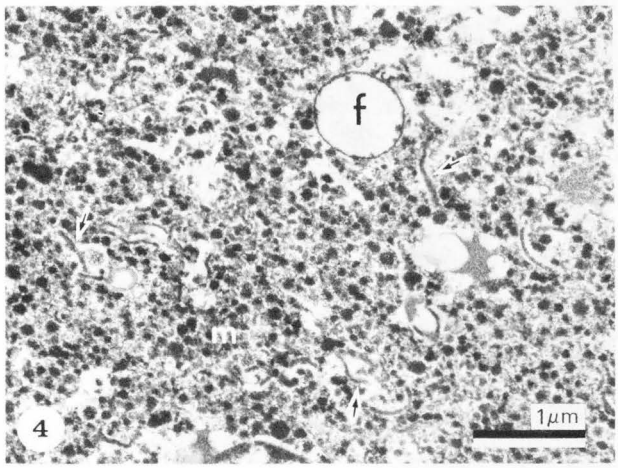

Fig. 4. Micrograph of an area in a meat binder made with a mixture of buttermilk and NDM solids (1:14) showing an accumulation of fat globule membrane fragments (arrows). f = *fat globule;* m = *casein micelles.*

However, Fig. 4 indicates that is was possible to detect buttermilk solids at a ratio of 1:14 (6.7%) in NDM in the presence of large amounts of wheat and mustard flours. The detection was possible even at the ratio of 1:19 (5% of buttermilk solids of the total milk solids) but this was considered to be the limit of sensitivity; it was not possible to detect buttermilk solids with certainty below this level and meat binders containing buttermilk and NDM solids at ratios of 1:24 and 1:29 were evaluated by the electron microscopist

as "pure NDM".

As there was a possibility that some kind of fibrous material present in the flours could simulate buttermilk solids, both kinds of flour were dispersed in water and processed for electron microscopy in the same way as the complete binders. The supernatants resulting from low-speed centrifugation were found to be only slightly opalescent and did not produce enough sediment by ultracentrifugation for embedding. Pellets obtained on a larger scale by doubling the volume of the supernatant were very compact and were difficult to section. However, because there was only a threshold concentration of the flour particles in the regular supernatants after low-speed centrifugation and because the dimensions and shapes of these particles were different from the fat globule membrane fragments, the flours posed no risk of misevaluating the electron micrographs. The particles were particularly scarce in supernatants obtained with mustard flour.

It is interesting to note that bacteria, anticipated to be present in large quantities in the NDM and buttermilk pellets (see Discussion with Reviewers in Reference 3), were not found in the middle portions of the pellets routinely used for the examination and were only occasionally encountered in the bottom portions known to accumulate the heaviest particles. It is assumed that if present in the milk powders, the bacteria were removed by low-speed centrifugation along with coarser constituents of the meat binders.

One of the samples of NDM contained a higher number of fat globule membrane fragments than would correspond to pure NDM. As was mentioned earlier[3], such isolated cases require additional studies. It has been known that microvilli and other membrane fragments are naturally present in cow's milk[8]. They sediment in the form of a so-called "fluffy" layer above the compact pellet composed of casein micelles.

In conclusion, it has been confirmed that the presence of fat globule membrane fragments in milk solids is indicative of buttermilk solids. The analytical technique presented in this study is a modification of an earlier electron microscopical technique[3] and has been developed to meet the requirements of the meat industry. The modification consists of the removal of ingredients other than milk solids from the meat binders by low-speed centrifugation, the separation being of such a degree that neither wheat nor mustard flours present in excess interfere. The initial technique[3] could be used by meat binder manufacturers to check the quality of their source NDM and the modified procedure could be useful to manufacturers of comminuted meat products in checking the quality of the meat binders supplied.

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Discussion with Reviewers

D.E. Carpenter: Aside from the membrane fragments present in the electron micrographs of samples prepared from buttermilk solids, the micelles appear to be different. In the case of the buttermilk solids, the micelles have less distinct edges compared to the micelles from nonfat dry milk solids. Does this have any relationship to the "hairy" micelles reported by others or is there another explanation for this?
Authors: We have no explanation for the "hairy" appearance of casein micelles in buttermilk. The micelles are more "hairy" in some buttermilk specimens than in others but we have not yet been able to correlate such appearance with manufacturing conditions. Also differences in the dimensions of casein micelles have remained unexplained.

J.R. Brunner: If microvilli represent extension of secretory cell plasmalemma similar to the plasmalemma surrounding the secreted fat droplet, why are they not sedimented into the NDM casein pellet as are the MFGM fragments?
Authors: Stewart *et al.*[8] stated that skim milk membranes found in the "fluffy" layer above the casein pellet were morphologically different from milk fat globule membranes; the latter ones

possessed an electron-opaque layer of neutral lipids bound to one face. This ultrastructural characteristic was not observed in thin sections of the skim milk membranes found in the "fluffy" layer.

D.E. Carpenter: It appears from the number of membrane fragments in the buttermilk solids that perhaps a membrane protein such as xanthine oxidase would be a good indicator of buttermilk solids. This or other chemical/enzymatic methods would give a more quantitative analysis of materials used in the meat binders. Are there efforts in these areas?
Authors: There have been some recent unsuccessful unpublished efforts to detect buttermilk solids in NDM by chemical methods. Analytical ultracentrifugation and electrophoresis have also been used. We were invited to participate in such studies using electron microscopy. Concerning xanthine oxidase, we are unable to predict how active this enzyme would remain in spray-dried materials stored in the presence of large amounts of a variety of ingredients including 10% salt. In other words, we are skeptical about an assay based on residual enzyme activity because this would be expected to be sensitive to both the processing and storing conditions.

J.T. Hynes: Dispersal, low-speed centrifugation, and ultracentrifugation over a minimum of 150 min could promote microbial growth at ambient temperature. Is this a matter of concern in fragment identification?
Authors: In spite of the relatively lengthy preparation of pellets prior to fixation, we have not encountered any microbiological problems. In fact, even the bottom parts of the pellets obtained by ultracentrifugation had low bacteria counts (Fig. 2 and 3). It is probable that most bacteria sedimented along with coarser particles and were removed by low-speed centrifugation.

J.T. Hynes: Homogenization of buttermilk prior to spray-drying would preclude the use of this technique. Do you agree?
Authors: We cannot answer this question because we do not know what changes would be caused in the appearance of the fat globule membrane fragments in buttermilk by homogenization. It is a good question and the only safe answer is that the method has been shown to work with buttermilk produced in Canada.

P. Jelen: Fillers other than wheat or mustard flour may be included in meat binders. Is the described method of buttermilk detection suitable for binders containing starch, bread crumbs, potato meal, or other fillers?
Authors: Wheat and mustard flours were used in our experiments as ingredients most likely to cause problems in buttermilk detection. Starch and bread crumbs are, in fact, other forms of wheat flour. Mustard flour is a ground whole seed and yet, in spite of its complex nature, it does not interfere with the determination of buttermilk solids in the binders. Potato meal is rarely used in meat binders and is also unlikely to interfere with the method. Fillers, which are completely sedimented by low-speed centrifugation, evidently do not contain submicroscopical particles which would resemble fat globule membrane fragments in both behaviour and appearance.

J.R. Brunner: This assay requires sophisticated equipment. How would it be implemented by the meat industry on a routine basis?
Authors: There is no need for routine implementation of this method. The fact that a method and the facilities to carry it out do exist, is believed to be a sufficient deterrent. In Canada, the vast majority of meat products is manufactured at government-inspected plants. Binders are either supplied as single ingredients or as complete units. Only several companies supply binder units. The binder business is very competitive and much effort is put into building trust. If this trust is broken, *e.g.* by discovering a false ingredient declaration, it would have a serious economic effect upon the violator.

P. Jelen: Would mixtures of buttermilk powder with other dairy fillers (*e.g.* caseinates, decalcified NDM, whey powder, or whey protein concentrates) behave similarly to NDM in typical meat applications?
Authors: Caseinates, whey powder, and whey protein concentrates are at present not being used in meat applications in Canada. Calcium-reduced NDM behaves in a similar way to NDM. Although casein micelles were found to be partially disintegrated and partly aggregated in calcium-reduced NDM (Fig. 5), they did not interfere with the detection of buttermilk solids in the binders.

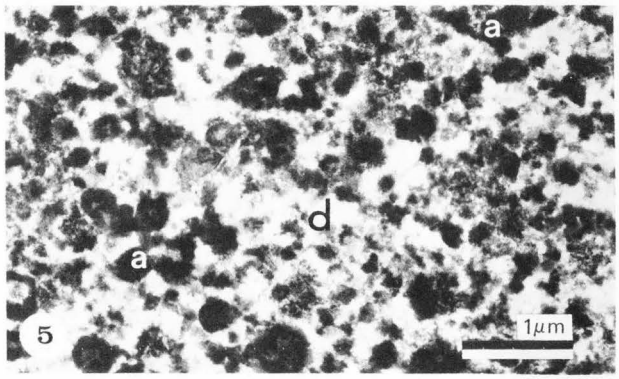

Fig. 5. Calcium-reduced NDM. Casein micelles are both aggregated (a) and disintegrated (d).

P.Jelen: Do you know of any reason why the meat processors do not like to use buttermilk solids despite their superior emulsifying capacity and lower cost as compared to NDM?
Authors: Our experience has been that the performance of buttermilk solids in comminuted meat products regarding texture and stability is similar to but not better than that of NDM. Comparable functionality at a lower price has been mentioned in our study. Buttermilk solids are being used by meat processors but skim milk powder has a better image because historically there have been serious problems with the quality of buttermilk solids, particularly with high bacteria counts, off-flavours, off-colours, and variable fat levels. Buttermilk solids are not always available, which is another problem since there is a reluctance to change ingredient labels on comminuted meat products.