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ARTEFACTS IN CONVENTIONAL SCANNING ELECTRON MICROSCOPY OF SOME MILK PRODUCTS

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Abstract

Artefacts develop due to changes in the microstructure of the sample under study because of inappropriate preparatory steps and/or due to distortion of the images of properly prepared samples during operation of the microscope. Of a wide variety of possible artefacts, only those occurring most frequently have been selected and illustrated with micrographs. In milk powders, the most common artefacts are the recrystallization of lactose in a humid atmosphere, "line" and "dark-area" charging artefacts, and electron beam damage. In moist milk products, artefacts may arise at any preparatory step, such as sampling, fixation, dehydration and critical-point drying or freezing and freeze-drying, mounting, metal coating, and microscopical examination. Images of the same subjects with artefacts either present or reduced to a minimum are compared and measures to avoid the development of the artefacts are suggested.

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Introduction

Scanning electron microscopy (SEM) in the conventional and cold-stage modes is well suited for studies of the microstructure of milk products. In this study, "conventional" SEM is characterized by the examination of metal-coated dried samples at ambient temperature using secondary electrons at an accelerating voltage between 5 and 30 kV.

Aggregation of casein micelles during the gelation of milk (16), protein matrices in yoghurt (29, 30) and curd (13), and the development of the microstructure in cheese (22, 33, 37, 41) have all been studied by SEM. However, with the exception of milk powders (8, 24, 25), SEM has not been employed with milk products to the extent which would fully exploit this technique. The reasons for this may be due to the very fine microstructure of some milk products and relatively difficult sample preparation required to obtain micrographs free from artefacts.

The corpuscular microstructure of milk is based on casein micelles, which are protein globules approximately 100 nm in diameter (32). Fat is also present in the form of globules, the dimensions of which depend on whether the milk has been homogenized or not (17). In the production of most milk products, the basic step is the coagulation of milk characterized by the aggregation of casein micelles into chains and clusters. Because of the small dimensions of the casein micelles, medium high magnifications are used to study them and their aggregates. The low content of structural solids (~6%) in gelled milk and the resulting high porosity of the protein matrix make the samples fragile and increase the probability of the occurrence of artefacts.

Techniques for the preparation of milk products were reviewed earlier (24, 26). In most published papers care is taken to minimize artefacts. For this reason it is not possible to present a collection of artefacts from the literature, although occasionally some have been published unintentionally. Artefacts in SEM are defined as products of sample preparation and imaging procedures which alter the subject under study contrary to the interest of the microscopist. This means, for example, that although the extraction of fat from a cheese sample alters the initial microstructure, it is not considered to be an

artefact if the intent of the microscopist is to study the cheese protein matrix free of fat. If, however, some fat is left in the sample due to incomplete extraction, which is not the intention of the microscopist, the image obtained is artefactual. Artefacts arise either from improper sample preparation or from improper operation of the microscope. There are many papers describing the appropriate execution of sample preparation as well as microscope operation techniques (4, 38-40) and the food scientist intending to study the microstructure of milk products should be familiar with such techniques.

The objective of this paper is to describe specific artefacts most commonly encountered in conventional SEM of milk products, explain their origin and nature, and suggest corrective measures in order to avoid them.

Materials and Methods

Milk products (spray-dried skim milk and buttermilk, Cottage cheese, Cheddar cheese, and yoghurt containing pregelatinized starch) of commercial origin as well as products made in the laboratory (yoghurt containing 10% nonfat milk solids and the same yoghurt containing 20% sugar or 0.2% pregelatinized Col-Flo 67 modified starch) (29) were used in this study.

Preparation of the samples for electron microscopy was varied to produce specific artefacts. Cambridge Stereoscan Mark II and AMR 1000 scanning electron microscopes operated at 20 kV unless mentioned otherwise were used to obtain micrographs, which were taken on 35-mm film.

Results and Discussion

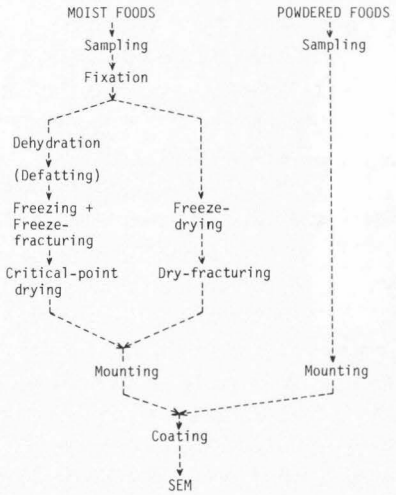
The development of artefacts was studied separately from two different points of origin, namely as produced by sample preparation and also as produced during the operation of the scanning electron microscope. Some artefacts, such as "charging", may develop as a result of both factors.

The artefacts are described in the order as they may develop during the preparation of the sample for SEM (see the simplified flow chart of sample preparation).

Sampling

In general, samples are taken from beneath the surface, where the food product is not affected by external forces such as pressure of packaging or evaporation from an exposed surface unless the interest of the microscopist is focussed on the surface layer, for example, on Cottage cheese granules or the surface of mold-ripened cheese. Even when a sharp blade is used when sampling, sample components are smeared on the cut surface and obscure the true microstructure of the sample under study (Fig. 1A); true microstructure is revealed by fracturing at a later stage of sample preparation to expose the undamaged interior (Fig. 1B).

Powdered milk products destined for SEM are maintained in a dry atmosphere to avoid changes in their appearance and microstructure due to humidity. In lactose-containing spray-dried milk powders, exposure to air having a relative humidity of 40% or more (43) leads to the hydration of amorphous lactose anhydride, which is uniformly distributed throughout the powder particles, and crystallization of the α-hydrate on the particle surface (Fig. 2); this results in the agglomeration of the spray-dried particles which otherwise occur as separate entities.

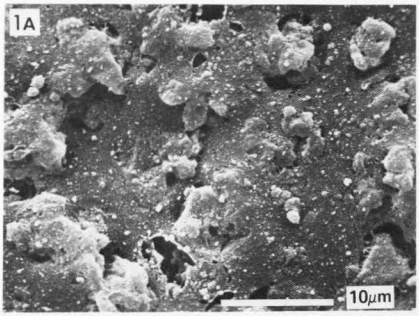


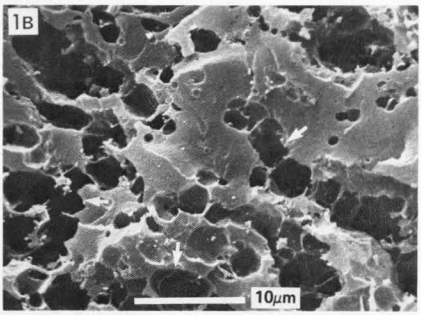
A simplified flow chart of sample preparation (27).

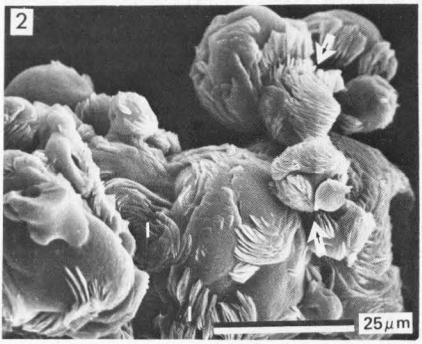
Fixation

Samples of milk products are usually fixed in a glutaraldehyde solution to stabilize the protein matrix and to facilitate the removal of soluble constituents such as lactose and whey proteins. Fixation of samples with glutaraldehyde, however, does not fix the fat component. Fat in the form of globules present in low-fat milk products which have a porous matrix such as yoghurt or Cottage cheese may be stabilized by postfixation with osmium tetroxide, particularly in the presence of imidazole (Fig. 3). However, in experiments aimed at preventing the fat from crystallization (1), fixation in imidazole-buffered OsO_4 at an elevated temperature of 40°C resulted in ruptured fat globule membranes and leakage of the fat (Fig. 4).

If the fat in the milk product is not protected by fat globule membranes, for example, in process cheese, conventional postfixation with osmium tetroxide stabilizes only the part of it, which contains unsaturated fatty acids. The rest of the fat is extracted from the sample during dehydration in ethanol and during critical-point drying from Freon or carbon dioxide. Remnants of







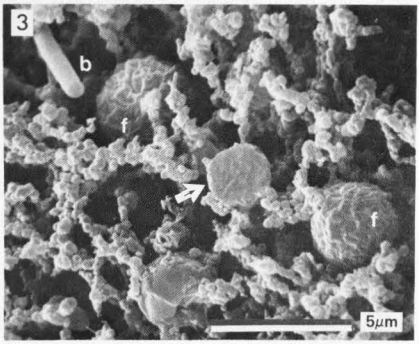
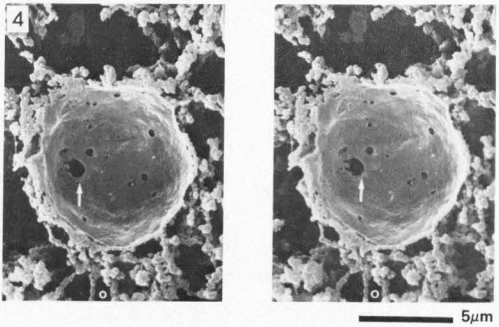


Fig. 1. Microstructure of Cheddar cheese. (A) The surface of the cheese particle was smeared with protein during cutting with a blade thus obscuring the microstructure of the sample. (Sample fixed with glutaraldehyde, washed, freeze-dried, and defatted in chloroform). (B) Fracturing reveals the internal microstructure of the sample. (Fixed sample dehydrated in ethanol, defatted in chloroform, freeze-fractured, and critical-point dried. Extraction of fat reveals cavities (arrows) in the protein matrix).

Fig. 2. Particles of spray-dried skim milk following exposure to a humid atmosphere. Exposure for 1 h resulted in the crystallization of lactose α-hydrate on particle surfaces and agglomeration of the particles. Insufficient electric contact between the agglomerate and the metal support resulted in a charging artefact in the form of a vertical line (arrows).

Fig. 3. Fat globules in whole-milk yoghurt preserved by postfixation with imidazole-buffered osmium tetroxide. Fat was revealed by freeze-fracturing (arrow). Other fat globules (f) are seen unfractured below the fracture plane; b = lactobacillus.

Fig. 4. Fat globule membrane ruptured during postfixation of whole-milk yoghurt at 40°C (stereo pair, 12° separation). Fat leaked out through openings (arrows) developed in the membrane at the elevated temperature. Two light circles in the micrographs are provided to facilitate focussing of the eyes.



stabilized fat are the source of an artefact characterized by the presence of fat residues in cavities, which had initially been filled with the fat before extraction (Fig. 5A). Unless the fat is destined for complete preservation (1), for example, by trypsin etching of the protein matrix (15, 48), it is better not to use OsO_4 and to extract the fat with chloroform or n-hexane (26, 27). However, even in a cheese sample which had not been postfixed with osmium tetroxide, incomplete extraction may leave residual fat in the form of droplets (Fig. 5B). After all the fat is removed, its initial distribution and the dimensions of the fat particles are determined indirectly from the distribution and the dimensions of the resulting void spaces (cavities) (Fig. 1B). Freeze-drying preserves fat in fixed and postfixed cheese samples, but this fat causes problems during mounting and metal-coating, melts easily when exposed to the electron beam, and obscures the protein matrix (Fig. 6) unless the sample is handled and examined at a low temperature (44, 45).

The removal of water-soluble constituents from the sample due to exposure to aqueous preparatory solutions means that even subjects of interest to SEM may be lost, for example, crystals of melting salts in process cheese (26, 41). Structures resembling the original crystals are, in fact, their imprints in the protein matrix after the crystals are dissolved and extracted (Fig. 7).

Freezing

Freezing is the prerequisite in the preparation of samples destined to for freeze-fracturing. Depending on which preparatory procedure follows, the sample is frozen with the aqueous phase present to be freeze-dried, or the aqueous phase in the sample is replaced with a cryoprotective medium such as 30% glycerol or with absolute ethanol and the sample is freeze-fractured, melted, and critical-point dried.

The most frequent artefact seen in frozen samples is the distortion of the protein matrix due to ice crystal development (Fig. 8). This artefact is caused by slow freezing and develops when the sample is too large or when a small sample is surrounded by excess water, when an inappropriate freezing agent (such as liquid nitrogen, dry ice with acetone etc.) is used, or when the speed, at which the sample is plunged into the appropriate coolant such as nitrogen slush (49) or freon cooled to its freezing point with liquid nitrogen (26, 27), is too slow.

Freeze-drying

Freeze-drying is one of two drying techniques most frequently used, the other being critical-point drying. In conventional SEM, the specimen is examined at ambient temperature. It must be dry in order to minimize generation of volatile vapours in vacuo inside the electron microscope. Problems associated with drying techniques were reviewed by Boyde (6).

The advantage of freeze-drying is that it preserves most food components including fat. In fixed samples not washed thoroughly with distilled water, the residual dissolved substances such as buffer salts or lactose may appear in the form of a fine efflorescence on the surface of the resulting freeze-dried sample; this efflorescence is usually noticeable under a dissecting microscope during the mounting of the samples and warrants disposal of the sample in question. Although freeze-drying distorts the gelatinized starch and alginates which are sometimes used as thickening agents in yoghurt (29), the distortion is less extensive than that caused by critical-point drying (9); this is evidently associated with the dehydration of unfixed polysaccharide gels in organic solvents.

An improperly functioning freeze-drier is a potential source of serious artefacts. Recrystallization of the frozen aqueous phase in the sample during freeze-drying is a serious risk to which the sample is exposed, particularly if the temperature of the sample rises above -80°C before all the ice has sublimed off; such artefacts are similar to those caused by slow freezing of the specimen. Contamination of the sample with an oil mist from the vacuum pump is another potential artefact; in this laboratory samples are kept from being contaminated in this way by installation of an activated alumina trap between the pump and the specimen chamber; the trap is freshly charged before each run.

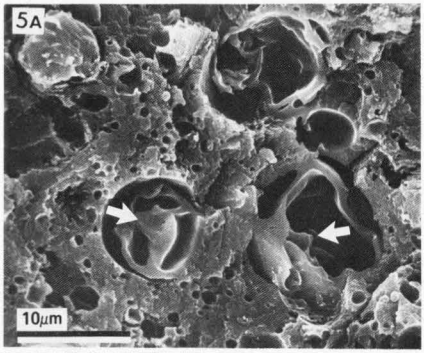
Freeze-fracturing

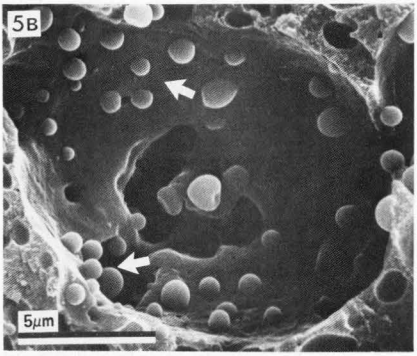
Freeze-fracturing exposes the internal structure of the specimen. A specimen may be freeze-fractured while impregnated with a cryoprotective agent such as 30% glycerol or after it is dehydrated in absolute ethanol. Both media decrease or even eliminate the risk of ice crystal development. There are, however, differences in the appearance of the fractured specimens: with 30% glycerol, the fracture planes run through lactic acid bacteria, fat globules, or casein micelles whereas with absolute alcohol, the fracture planes run between bacteria, fat globules, and casein micelles (26). This indicates that the sample reacts differently under different conditions and that this behaviour should be considered when the micrographs obtained are evaluated.

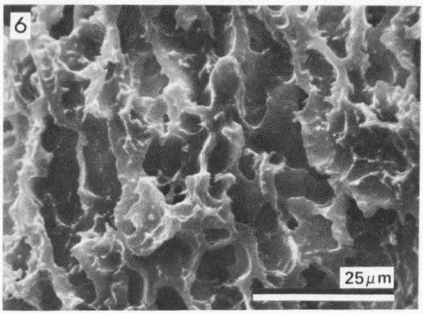
Dehydration

In general, the term "dehydration" means any removal of water from the specimen, but here it is used more specifically to mean substitution of organic solvents for water. The objective of dehydrating the fixed specimen is to prepare it for critical-point drying or for freeze-drying from alcohol. The aqueous phase in the food specimen is first replaced with an organic solvent miscible with water, for example, ethanol or acetone. Being less efficient in extracting fat than acetone, ethanol in the form of a graded series of concentrations is more frequently used to dehydrate fixed fat-containing milk products. The final step consists of impregnating the sample with 100% ethanol (absolute alcohol). Incomplete removal of water may be a source of artefacts, although dehydration shortened to only 10 min of total exposure to the dehydrating agent failed to demonstrate any apparent artefacts.

Chemical dehydration in acidified 2,2-dimethoxypropane is a rapid, single-step technique, in







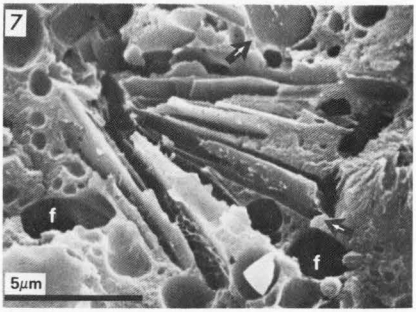
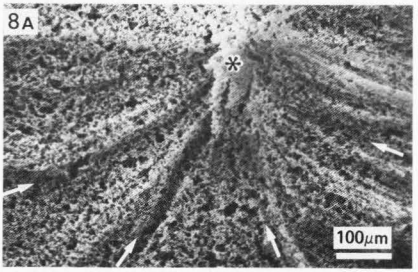


Fig. 5. Residual fat in process cheese. (A) Postfixation of the sample with osmium tetroxide prevented a part of the fat (arrows) from extraction with chloroform. (B) In a cheese sample which had not been postfixed with OsO_4, incomplete extraction of the fat with chloroform has left residual fat in the form of droplets (arrows).

Fig. 6. Cheese protein matrix obscured with fat. A fixed (glutaraldehyde) cheese sample was freeze-dried and dry-fractured; fat was retained in the sample.

Fig. 7. Crystals of melting salt in process cheese. Water-soluble crystals of sodium citrate were removed during fixation in an aqueous glutaraldehyde solution; their imprints were left in the protein matrix (arrows). Globular void spaces (f) indicate the presence of fat before extraction.



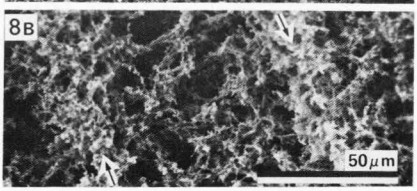


Fig. 8. Yoghurt sample frozen in liquid nitrogen. (A) Ice crystal (arrows) development towards the centre (asterisk) of a fixed and washed yoghurt sample during slow freezing distorted the casein micelle matrix. (B) Detail of the damage to the yoghurt matrix caused by the ice crystal development. Protein was compacted in the form of ridges (arrows).

which water is quantitatively consumed to hydrolyze the reagent into methanol and acetone; the hydrolysis is catalyzed by hydrogen ions (35). A comparison was made between the results obtained with the dehydration of cells in culture using ethanol and 2,2-dimethoxypropane (21) but no systematic studies have been carried out with milk products; no differences were noticed when the two dehydrating agents were used with yoghurt in this study.

Defatting

It is preferable to remove fat from the sample unless it can be adequately retained for SEM (1, 15, 48). Dehydration and defatting may be combined in one operation by using acidified 2,2-dimethoxypropane. Because the combined resulting molarities of methanol and acetone in the dehydrating medium correspond to the total amount of water in the sample at the completion of dehydration, 2,2-dimethoxypropane which exists in great excess is practically the only lipophilic solvent affecting the sample.

Another defatting procedure consists of transferring the sample, dehydrated in absolute alcohol or acetone, into chloroform. Following several changes of the latter solvent, the extracted sample is returned to absolute alcohol for subsequent critical-point drying.

Micrographs of cheese samples chemically dehydrated and defatted in 2,2-dimethoxypropane and samples of the same cheese conventionally dehydrated in a graded alcohol series and defatted in chloroform are virtually indistinguishable from each other. It was already mentioned in the section dealing with fixation that it is preferable to omit postfixation with osmium tetroxide if the sample is destined for defatting.

Critical-point drying

Principles and practical use of critical-point drying were described elsewhere (2, 10, 26, 27). In general, the sample impregnated with an organic solvent such as ethanol, acetone, or amyl acetate is placed in a pressurized cell where the organic solvent is replaced at a low temperature with a transitional fluid (Freon or carbon dioxide). Heating of the cell contents above the critical temperature (31.3°C for CO_2) converts the transitional fluid into gas; after the pressure is reduced, the sample is dry without having passed through any phase boundary.

In spite of this advantage, critical-point drying is not free from producing artefacts (6). This technique was reported to cause shrinkage of some fine biological structures such as animal tissues (5), but no measurements have yet been made on this effect with regard to milk products. The effects of critical-point drying on milk products which contain gelatinized starch and alginates as thickening agents have been reported (29). These thickeners are not fixed by glutaraldehyde and undergo structural changes (shrinkage and distortion) in the organic solvents used to dehydrate the specimen for subsequent critical-point drying.

A set of micrographs (Figs. 9-12) demonstrates differences in the images of a yoghurt consisting of 10% milk solids and 2% pregelatinized Col-Flo 67 modified starch. The starch was not of "instant" quality and the granules were not completely gelatinized during the heat treatment

Legends to figures on facing page:

Fig. 9. Freeze-drying of an unfixed yoghurt (10% milk solids) containing 2% pregelatinized starch. The microstructure of starch (large arrow) was severely distorted whereas the protein matrix was affected to a lesser extent (small arrow).

Fig. 10. Yoghurt containing 2% pregelatinized starch, fixed with glutaraldehyde, dehydrated in ethanol, freeze-fractured, and critical-point dried.
Arrows point to starch particles; b = lactic acid bacteria.

Fig. 11. Yoghurt containing 2% pregelatinized starch, fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated in ethanol, freeze-fractured, and critical-point dried.
Arrows point to starch particles.

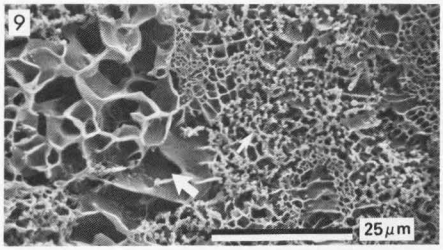
Fig. 12. Yoghurt containing 2% pregelatinized starch, fixed with glutaraldehyde, freeze-fractured, and freeze-dried.
(A) Starch particles (S) appear to be in a good contact with the protein matrix (p) but suffer from the characteristic ice crystal damage; b = lactic acid bacteria.
(B) Detail of contact between pregelatinized starch (s), protein matrix (p), and lactobacilli (l) is shown at a higher magnification.

Fig. 13. Commercial yoghurt containing pregelatinized starch treated in the same way as the laboratory-made yoghurt shown in Fig. 10.
Arrows point to starch granules. Protein matrix (p) is considerably more compact than in the laboratory-made yoghurt (Figs. 10-12) which contained only 10% total milk solids; b = lactic acid bacteria.

Fig. 14. Yoghurt containing 10% total milk solids and 10% sucrose.
Crystals (arrows) of unknown composition developed in fixed yoghurt samples dehydrated in ethanol and critical-point dried; c = lactic acid bacteria (streptococci).

Fig. 15. Yoghurt (10% total milk solids) dried from Freon 113 in a desiccator at a reduced pressure.
The sample was fixed, dehydrated, freeze-fractured, and melted in ethanol; Freon 113 was substituted for ethanol and the sample was dried in a desiccator at a reduced pressure. Local compaction of the casein micelle matrix is apparent in comparison with another sample of the same yoghurt which had been critical-point dried (Fig. 16).

Fig. 16. Critical-point dried yoghurt sample (10% total milk solids).
The image is assumed to be free of artefacts; l = lactic acid bacteria (lactobacilli).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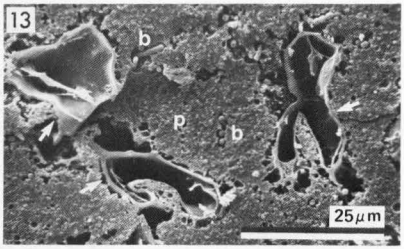
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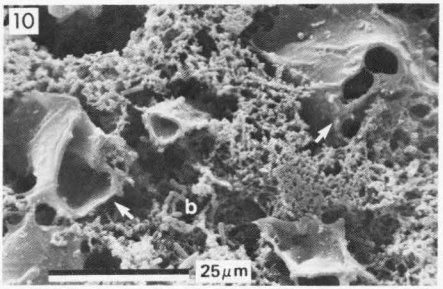
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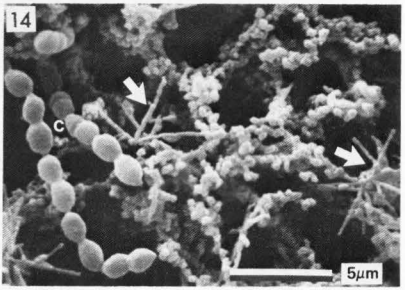
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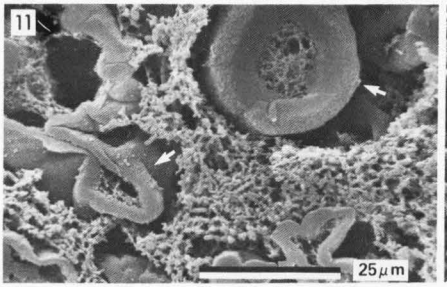
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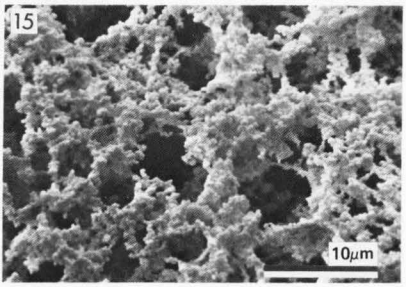
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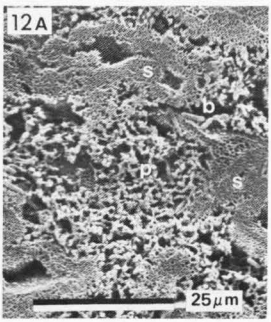
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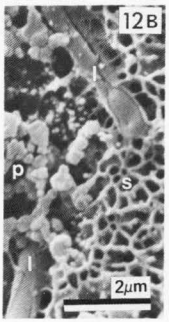
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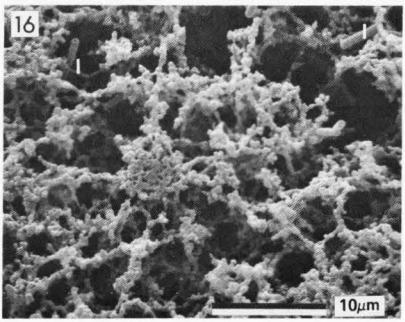
2μm


16
l
l
10μm

of the milk used. However, the appearance of the starch was the same in this laboratory-made yoghurt and commercial yoghurt when the same SEM techniques were used (Fig. 13). Fig. 9 shows the former yoghurt frozen and freeze-dried without any preliminary preparatory steps. Artefacts caused by the development of ice crystals and by the presence of soluble constituents render the micrographs unsuitable for any study. Fig. 10 was obtained with a sample fixed in glutaraldehyde, dehydrated in alcohol, freeze-fractured, and critical-point dried. The casein micelle network is interspersed with irregularly shaped starch particles; there are large void spaces between starch and casein indicating that the micrograph does not reflect the original microstructure. In yoghurt which was impregnated with 30% glycerol following fixation in glutaraldehyde, freeze-fractured, melted in 30% glycerol, dehydrated in alcohol, and critical-point dried, the starch particles were severely shrunken and diffused into the protein matrix at the same time. Postfixation with OsO_4 followed by dehydration in alcohol, freeze-fracturing, and critical-point drying resulted in a lesser distortion (shrinkage) of the starch granules as judged from smaller void spaces between starch and casein (Fig. 11). The tightest microstructure of this yoghurt was found when it was fixed with glutaraldehyde, washed with water, and freeze-dried (Fig. 12A); the starch itself, however, appeared porous, apparently because of the development of fine ice crystals inside the gelatinized particles during freezing and their subsequent sublimation during freeze-drying. Otherwise, the initial dimensions of the starch particles seem to be unchanged and their contact with casein micelles and lactic acid bacteria preserved (Fig. 12B). Freeze-fracturing of the original unfixed yoghurt followed by replication with platinum and carbon and examination of the replicas in a transmission electron microscope is needed to identify the SEM preparatory technique which produces the minimum of artefacts with this milk product. Fig. 13 shows a commercial yoghurt with the declared presence of pregelatinized starch, which was fixed in glutaraldehyde, dehydrated in alcohol, freeze-fractured, and critical-point dried; thus, it corresponds to Fig. 10 except that the casein matrix in the commercial yoghurt appears to be considerably denser than in the yoghurt made in the laboratory.

Yoghurt made with 10 and 20% sucrose, fixed in a 3.5% glutaraldehyde solution, dehydrated in ethanol, and critical-point dried from carbon dioxide appeared to contain crystal-like aggregates in the protein matrix (Fig. 14), the formation of which was reproducible. Crystals were also found by Katsaras and Stenzel (31) in beef muscles dehydrated in ethanol and critical-point dried; the crystals were not present either in yoghurt or in beef muscles when ethanol-dehydrated samples were dried in vacuo or when acetone, isopropanol, or 2,2-dimethoxypropane were used to dehydrate the samples. The chemical nature of the crystals is not known.

Drying in air

Drying of fixed biological samples in air without preliminary dehydration is not recommended. Acceptable but not good results have been obtained, however, by air-drying samples impregnated with organic solvents such as ethanol or acetone.

An alternative method to freeze- and critical-point drying, designed to retain fat, consists of postfixing the sample in an osmium tetroxide solution, dehydrating in ethanol, substituting Freon 113 for ethanol, and drying the sample at a reduced pressure (3, 34) in a desiccator. In meat emulsions dried by this technique (3), preserved fat globules were found to be displaced from their original positions in the protein matrix, which should be considered to be an artefact.

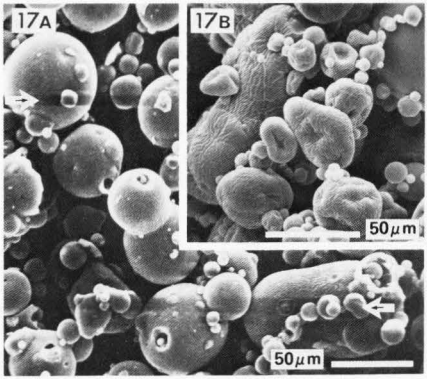
A similar procedure was tested with nonfat yoghurt; it consisted of fixing the specimen, dehydrating it in ethanol, freeze-fracturing, thawing in ethanol, substitution with Freon, and drying in a desiccator. The casein micelle matrix appears to be more compacted (Fig. 15) than the matrix of freeze-dried or critical-point dried specimens (Fig. 16). This technique cannot, therefore, be recommended for yoghurt; its usefulness in drying more compact milk products such as cheeses has not been explored.

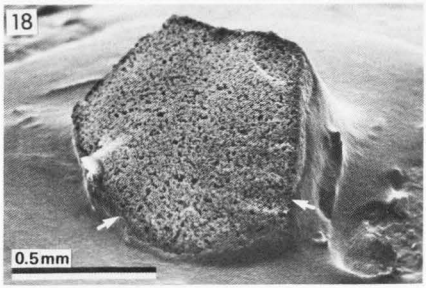
Mounting

Mounting on a metal stub prepares the sample for coating with a thin metal layer and for subsequent insertion of the sample into the electron microscope.

Preparatory methods for SEM of various powders were reviewed by Johari and DeNee (20) and by DeNee (11). They are also useful in preparing food samples. Mounting of powders and solid particles for SEM was recently reviewed in great detail by Murphy (36) who has described many very useful techniques. Their use in food microscopy is highly recommended. Powdered foods have routinely been mounted on stubs covered with a double-sided sticky tape (27, 28), the edges of which had been painted with a silver cement to improve conductivity between the tape and the stub. To minimize the development of charging artefacts, particularly if gold coating will be done by vacuum evaporation, the powder particles should be in a single layer. If too many particles are present, some of them lack electric contact with the stub and this leads to an imaging disturbance called "charging" (Fig. 17A) (46). Areas, which appear considerably lighter on the screen than other properly mounted structures at low magnifications, usually cause severe imaging problems when examined at a higher magnification. When studying particle size distribution, it is better to apply only a small amount of the milk powder to the stub and retain all the particles than to start with too much powder and to remove excess particles by shaking them off or blowing them away in accordance with an earlier suggestion (26, 27). Such thinning may preferentially remove the larger particles. It is relatively easy to obtain good images of thinly mounted powder particles. However, the charging artefact is less likely to develop in densely packed particles provided that sputter coating rather than vacuum evaporation is used (Fig. 17B).

Solid samples are mounted directly on SEM stubs. Low-mass stubs, for example those made of aluminum or carbon are best because they produce minimal interference with the sample during SEM





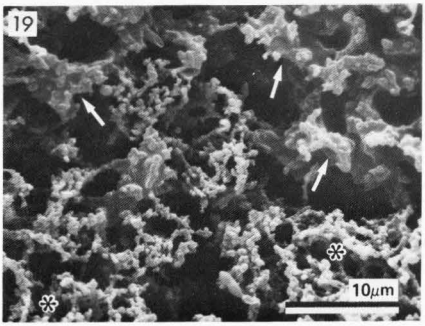
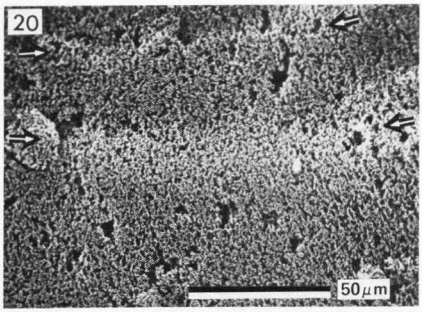


Fig. 17. Scanning electron microscopy of spray-dried skim milk particles.
A = Charging artefact in the form of streaks (arrows) developed due to insufficient electric contact between the milk particles and the metal stub (gold coating by vacuum evaporation). B = Sputter coating of densely packed skim milk particles with gold resulted in artefact-free images.



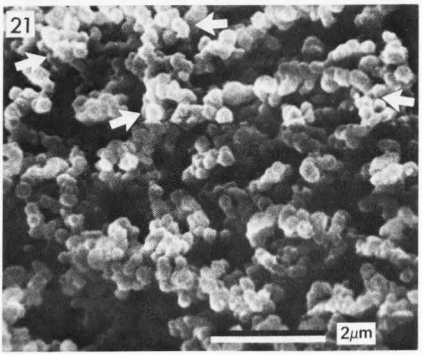


Fig. 18. Example of a yoghurt particle properly mounted for SEM.
The particle walls were painted with silver cement up to the freeze-fracture plane (arrows) to aid conductivity in the porous sample.

Fig. 19. Yoghurt matrix collapsed by the penetration of thin silver cement.
Arrows point to the collapsed matrix, asterisks show an area less affected by the cement. Intact yoghurt matrix is shown in Fig. 16.

Fig. 20. Freeze-fracture plane of a yoghurt sample coated with a portion of gold evaporated at a very low angle.
Light streaks (arrows) indicate areas which were insufficiently coated with gold due to the evaporation angle and would suffer from more severe charging artefacts if examined at a higher magnification.

Fig. 21. Charging artefact caused by insufficient gold coating.
Some areas in the smooth fracture plane of this Cottage cheese sample are excessively light (arrows); this artefact is not as easy to recognize as the streaks in Fig. 17.

(40). Although beryllium was mentioned by Brown and Teetsov (7) as a suitable material for the stubs, it cannot be recommended for general use because of its toxicity.

The appropriate adhesive (cement) selected to mount the sample should have the characteristics listed by Murphy (36). The characteristics most important for the mounting of milk product samples are adequate viscosity to prevent the collapse of porous samples, sufficient tackiness to provide proper bonding of the sample to the stub, resistance to heat generated during metal evaporation or sputtering and by the electron beam without altered surface morphology of the adhesive, a resultant smooth surface upon drying, and ease of application with reasonable drying time and a low vapour pressure after drying in order not to contaminate the vacuum system of the microscope. Suitable cements have been listed by Murphy (36).

Porous samples, such as coagulated milk or yoghurt, require special handling for best results. Dried freeze-fractured fragments are mounted with their fractured planes facing up. The cement is extended up the sides of the specimens as close to the fracture as possible (Fig. 18). Four to six particles can easily be mounted on a stub in this way. Inspection of each particle for proper mounting with the stub tilted and a few finishing touches with the cement will contribute to successful SEM.

Improper mounting may be the source of several artefacts. Failure to provide good electric contact between the sample and the stub leads to charging artefacts (46). The reason may be an insufficient amount of the adhesive used, or its improper consistency (viscosity): a cement too thick does not adhere properly to porous particles whereas a cement too thin penetrates the particle and alters its microstructure (Fig. 19). Instruments used to manipulate the sample leave their imprints especially on porous samples (23). It is also advisable to remove any debris, resulting from fracturing, which may adhere to the surfaces due to static electricity generated during the mounting of the samples; this cleaning may be accomplished with a gentle stream of dry air.

Coating with metal

This procedure renders the specimen electrically conductive. It is accomplished by vacuum evaporation or sputter coating but other techniques are also available (12). In biology, effects of the different coating techniques on specific subjects have been studied and published (18, 19, 42). Triode sputter coating, which employs a water-cooled stage, may be better suited for coating fat-containing specimens than vacuum evaporation, during which the specimen may be excessively heated. A comparison of both techniques has not yet been made with milk products. Micrographs of yoghurt obtained in this study appeared to be similar irrespective of the coating technique used.

Gold or a gold-palladium alloy is used alone with compact samples such as cheese but preliminary coating with carbon reduces the risk of charging artefacts in porous samples such as milk gels. The reason for this behaviour is still a point of discussion. However, if carbon is allowed to sputter even briefly during evaporation because an excessively high electric current is used, minute carbon particles may contaminate the sample surface, and are clearly seen upon observation in the microscope. If carbon sputtering is a problem, it can be prevented by using indirect evaporation in a gas at a very low pressure of approx. 130 mPa (1.3×10^{-3} mbar or 1×10^{-3} torr).

For metal coating by vacuum evaporation in the author's laboratory, the gold is divided into two portions deposited sequentially on the sample at two different angles while the sample is rotated. If the fracture plane of the specimen runs parallel to the stub base, evaporation of gold at a very acute angle ($<20^{0}$) cannot be recommended for particles, which had been thoroughly painted with the mounting cement. Most of the gold would be deposited on the sides of the particles, which is unnecessary, whereas very little of the gold would be deposited on the fracture faces at such low angles, and charging would still result. In fact, this coating may be the source of a shadowing artefact when the fracture is uneven. SEM examination at a low magnification indicated that gold evaporated at a very low angle did not coat depressions in the fracture. A difference in the amount of gold deposited in the shaded and exposed areas is noticeable even after the other portion of gold was evaporated at a greater angle (Fig. 20).

Similar to improper mounting, insufficient coating leads to charging artefacts (Fig. 21); samples such as milk gels, which have a large surface area due to their porosity, require more gold to be evaporated onto the fracture face to achieve proper coating than do compact samples, such as cheese, which have a smaller surface area. However, excessively large amounts of metal obscure surface details and result in overcoating artefacts. The ideal thickness of the metal coating in conventional SEM is approximately 20 nm.

During evaporation in vacuo, metal atoms travel in straight lines. When several samples are coated at the same time using a rotary table, the metal is deposited according to a certain pattern

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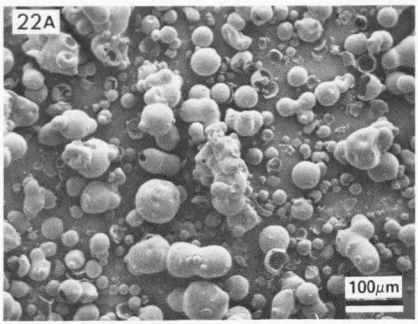
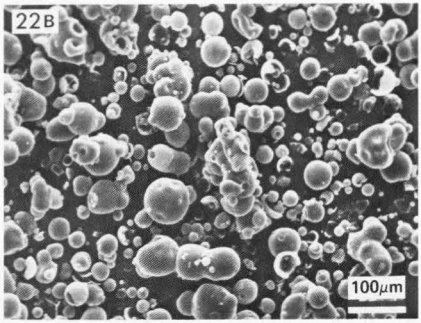
Fig. 22. Spray-dried skim milk powder examined at an accelerating voltage of 5 kV (A) and 30 kV (B) at a low magnification.

Fig. 23. Yoghurt examined at an accelerating voltage of 5 kV (A) and 30 kV (B) at a high magnification.
At the higher magnification used (6,000 X), the low accelerating voltage was insufficient to provide an acceptable image (B).

Fig. 24. Electron beam damage in a spray-dried buttermilk particle.
(A) Initial intact spray-dried buttermilk particle; (B) A raised rectangle (large arrows) developed by focussing the electron beam (20 kV, 180 A) in the TV-mode; line artefact (small arrows) developed by focussing the electron beam using a stationary line scan. (C) The particle was ruptured by the electron beam at 30 kV. The crack (arrow) was caused by a stationary line scan.


22A
100μm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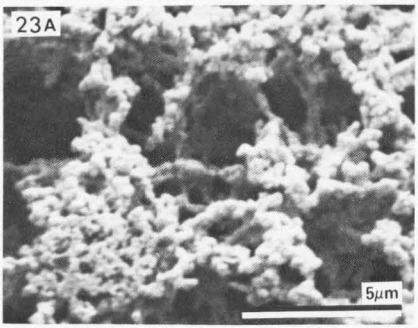
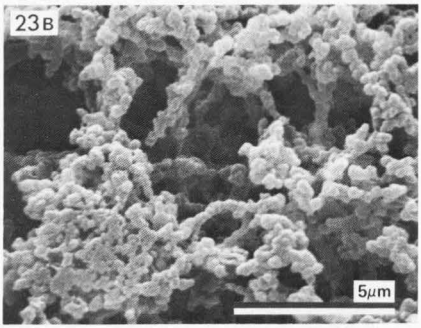
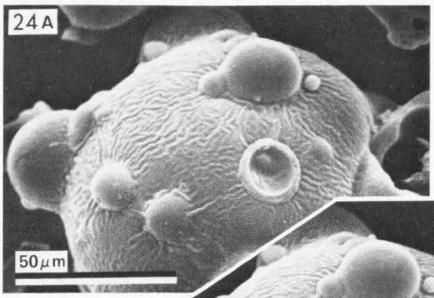
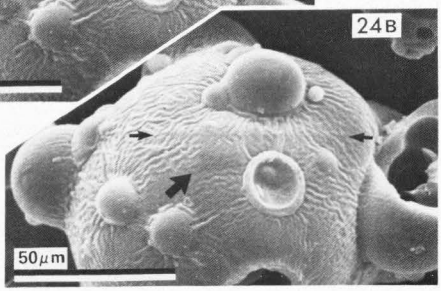
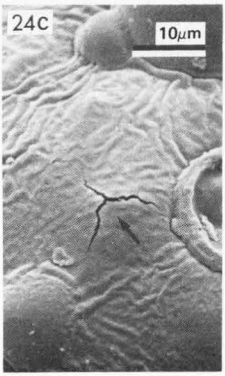
100μm


23A
5μm


23B
5μm


24A
50μm


24B
50μm


24C
10μm

which does not ensure that crevices in porous structures will be coated. One way to improve the uniform coating is to rotate the sample around two axes at the same time. Another way is to coat the sample in a somewhat inferior vacuum of 13 to 133 mPa. At this pressure, there is a higher incidence of collisions between gas molecules present in the evaporation chamber and the vaporized metal atoms leading to the deflection of the latter ones from their straight line path and their deposition on the sample in areas which otherwise would remain uncoated (26).

SEM examination

Operation of scanning electron microscopes has been described in many handbooks, some of which were listed earlier (27). Every scanning electron microscope offers a variety of conditions under which the sample may be examined. Assuming that images of milk products, which are produced, are due to secondary electrons, the electron microscopist may vary the accelerating voltage, working distance, tilt, beam current, aperture, lens currents, and microscope contrast. When taking micrographs, additional conditions must be selected, for example, brightness, camera contrast, line density, and scan rate on the monitor and format and sensitivity of the film used. All these and additional factors were reviewed by Pfefferkorn et al. (40) in a highly informative paper. The authors have provided valuable advice on how to obtain optimum results. Their emphasis is on adjusting the variables depending on the nature of the sample and on the objective of the study.

Accelerating voltage

It is believed, in general, that a higher accelerating voltage gives better resolution. High accelerating voltage (20 kV and higher), however, is not only unnecessary when samples such as milk powder or cheese are examined at low magnifications (<1,000 X), but it may even produce inferior micrographs if the specimen is susceptible to charging. Electrons penetrate deeper into the specimen at a higher accelerating voltage and images of structures located below the sample surface may interfere with images from the surface, producing a somewhat inferior and less informative image. Differences in the images obtained at a low (5 kV) and a high (30 kV) accelerating voltage with two different samples at two different microscope magnifications are shown in Figs. 22 and 23. At the low magnification (120 X), the low accelerating voltage produced good images of spray-dried milk powder particles (Fig. 22 A), whereas at the higher magnification (6,000 X), the image of a yoghurt sample taken at the low accelerating voltage was of unacceptable quality (Fig. 23A).

As the kinetic energy of the electrons is increased with increasing accelerating voltage, the risk of electron beam damage to the sample is increased (4, 47). Milk powders are very susceptible to this kind of damage (8, 25, 28) which is evident particularly when the focussing at a higher magnification (>2000 X) takes too much time, i.e. several seconds (Fig. 24). The electron beam may conveniently be focussed by operating the microscope in the TV-mode using a small selected area on the screen. This kind of focussing also may reveal deficiencies in astigmatism correction. Another way of focussing uses a line scan: the oscilloscope pattern of the beam line is adjusted with the focussing control to produce the sharpest peaks. This way of focussing is rapid but does not provide information about astigmatism. It may cause beam damage artefacts in the form of cracks in the sample (Fig. 24C) or may imprint lines on its surface, which subsequently show up on the micrographs (Figs. 24C and 25). The severity of the electron beam damage can be reduced by lowering the beam current, by focussing as rapidly as possible, by scanning the sample during photography at a shorter frame time or in the TV-mode, if possible, or by reducing the accelerating voltage.

Working distance

Working distance affects both the resolution and the depth of focus. If the design of the microscope allows the working distance to be adjusted by moving the specimen, that distance should be as short as possible for best resolution whereas it should be as long as possible for maximum depth of focus. The dimensions of the sample may limit the usable range of working distance. Because working distance affects magnification, it should be maintained constant if the

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Fig. 25. Imprints of a line scan on an insufficiently cleaned gold replica of a freeze-fractured yoghurt.
Arrows point to vertical and horizontal line scan artefacts; w = cell walls of flattened bacteria.

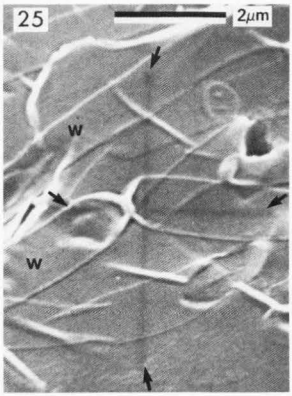
Fig. 26. A tilted yoghurt sample.
The same area as that shown in Fig. 16 has been tilted 30°. Areas moved upwards (U) and downwards (D) by the tilting are out of focus.

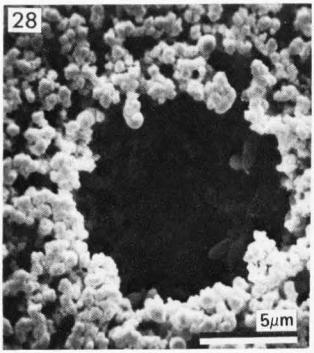
Fig. 27. Edge of fractured Cottage cheese particles.
(A) The angle at which the sample had been tilted to view the edge of the particle causes the edge to appear very light (arrows) and makes its examination difficult. (B) A lower tilt angle makes it possible to examine the edge (arrows) of another particle.

Fig. 28. Edge of a void space in a yoghurt sample.
Casein micelles forming the edge appear lighter than casein micelles farther away from the edge.

Fig. 29. Effect of astigmatism on the image of casein micelle network in a yoghurt sample.
(A) Image corrected for astigmatism. (B) Uncorrected underfocussed image. (C) Uncorrected overfocussed image.

Fig. 30. A very high setting for microscope contrast transforms the image of a yoghurt sample (Fig. 16) into a black-and-white pattern.
White areas: protein matrix of the yoghurt and lactic acid bacteria; black areas: void spaces in the matrix initially containing the aqueous phase.


25
2μm
W
W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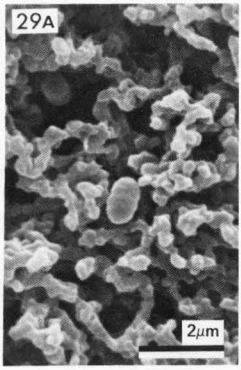
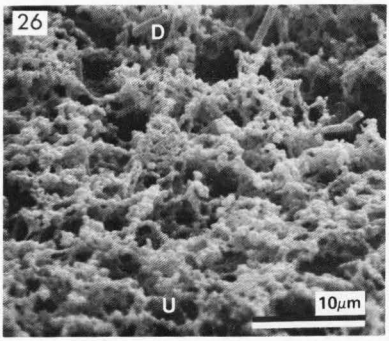
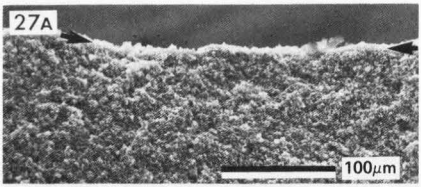
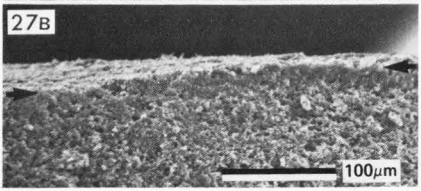

29A
2μm

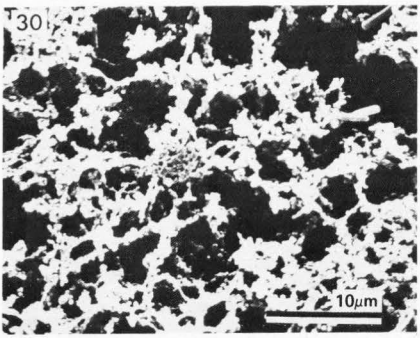

26
D
U
10μm


29B
2μm


29C
2μm


27A
100μm


27B
100μm


30
10μm

resulting micrographs are destined for comparison, unless the same magnification is adjusted by the zoom, and using the rotation unit to scan parallel to the x-axis after changing the working distance of the tilt axis.

Tilt

Tilting the sample makes it possible to change the angle of the incident electron beam to the sample surface. Undistorted images are obtained if the angle is 90^{0} (27). Otherwise, dimensions perpendicular to the axis of tilt are shortened in the micrographs approximately according to the formula:

$$d_i = d_r \times \mathrm{tg}\,\alpha$$

where d_i is the length of the image, d_r is the real length, and α is the angle of tilt.

This formula does not take the effect of changed working distance into consideration: that distance is either shorter or longer for structures located at opposite sides of the axis of tilt. This all makes the interpretation of the micrographs more difficult (compare Fig. 26 with Fig. 16); in addition, tilting moves different parts of the sample in and out of focus.

Samples examined for microstructure in cross fractures immediately below the surface usually need to be tilted. The tilt, which positions the sample at a 90^{0} angle to the incident electron beam, however, produces an artefact characterized by an excessively light edge of the sample (Fig. 27A). This impedes examination of the subjects of the greatest interest, for example, the hypothetical "skin" on Cottage cheese granules (13, 24); a slight decrease in the angle of tilt prevents the "edge phenomenon" (Fig. 27B). Similar excessively light edges are also encountered when cracks or cavities are present in the sample (Fig. 28); characteristic examples are void spaces in yoghurt developed by the action of lactic acid bacteria and cavities in cheese caused by the extraction of fat droplets or crystals of melting salts. This phenomenon can be sometimes caused by defects in electric conductivity and consequent charge build-up.

Astigmatism correction

Magnetic lenses in the electron microscope do not have a perfect symmetry. If the symmetry is elliptical rather than circular, a single point focusses, as the lens current is varied, to two separate line foci instead of to a single point. This means that underfocussed and overfocussed spheres (such as casein micelles below and above the focus plane) appear elongated at right angles to each other (Fig. 29). To correct this defect, the magnetic field of the final lens is brought to the desired symmetry. The objective is to correct the primary beam with a stigmator to have the smallest diameter possible and to have a circular cross section, in order to obtain the best resolution in all directions. Two major controls are usually used: the stigmator magnitude and orientation are adjusted alternately and repeatedly until the sharpest image is obtained. This correction is possible only for the asymmetry in the magnetic field of the final lens but not for effects having another origin such as an incorrect alignment of the filament, dirty aperture etc. (14).

Lens current

Lens current is related to the signal-to-noise ratio. The optimal ratio can be obtained by manipulating the controls and examining the effects of that manipulation. Adjustment can be learned only through experience (40) and differs for each accelerating voltage value.

Microscope contrast

Fractured milk product samples can usually be examined in the SEM without any change in the contrast setting. However, very flat samples, for example, ultrafiltration membranes, may show details better at an increased contrast setting, particularly when examined at 90^{0} to the incident electron beam. An excessively high contrast setting eliminates halftones and converts the micrographs into black-and-white two-dimensional patterns. Such micrographs (compare Figs. 30 and 16) do not properly represent the three-dimensional structure of the sample. However, because the depth of focus is suppressed and the surface structures are highlighted, such micrographs may be used in digital image analysis, at least for comparative purposes, since regular SEM micrographs are not considered to be suitable for this analysis (50).

Conclusion

Electron microscopy is well suited for studies of microstructure in milk products. Scanning electron microscopy, in particular, is capable of providing solutions to some problems quite rapidly. The usefulness of the SEM results depends to a great extent on a proper preparation of the samples and a correct operation of the microscope. The possibility that artefacts may develop needs to be considered and measures aimed at reducing their incidence have to be implemented. Modern instruments and accessories (high-resolution microscopes, sputter coaters, charge neutralizers) as well as new preparatory techniques (procedures rendering the specimens electrically conductive) may diminish the incidence of artefacts in conventional SEM but may also produce new artefacts. Even if all efforts are made to reduce the introduction of artefacts of any origin, it is advisable to use other microscopical techniques, for example, transmission electron microscopy, to confirm the findings made using SEM.

Acknowledgments

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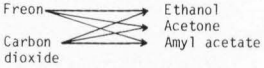
Discussion with Reviewers

T. Makita: What concentration of imidazole combined with osmium tetroxide is suitable for the preservation of fat?
Author: The postfixative used with whole-milk yoghurt (1) was prepared by dissolving 0.5 g of crystalline OsO_4 in 50 mL of a 0.05 M veronal-acetate buffer (pH 7.4) and mixing this stock solution with an equal volume of a 0.2 M imidazole solution adjusted to pH 7.4 with 1 N HCl.

T. Makita: What combination:



or which reagents in critical-point drying do you recommend best for milk products?
Author: I use ethanol and carbon dioxide.

T. Makita: What combination of metal coating (gold + carbon; palladium + carbon; platinum + carbon) would be the best for milk products?
Author: In conventional SEM, gold alone or in a combination with carbon produces good results. In high-resolution SEM, platinum should be used.

T. Makita: As far as the charging artefacts are concerned, you appear to regard the electric contact of the specimen to the stub to be the most important factor. Moisture or the presence of gas as you have mentioned, would also be key factors. In this respect, you have emphasized too much to shorten the dehydration ("... although dehydration shortened to only 10 min of total exposure to the dehydrating agent failed to demonstrate any apparent artefacts"). Can you recommend a minimal required dehydration time for a given size of the specimen? As you have mentioned it, dehydration is the substitution of organic solvents for water, can you explain how important it is to remove the organic solvents before metal coating, because it is also a key factor in charging?
Author: Metal coating is carried out at a reduced pressure. If a volatile substance such as water or an organic solvent is present in the sample, the desired low pressure (<13 mPa) is achieved only after that substance has been evaporated.

T. Makita: The magnification attainable by SEM has been increased to 100,000-300,000 X. Preparation of the specimens, especially metal coating, has to be adjusted to the higher resolution. Sooner or later, milk products will also be subjected to such high magnifications. Thus, more information in this respect would be appreciated.
Author: The question is, what the very high magnifications are expected to reveal in milk products, in which casein micelles (100 nm in diameter) are the structural protein units and fat globules (>>100 nm in diameter), lactic acid bacteria, salt crystals etc. are present in addition. At present, the mutual relations of all these components are of interest and the magnifications used have not exceeded 24,000 X.

S.H. Humphreys: The preservation of fat with imidazole-buffered OsO_4 would seem to involve formation of different complexes as "traditional" understanding of OsO_4 fixation does not invoke participation of the buffer in fixing. This striking preservation of fat is well worth a precise protocol, especially as the reference cited does not give a precise protocol.
Author: The reference (1) provides sufficient information on the preparation and use (time and temperature) of the fixatives. It has been stated that fat globules postfixed with an imidazole-buffered 0.5% OsO_4 solution for 24 h at 22°C were well preserved, that a 2% OsO_4 solution emphasized details in the fat globule membrane but led to an excessive deposition of Os in casein micelles present in the sample, and that postfixation of the samples at 44°C resulted in the disintegration of the fat globules. The study indicates that the conditions for optimal postfixation of other milk products may differ from those established for whole-milk yoghurt.

S.H. Humphreys: What does it mean that chemical dehydration of samples in acidified 2,2-dimethoxypropane is a "rapid" technique?
Author: There is no need to carry out the dehydration with 2,2-dimethoxypropane in steps and change the dehydrating agent such as ethanol or acetone several times in order to remove all the water present. Water reacts chemically and quantitatively with acidified 2,2-dimethoxypropane and produces methanol and acetone. This procedure is less laborious and is accomplished within a shorter period of time than dehydration using ethanol or acetone.

W. Buchheim: Chemical fixation (e.g. in glutaraldehyde) is a common preparatory step in order to stabilize protein aggregates and matrices. How do you estimate the degree of shrinkage induced by this treatment, especially with samples like yoghurt?
Author: No studies have been reported on this subject, although a combined effect of fixation and dehydration on Cheddar cheese (overall linear shrinkage of 19.5%) has been mentioned (52).

W. Buchheim: Please comment on the folded surface of the two unfractured fat globules in Fig. 3. Is this an artefact?
Author: Compared to the images of fat globules in milk which you have obtained by freeze-fracturing and freeze-etching (51), fat globules in Fig. 3. appear as if covered with wrinkled fat globule membranes. Adsorbed proteins and the gold coating may be factors contributing to this appearance but may not explain it fully. If the wrinkled surface is an artefact, its origin and nature have yet to be explained.

W. Buchheim: Could the openings in the fat globule membranes (Fig. 4) perhaps represent locations of previously adsorbed casein micelles which desorbed during the dehydration step?
Author: In the absence of experimental proof, such a hypothesis would be unfounded.

W. Buchheim: It appears unlikely that Fig. 13 represents the true original structure of a yoghurt sample because of the high water content (~90%). Could it perhaps represent an atypical particle of highly aggregated protein within this yoghurt?
Author: The appearance of that commercial yoghurt certainly is surprising and the study of its microstructure could produce interesting results provided that the composition and manufacturing procedure are known. However, this is usually not the case with commercial products. The microstructure shown in Fig. 13 was consistently obtained with samples irrespective of the sampling site and is not related to highly aggregated protein areas such as the one mentioned earlier (24).

G.E. Pfefferkorn: The possibility of backscattered electron (BSE) images at a low magnification is not mentioned; the method of Volbert (53), who subtracts the BSE image from the normal secondary electron image to show only the topography of the surface should be discussed.
Author: Signal mixing techniques using a 2-detector system make it possible to show the "true" surface topography in multicompositional specimens without any artefacts. However, there are no reports on their being used in scanning electron microscopy of milk products and I have no practical experience with them.

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