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AN APPARATUS FOR A NEW MICROCUBE ENCAPSULATION OF FLUID MILK IN PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

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Abstract

A simple apparatus has been developed for a new "microcube" encapsulation of fluid milk samples in their prefixation preparation for electron microscopy. The new technique is based on making cubic wells in an agar gel layer, filling them with fluid milk samples, and sealing them with another agar gel layer. The individual wells are then separated by cutting from the initial block, providing 0.5 mm walls around the samples. The embedded material (milk, buttermilk, yogurt, etc.) is fixed, dehydrated, and embedded in a resin for transmission electron microscopy. The procedure is simpler, and more versatile, reliable, and reproducible than other encapsulation methods used to prepare similar food samples. Agar gel tubes used in the other methods have several disadvantages such as the need for manual dexterity of the experimenter to make them, and difficulty in sealing the filled capsules properly. Results obtained by the microcube procedure were compared with results obtained by two methods using agar gel tubes and also by mixing a warm agar sol with fluid food samples. This latter method is simpler than agar encapsulation but shows agar strands in the micrographs of the milk samples, which is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT (ultra-high temperature-treated) milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

Key Words: Agar gel, apparatus, buttermilk, dairy products, embedding, encapsulation, fluid milk, microcube encapsulation, transmission electron microscopy, yogurt.

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Introduction

Preparation of fluid milk for electron microscopy requires the use of specific techniques in order to obtain artifact-free images of the samples (Carroll *et al.*, 1968; Stewart *et al.*, 1972; Andrews *et al.*, 1977; Davies *et al.*, 1978; Kalab, 1983; Heertje *et al.*, 1985; Farah and Ruegg, 1989). Fluid or gelled milk samples destined for electron microscopic examination must not disintegrate while they are fixed, dehydrated, and mounted or embedded. Various methods have been employed to achieve this objective both in scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Mixing of fluid milk with agar sol followed by gelling the mixture and treating the resulting gel as a solid sample has often been used in TEM because this procedure is simple. However, dilution of the sample with the agar sol alters the distribution of structural elements in the sample and agar strands present in the gel contaminate the sample and are visible under the electron microscope (Kalab, 1981; Harada *et al.*, 1991). Their images may interfere with other structural components such as fat globule membrane fragments (Kalab, 1980). Enclosing a small volume of the sample in a capsule permeable for fixatives and dehydrating agents promised to avoid some of the problems. Agar encapsulation was pioneered by Salyaev (1968) and his technique has been adapted and modified by several authors (Henstra and Schmidt, 1970, 1974; Jewell, 1981; Allan-Wojtas and Kalab, 1984; Kalab, 1987, 1988; Veliky and Kalab, 1990), particularly for use in SEM. In the original and modified procedures, the sample is aspirated into a narrow agar tube (Henstra and Schmidt, 1970, 1974; Jewell, 1981; Allan-Wojtas and Kalab, 1984). The sample may alternatively be aspirated into a Pasteur pipette which is subsequently coated with agar gel and the sample is transferred from the Pasteur pipette into the gel tube formed by withdrawing the pipette from the agar gel coating (Kalab, 1987, 1988). The sample-containing gel tube is sealed with warm agar sol.

Other hydrocolloids (sodium alginate) with low-temperature gelling properties have also been used to encapsulate viscous food samples (Veliky and Kalab,

1990). A somewhat different encapsulation procedure for SEM was used by Teggatz and Morris (1990). Holes (2 mm diameter x 2 mm depth) drilled into aluminum SEM stubs, were filled with fluid food samples and the stubs were then dipped into 3% agar sol at 45°C which subsequently solidified. After fixation and dehydration, the agar layer on top of each stub was lifted off and mounted upside-down on a clean SEM stub using double-coated tape. The samples were surrounded by a coat of carbon paint and coated with a layer of gold-palladium before being viewed in a scanning electron microscope at 6 kV (Teggatz and Morris, 1990).

All reported agar encapsulation procedures require a relatively high degree of manual dexterity of the experimenter both during preparation and sealing of the capsules, particularly because capsules used in TEM are considerably smaller than those used for SEM. Perfect sealing of fluid milk samples is difficult to achieve and the samples may slowly leak out.

A new method for encapsulation of fluid milk samples of both low and high viscosity is described in this paper. The problematic step of aspiration is eliminated and the samples are sealed in a different manner. Results obtained using this new method are compared with those obtained by other methods.

Materials and Methods

Reagents

Agar was purchased from Difco Laboratories (Detroit, Michigan, USA); glutaraldehyde, osmium tetroxide, propylene oxide, and Epon-Araldite epoxy resin were obtained from Electron Microscopy Sciences (Fort Washington, Pennsylvania, USA). All other chemicals were of analytical reagent grade.

Samples

Whole milk, stirred yogurt, and 8-month-old ultra-high temperature (UHT) processed ultrafiltration milk retentate (concentrated 3x by volume) were obtained from the Dairy Products Laboratory at Utah State University. Cultured buttermilk was of commercial origin.

Microcube encapsulation compared with encapsulation in agar gel tubes (Experiment a)

Apparatus: A kit for microcube encapsulation was designed and constructed. It consists of (**a**) a stainless steel mold (100 mm in length) with a row of 24 teeth of dimensions 1 x 1 x 2 mm (height x width x length) separated by 2-mm gaps (in Figure 1 a shorter model, 52 mm in length, containing 12 teeth is shown) for simultaneous preparation of 24 wells in agar gel, and (**b**) a fiberglass mask (bottomless rectangle) of inner dimensions 2 x 10 x 100 mm (height x width x length) for casting a block of agar gel of similar dimensions. The ends of the mold project 1 mm below the teeth so that when positioned over the block of agar gel, there is a 1-mm thick agar gel layer left beneath the bottoms of the wells.

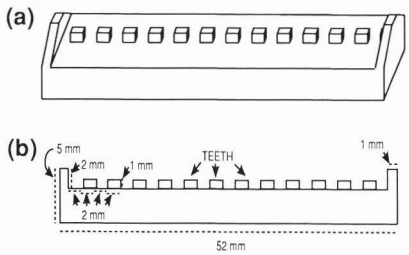

Figure 1. Stainless steel mold (shorter version of the 100 mm long one used in the study) used to prepare microcube samples (**a**) Stereographic view, (**b**) lateral view.

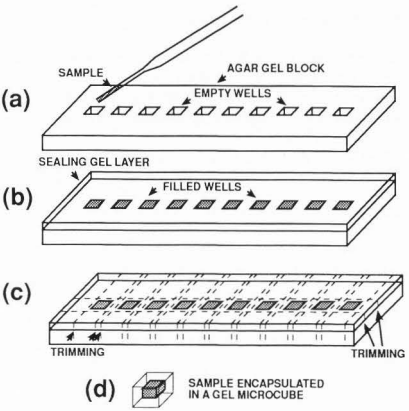

Figure 2. Steps involved in the preparation of microcube samples: (**a**) Filling empty cubic wells impressed in the agar gel block; (**b**) Sealing filled wells in agar gel block; (**c**) Trimming 'cubes' from agar gel block; (**d**) Gel 'microcube' with encapsulated sample.

Microcube encapsulation: The fiberglass mask was placed on a square sheet of fiberglass (side: 200 mm) and the joints were sealed with a 4% agar sol kept at 50°C and stirred continuously with a magnetic bar. Two ml of the same agar sol was then deposited in the opening of the mask using a plastic transfer pipette. After 5 seconds when gelation had just begun, the mold was pressed into the 2 x 10 x 100 mm (height x width x length) block and held in place for 5 seconds before being carefully removed to leave cubic wells impressed in the agar block (Figure 2a). All the wells in the block were then filled with 2 mm^3 aliquots of one fluid food sample through a 25G 7/8 needle (Becton Dickinson,

New Jersey, USA) from a 1 ml syringe. In each case, the sample was drawn into the syringe before attaching the needle to dispense materials into the wells. Preliminary studies compared results of filling wells with different gauge needles to determine if the shear forces on sample using the smaller bore needles affected the extended structures of samples. No detected differences in the extended structures of the samples were observed in samples dispensed with 16G 1, 21G 1/2 and 25G 7/8 needles. The 25G 7/8 needles were subsequently used because they filled the wells more efficiently than the other needles. After the last well was filled, another layer of agar sol was placed over the block to a depth of 1 mm where it gelled, thus covering and sealing the samples in the wells (Figure 2b). A razor blade was used to trim the agar seal from the top of the mask and the entire solidified agar gel block was removed from the mask. The block was then cut with a razor blade 0.5 mm around each well (Figure 2c) to produce 'cubes' with 2 mm^3 of the sample (Figure 2d). The 'cubes' were examined for leaks and other defects, then fixed and processed for TEM as described below in the Electron Microscopy section. Preliminary studies had shown that (a) agar sol concentrations < 4% were unsuitable for sealing the wells since they displaced the sample (especially the less viscous ones) from the wells and (b) 3 x 3 x 2 mm agar 'cubes' with 2 mm^3 of milk sample were small enough to allow satisfactory fixation and dehydration.

Encapsulation in agar gel tubes - Salyaev method: Capsules were made by dipping a stainless-steel rod (0.5 mm in diameter) repeatedly into a 4% agar sol (stirred continuously with a magnetic bar at 45°C) and manipulating the rod to form a thin (~0.5 mm) agar gel tube around it. The lower end of the solid tube was then cut off with a razor blade. Milk samples were aspirated into the tube by immersing its open end into the sample and pulling the steel rod upwards as a piston. After enough milk sample had been aspirated into the tube (a length of approximately 12 mm), its bottom end was wiped with tissue paper and the tube was sealed with warm (45°C) agar sol producing a capsule. A 4-mm length portion of the capsule was then cut off from the bottom and a drop of agar sol was applied with a transfer pipette to seal the upper end, producing a microcapsule. This was repeated to produce three microcapsules from each capsule. All microcapsules were examined for leaks and other defects.

Encapsulation in agar gel tubes - Kalab method: A glass Pasteur pipettes with inner diameter of 1.0 mm was drawn out into a capillary tube with inner diameter of ~ 0.5 mm. The milk sample was aspirated into the capillary tube to a length of ~ 2 mm. The lower end of the tube was wiped clean with tissue paper and sealed with a droplet of the agar sol. After the sealed end had solidified, the capillary tube was dipped into the agar sol and then manipulated to form a thin layer of agar gel on the glass surface around the sample. Dipping was repeated twice to form a uniform agar gel layer around the glass surface. This agar gel sleeve was then trimmed at the upper end of the sample and removed. The capillary tube was then withdrawn from the agar gel sleeve resulting in the sample sliding from the glass tube into the gel tube. The freed upper end of the agar gel tube was then trimmed with a blade approximately 0.5 mm above the sample and sealed with a droplet of the agar sol producing a microcapsule. This was repeated for each milk sample to produce the desired number of microcapsules. All microcapsules were examined for leaks and other defects.

Electron microscopy: Agar gel capsules obtained by three encapsulation methods (Salyaev, 1968; Kalab, 1987, 1988; and our new microcube method), which contained whole milk, buttermilk, and yogurt samples, were fixed at 20°C for 24 hours in 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.6 for whole milk, and pH 4.5 for buttermilk and yogurt). After the fixation had been completed, these samples as well as samples obtained by mixing milk with agar sol were washed with 0.1 M phosphate buffer, pH 6.6, and post-fixed with a buffered (0.1 M phosphate buffer, pH 6.6) 1% osmium tetroxide, dehydrated in a graded ethanol series of 30, 50, 70, 95, and 100% ethanol, infiltrated with propylene oxide, and embedded in Epon-Araldite epoxy resin. Thin sections (90 nm thick, Sorvall MT-2 Porter-Blum ultramicrotome) from the epoxy resin blocks were then stained with a uranyl acetate solution in methanol for 15 minutes (Youssef, 1985), followed by lead acetate staining for 5 minutes (Youssef, 1985). TEM was carried out using a Zeiss CEM 902 electron microscope operated at 80 kV. Micrographs were taken on Kodak SO 163 sheet film and printed on Kodak polycontrast III RC paper.

Microcube encapsulation compared with agar sol mixing (Experiment b)

Microcube encapsulation: Microcube encapsulation of UHT concentrated milk retentate and yogurt was performed as described in Experiment (a) above. Half of these samples were placed in vials containing 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 4°C for 1 hour and subsequently refrigerated at 6°C for 24 hours in a fresh 1.5% glutaraldehyde solution. The other half were placed in vials containing 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 45°C for 1 hour and subsequently refrigerated at 6°C for 24 hours in a fresh 1.5% glutaraldehyde solution.

Mixing with agar sol: UHT concentrated milk and yogurt were each divided into two 5-ml aliquots and prepared as follows:

(1) **Fixation with glutaraldehyde at 45°C followed by solidification with agar**: The samples were fixed in a 4.5% glutaraldehyde solution by mixing 5 ml of sample with 0.5 ml of a 50% aqueous glutaraldehyde solution. Fixation proceeded for 10 minutes at 20°C. The samples were then heated to 45°C and 5 ml of a

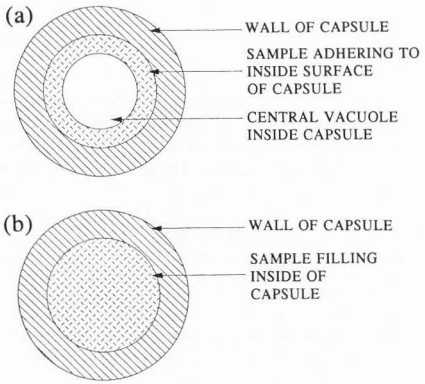

Figure 3. Diagram comparing (**a**) defective microcapsule with (**b**) non-defective microcapsule. Defective microcapsule shows large vacuole within the sample matrix.

Figures 4-6. Transmission electron micrographs of whole milk (**Fig. 4**), cultured buttermilk (**Fig. 5**) and stirred yogurt (**Fig. 6**) prepared using Salyaev method (**Figs. 4a, 5a, 6a**), Kalab method (**Figs. 4b, 5b, 6b**), and microcube method (**Figs. 4c, 5c, 6c**). c: casein micelles; small arrowheads: fat globule membrane fragments; large arrowheads: intact fat globules.

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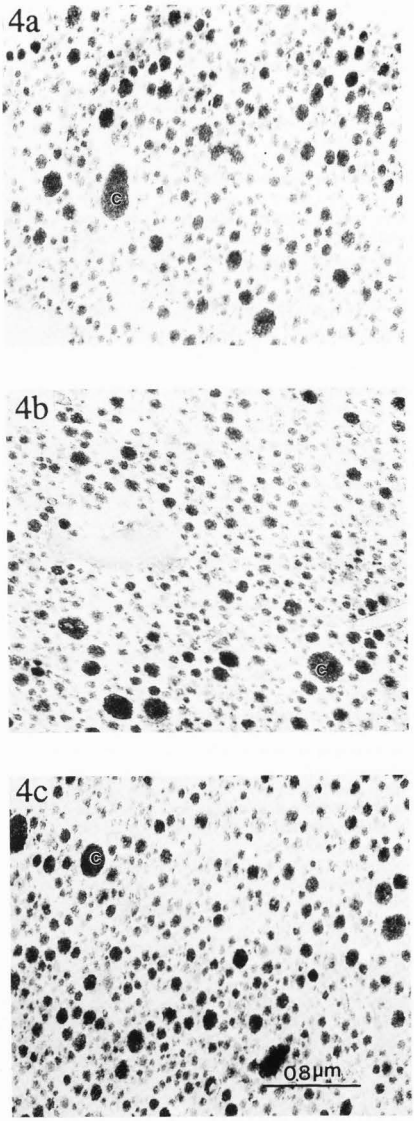
warm (45°C) 3% agar solution were added. The mixtures were mixed on a vortex mixer, poured into petri dishes and allowed to solidify. The sample-agar gel was then cut into 1 mm^3 cubes and placed in labeled vials containing 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.6 for UHT milk and pH 4.5 for yogurt) at 6°C for 24 hours.

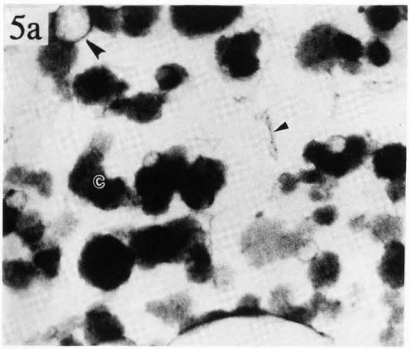
(2) **Solidification of samples with agar followed by glutaraldehyde fixation at 4°C**: The UHT milk and yogurt samples were mixed with 3% agar sol at 45°C, solidified by cooling in petri dishes and cut into 1 mm^3 cubes. These cubes were fixed with 1.5% glutaraldehyde at 4°C for 1 hour and subsequently refrigerated at 6°C in a fresh 1.5% glutaraldehyde solution for 24 hours.

Results and Discussion

Microcube encapsulation compared with encapsulation in agar gel tubes (Experiment a)

The first criterion used to assess the quality of encapsulation was to evaluate how well the samples were sealed inside the agar gel capsules. Results obtained by the microcube encapsulation method were compared with results obtained from the methods of Salyaev (1968) and

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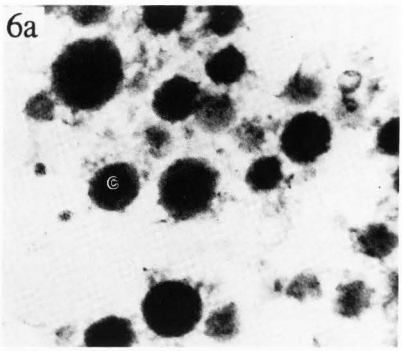
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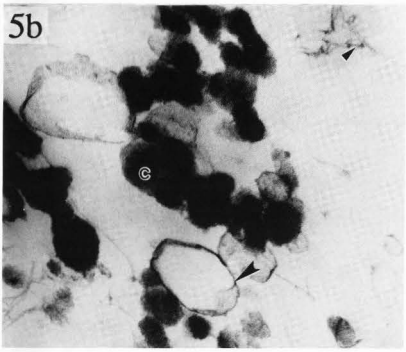
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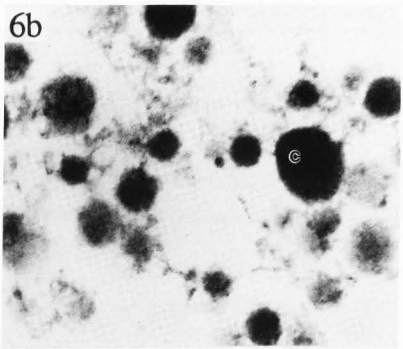
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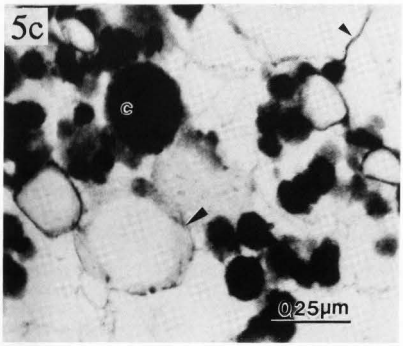
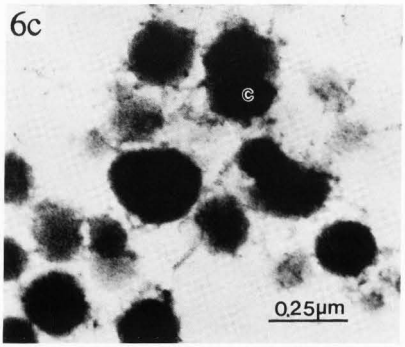

5c
C
0,25µm


6c
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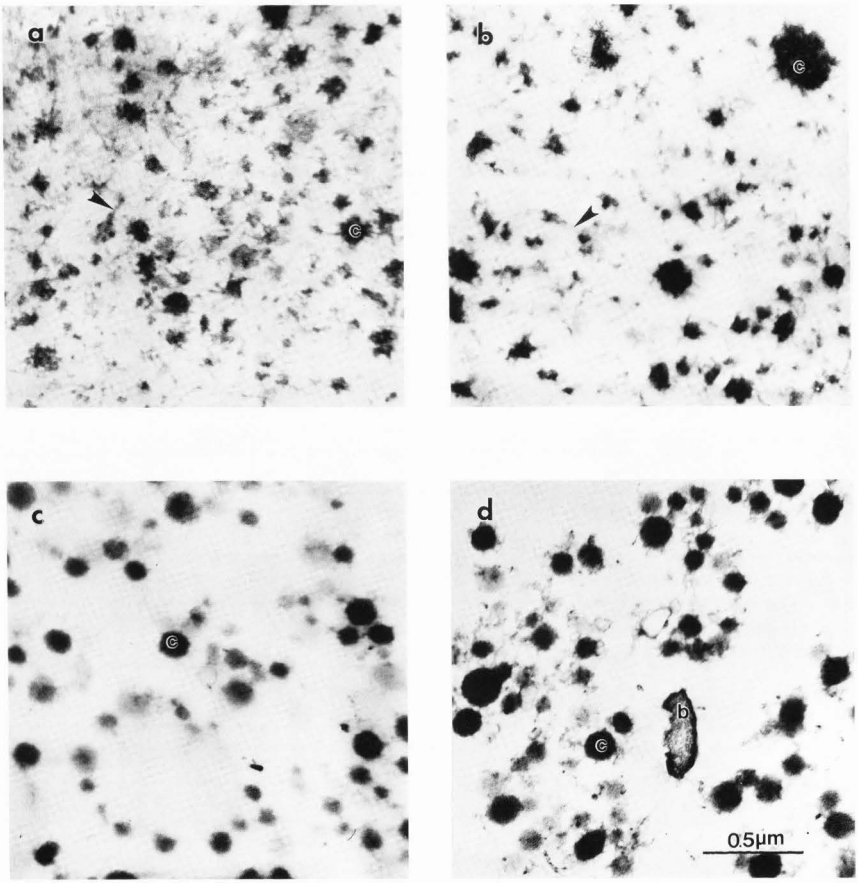

Figure 7. Transmission electron micrographs of **(a)** UHT milk concentrate fixed at 4°C; **(b)** UHT milk concentrate fixed at 45°C; **(c)** yogurt fixed at 4°C; and **(d)** yogurt fixed at 45°C, all prepared by microcube encapsulation. b: bacteria; c: casein micelles; arrows: tendrillar appendages connecting casein micelles.

Kalab (1987, 1988). The numbers of leaky capsules detected after glutaraldehyde fixation for each method are shown in Table 1. The χ^2 analysis of these data shows the proportion of intact capsules to be significantly higher ($P = 0.05$) for the microcube method when compared to the other methods singly and collectively. There was no significant difference between the Salyaev and Kalab methods. After the production of milk-filled agar gel capsules using both methods, buttermilk and yogurt samples were found adhering to the inner walls of the microcapsules in the form of rings rather than uniformly filling the capsules (Figure 3). This phenomenon may be attributed to inadequately filled capsules. A similar problem was observed with milk as one of the less viscous samples. This was due to the relatively low total solids content of milk. Replacement of the milk

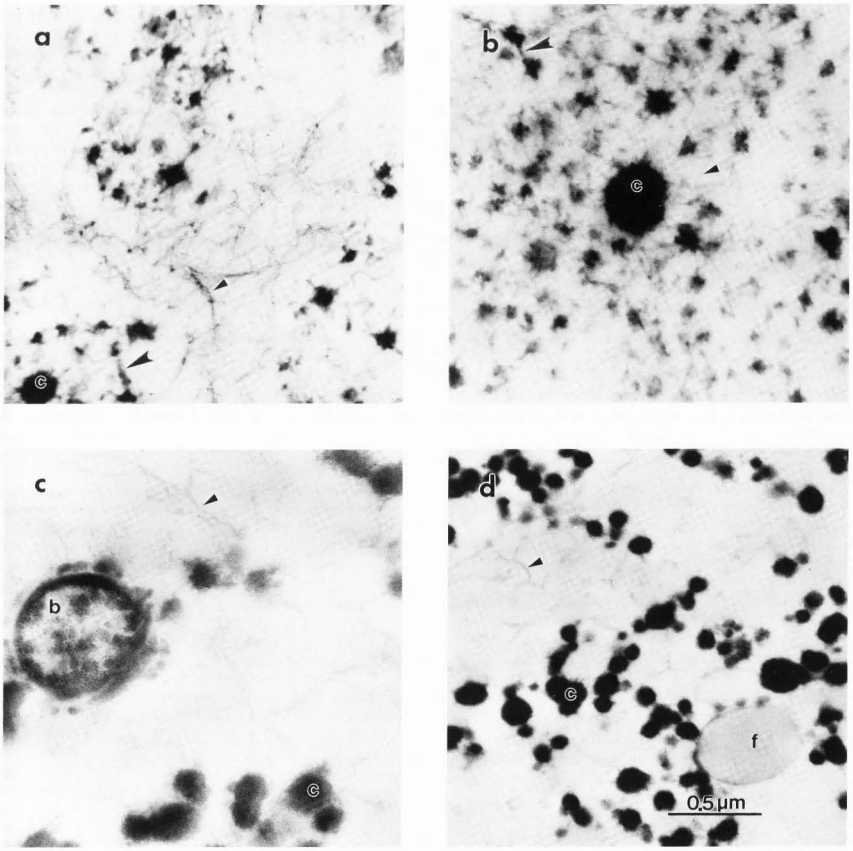

Figure 8. Transmission electron micrographs of **(a)** UHT milk concentrate fixed at 4°C; **(b)** UHT milk concentrate fixed at 45°C; **(c)** yogurt fixed at 4°C; and **(d)** yogurt fixed at 45°C, all prepared by mixing with agar sol. b: bacteria; c: casein micelles; f: fat globules; large arrows: tendrillar appendages; small arrowheads: agar strands.

serum with aqueous glutaraldehyde during fixation and, in particular, dehydration in organic solvents destabilized the casein micelle suspension and resulted in sedimentation and adherence of the casein micelles to the well inner walls. This phenomenon was not evident with the microcube method since it apparently produced sedimentation only onto the 'bottom' walls of the cubes resulting in intact samples. Throughout the fixation and dehydration processes, the 'microcubes' were kept in a fixed position as the liquids were removed and added using transfer pipettes. This method was also used with the capsules but their shapes resulted in shifts of position which could represent sedimentation of sample around the insides of the capsules.

Of the 50 blocks per method examined, the proportions that displayed this defect are shown in Table 2.

Table 1. Percentage of intact capsules produced from three methods of encapsulating milk samples.

	Salyaev Method	Kalab Method	Microcube Method
Intact Capsules	28	33	50
Leaky Capsules	22	17	0
Percentage Intact	56%	66%	100%

Table 2. Percentage of good blocks produced from three methods of encapsulating milk samples.

	Salyaev Method	Kalab Method	Microcube Method
Good Blocks	37	38	50
Defective Blocks	13	12	0
Percentage Good	74%	76%	100%

From χ^2 analysis, the proportion of defective blocks was significantly lower ($P = 0.05$) for the microcube method compared to the other methods singly and collectively. There was no significant difference between the Salyaev and Kalab methods. Micrographs produced from the three techniques were quite similar (Figures 4 to 6). Distortion of fat globules shape observed in Figure 5 is due to melting of fat globules when the microcapsules and microcube were sealed using hot agar. In addition to being simpler than the techniques of Salyaev and Kalab, the microcube method proved more reliable. It does not require a high degree of manual dexterity, produces reproducible results, and enables encapsulation of a wider viscosity range of fluid dairy foods.

Microcube encapsulation compared with agar sol mixing (Experiment b)

The agar sol method was simplest of all the techniques examined. Problems associated with Salyaev and Kalab encapsulation techniques include difficulties in aspiration (especially with viscous materials), sealing the capsules, and handling bulky capsules resulting from strengthening them with additional agar gel. These problems can adversely affect the quality of micrographs. The agar sol mix method avoids these problems but it has other drawbacks such as dilution of the samples and presence of visible agar strands.

Both methods under study showed casein micelles in aged ultra-high temperature (UHT) treated milk samples (Figures 7a, 7b, 8a and 8b) to be connected by strands (tendrillar appendages), giving them a characteristic star shape while casein micelles in yogurt were seen as discrete round structures (Figures 7c, 7d, 8c and 8d). Agar strands were noticeable in the micrographs of agar sol mix samples (Figure 8) and in the case of the UHT milk samples (Figures 8a and 8b) it was difficult to distinguish the tendrillar appendages (connecting micelles) from these agar strands. These strands were present even when purified agarose (electrophoretic grade) was used to solidify milk. No artifacts caused by polymerization of glutaraldehyde through heating to 45°C were observed.

No artifacts of electron-dense granules as observed by Harwalkar and Kalab (1986, 1988) and McMahon *et al.* (1991) were found in any of the micrographs of Experiments (a) or (b). The granules have been shown to be a complex of osmium tetroxide and glutaraldehyde (Parnell-Clunies *et al.*, 1986). The use of low concentrations of the fixatives (1.5% glutaraldehyde and 1% osmium tetroxide) are believed to be partially responsible for this result.

Conclusion

The microcube encapsulation method is a simple, versatile, reliable, and reproducible method for prefixation preparation of fluid dairy products. It is more reliable than the agar gel tube methods of Salyaev and Kalab in sealing the encapsulated fluid and does not require the production of individual agar gel tubes into which the sample is aspirated. The microcube method will prove beneficial to researchers in structural studies of fluid foods because it allows a greater number of samples to be prepared in a simpler way and ensures a considerably lower incidence of sample leakage.

There was no apparent effect of fixing with glutaraldehyde at 45°C rather than at 4°C. Fixation at 20° to 25°C is therefore adequate unless the state of the material at another temperature is under investigation.

Acknowledgments

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Discussion with Reviewers

B.L. Armbruster: The agar well or enclosure is essentially acting as a solid dialysis chamber for the exchange of fixatives, solvents and embedding media. Have you tried to process fluid dairy samples in dialysis tubing and compared those results with those derived from the 'microcube' technique?
Authors: No, we did not try the process using dialysis tubing.

B.L. Armbruster: Agar fibers are most noticeable in an embeddment when the agar is not completely dissolved or the agar is not pure. What grade of agar did you use and how long was it heated at 45°C before use?
Authors: The agar used was Bacto-Agar obtained from Difco Laboratories (Detroit, Michigan, USA). This is purified agar prepared especially for use in microbiological culture media, by a process in which extraneous matter, pigmented portions and salts are reduced to a minimum. It is in fine granular form so that it dissolves rapidly giving clear solutions. The agar powder and distilled water were thoroughly mixed and this mixture heated (2-4 minutes) until it boiled and the agar powder was completely dissolved. The sol was then cooled to 45°C and used immediately.

H.D. Goff: Is the reason why the microcube did not leak because they were left standing upright as per your discussion (concerning the adherence of sample to the inside of the microcapsules)?
Authors: For statistical analysis, microcubes and microcapsules previously determined as not leaking were retested for leaks after aldehyde fixation. The encapsulated specimens were not subjected to any change of position during this fixation process so the explanation given for lack of sedimentation in the microcubes is not valid here. That explanation states that the microcubes remained in a 'fixed position' but it was not established

that they were 'standing upright' as you suggested.

H.D. Goff: In order to preserve the fat globules and particularly the fat crystals within them when fixing ice cream mix for TEM, we found it necessary to maintain refrigerated temperatures in the samples during the fixation. Otherwise, the fat globules appear melted and distorted. I see the same problem in your Figure 5. Thus, we utilized a low gelling temperature agarose (Goff *et al.*, 1987; Liboff *et al.*, 1988). Would your procedure be suitable for sample encapsulation of cold samples or would the pouring of the top layer of agar be enough to cause the sample temperature to rise and thus melt some of the fat?
Authors: The top layer of agar in this procedure cools rapidly after being poured but some melting of fat does occur as is evident in Figure 5. The microcube technique can, however, be used with low gelling temperature agarose and (with slight modification) ultralow gelling temperature agarose. With these agaroses, the procedure would be suitable for sample encapsulation of cold samples.

M. Rosenberg: The exposure of the sample to agar of a relatively high temperature seems to be extensive in the described method. Were the potential effects of this aspect evaluated by the authors?
Authors: No, the potential effects of this aspect were not evaluated in this study.

M. Rosenberg: Why did you not use imidazole buffered osmium tetroxide to fix the fat? It has been demonstrated in many publications that this reagent is more effective than the imidazole free buffered osmium tetroxide.
Authors: We were primarily interested in comparing methods of prefixation preparation of milk samples and were not particularly concerned with the stabilization of fat present. Although imidazole-buffered osmium tetroxide has been shown to be more effective as a fixative of fat than the imidazole free variety, we doubt that an improved structure of fat globules would have been observed in our study since the melted and distorted appearance of the fat globules is attributed to heating administered during the prefixation preparation.