Structure of Dairy Products

Edited by

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Structure of Dairy Products
1 Overview of Microscopical Approaches

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For more than 60 years, the Society of Dairy Technology (SDT) has sought to provide education and training in the dairy field, disseminating knowledge and fostering personal development through symposia, conferences, residential courses, publications, and its journal, the *International Journal of Dairy Technology* (previously known as the *Journal of the Society of Dairy Technology*).

In recent years, there have been significant advances in our understanding of milk systems, probably the most complex natural food available to humanity. Improvements in process technology have been accompanied by massive changes in the scale of many milk/dairy processing operations, and the manufacture of a wide range of dairy and other related products.

The Society has now embarked on a project with Blackwell Publishing to produce a technical series of dairy-related books to provide an invaluable source of information for practising dairy scientists and technologists, covering the range from traditional to modern large-scale operations. This fourth volume in the series, on the *Structure of Dairy Products*, under the editorship of Dr Adnan Tamime, provides a timely and comprehensive review of our understanding of the microstructure of milks and milk products. Subtle changes in microstructure as a result of compositional or process changes can lead to shifts in the stability, texture and rheology of products, all of which are closely related to their acceptability and hence of vital interest to the industry.

Andrew Wilbey  
President, SDT  
September 2006
Preface

One of the main aims of the Society of Dairy Technology (SDT) in the United Kingdom is ‘the advancement of education in dairy science and technology, food technology and management of resources in all branches of the industry by the dissemination and application of knowledge gained from experience and experiment’. Such knowledge is disseminated to the Society’s members through meetings at regional level, symposia, conferences and residential courses, publishing a scientific journal (International Journal of Dairy Technology) in conjunction with Blackwell Publishing Ltd, and providing technical books covering selected aspects of dairy technology.

Structure of Dairy Products is the fourth book of the Technical Series, where the structures of the main dairy products, including analytical methods, are reviewed by experts from many different countries. However, the last 30 years have witnessed great interest in the microstructure of dairy products, and during the same period different techniques have been developed to visualise clearly the properties of these products. Hence, different microscopy techniques have been used as complementary methods in quality appraisal of dairy products, and have been employed for product development and in troubleshooting wherever fault(s) arises during the manufacturing stages and storage period.

The topic is, therefore, of intense interest to everyone from the manufacturers of dairy products through to dairy consultants and scientists who are involved in product development and troubleshooting. The topic has been extensively researched, and the result of this widespread interest is that many articles on the structure of different dairy products have been published in scientific journals targeted at very specific groups of scientists. In addition, some of the same information has been published in chapters in different textbooks. It is therefore timely to produce a multi-author text (with contributions from the UK, Europe, Canada, USA and New Zealand) containing chapters that summarise recent findings for each dairy product; the aim of this present book is to provide such an overview. While most chapters of the book have been referenced extensively, Chapters 1 and 10 also include a comprehensive bibliography for further reading regarding many aspects of microscopy.

It is hoped that this text will become an important component of the Technical Series promoted by the SDT.

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1 Overview of Microscopical Approaches

D.F. Lewis

1.1 Introduction

Microscopy is the most fundamental of observation techniques for understanding the behaviour of foods. In essence it uses those natural abilities which allow us to live our everyday lives. Consider Fig. 1.1: even without the additional benefit of colour pictures, most people will have no difficulty in recognising the two pictures of cows and distinguishing between them. Human ability for recognition is still more sensitive than automatic recognition with regard to everyday objects. Consider how a cartoonist can represent a celebrity with a few strokes of a pencil. Microscopy capitalises on this human ability to recognise. In everyday life, shape and size are important, but other characteristics, such as visual texture and colour, are used for identification; microscopy uses these same attributes but allows finer detail to be visualised and allows other optical characteristics to be used to supplement the identification.

The aim of this chapter is to give an overview of how the different approaches to microscopy can aid the process of identification and is based on the author’s experience. It is not intended to be a ‘methods manual’, and the bibliography indicates useful sources for those wishing to further develop the techniques mentioned in this chapter. Chapter 2 gives a more thorough technical description of microscopy techniques, and the remaining chapters illustrate the application of these techniques to explain the mysteries of dairy technology.

Fig. 1.1  Examples of two breeds of cattle: (a) Highland cow; (b) Ayrshire cow.
1.2 Light microscopy

At its simplest, microscopy consists of obtaining a magnified image of an object in order to allow finer detail to be made visible to the eye. A simple hand lens or jeweller’s loupe will readily allow a magnification of around ×10 to be used and, for some purposes (e.g. identification of insect parts), this may be all that is required. More helpful is a ‘stereo microscope’, which typically allows magnifications around ×50 to be used. An examination with a stereo microscope is generally a good start to any investigation, and particularly so in the identification of foreign bodies. Generally, objects examined by stereo microscopy need little preparation and are not altered by the examination, and this is a profound benefit. However, the amount of detail that can be seen is somewhat less than with more sophisticated techniques. Typically, the resolution of a stereo microscope will be in the region of tens of microns. The use of a compound microscope can improve the resolution and, hence, the useful magnification to a theoretical limit of about 0.2 μm.

In addition to improved resolution, the compound microscope allows a range of contrast techniques to be used that are based on the optical properties of the specimen, and which consequently add to the visual information provided in the image. However, making use of the advantages of the compound microscope normally requires sample preparation that can cause changes in the specimen. The microscopist is confronted with the issue of artefacts or features that are produced by the process of microscopy. In reality, all scientific observations are artefacts – the result of a chemical analysis for a metal or for a complex organic compound is dependent on the procedure used. Hence, two different extraction procedures will often give different values, and the presence of other materials may alter the value obtained. Even an apparently simple physical determination, such as viscosity, will give different values according to the method used and the behaviour of the sample under shear forces. Hence, all results of scientific measurements need to be interpreted, and microscopy is no exception. Every image has to be considered in the light of the procedures that have been used to produce the image. Wherever possible, the least obviously intrusive techniques should be used and, where important conclusions are being drawn from one microscopical approach, it is wise to confirm key observations by a technique based on different preparation principles.

1.2.1 Compound light microscope

Most useful observations on foodstuffs with the compound light microscope are made by transmitted light, and this requires that the sample under observation should be reasonably thin; techniques for achieving this are detailed in Chapter 2.

For all microscopy techniques, visualisation depends on two characters – resolution and contrast. Resolution indicates the degree to which two points must be separated to be seen as two points; for light microscopy, the theoretical limit for resolution is around 0.2 μm. Contrast is the difference in intensity between a point and its background and, generally, a difference in intensity of about 16% is the limit for the eye to distinguish.

The normal illumination technique for bright field microscopy is known as Koehler illumination, and is aimed at optimising resolution and light intensity. The condenser diaphragm or aperture affects contrast. Normally, a setting where the condenser aperture fills about 75% of the back of the objective is the best compromise for contrast and resolution. Closing
the aperture increases contrast, but reduces resolution and causes fringes to become visible. Figure 1.2 is a diagram of a microscope with the terms used in the following explanations illustrated.

Dark field and phase contrast

The advent of brighter light sources and the widespread adoption of Koehler illumination have enabled a number of other contrast techniques to be applied with good effect to food systems. The continued development of light-emitting diodes (LEDs) as light sources will undoubtedly simplify microscope design and may well allow advances in imaging techniques. In simplistic terms, contrast and detail are provided by light, which has been altered in some way by interaction with the specimen. In normal bright field, the altered light can be ‘swamped’ by light, which has passed directly through the sample without change, and so most contrast techniques involve reducing the intensity of the ‘direct’ light without much reducing the ‘altered’ light. The most obvious of these contrast techniques is known as dark field. In this case, a dark central disc is placed in the front focal plane of the condenser so that no direct light enters the objective lens. Consequently, the image is made up entirely of light, which has been reflected, diffracted or refracted by the specimen. This technique tends to provide very high-contrast images, and is generally most useful for visualising small particles, e.g.

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Fig. 1.2 Diagram of a light microscope; note that important focal planes of the microscope are shown in capital letters. Source: Lewis (1978) Manual of Microscopical Methods, Leatherhead Food Research Association.
diatoms or bacteria at relatively low magnifications. A modification of the dark field technique, which can impart colour to features with different orientations, is called Rheinberg illumination (http://www.nhm.ac.uk/hosted_sites/quekett/Technical/Rheinberg.html).

Dark field microscopy eliminates all light that is not modified by the specimen and so produces a very high-contrast image. Phase contrast reduces the intensity of the direct light, but also introduces a phase shift between the direct and altered light so that interference can occur, and the lightness or darkness of the image depends on the specimen thickness and refractive index. This allows differentiation of cellular structures up to high magnifications. In food studies, it is particularly useful for distinguishing phases, e.g. in emulsions or for examination of live micro-organisms. The main disadvantage of phase contrast is that it tends to produce a ‘halo’ around boundaries with a sharp refractive index difference between the phases.

Polarised light and differential interference contrast

Possibly, the most useful single contrast technique for light microscopy of foods is polarised light. In basic terms, light can be considered as vibrating in all planes around the direction of travel. A polarising filter restricts the directions of vibration to a single plane. Some materials, notably crystals and fibres, are birefringent, which means that light passing through them vibrates in different planes and travels at different speeds or, in other words, the material has different refractive indices according to the direction of vibration of the light. When a beam of plane polarised light passes through a birefringent material, the light can be resolved into two vectors with light travelling at a different speed along each vector. If these beams are recombined by another polarising filter, then interference occurs between the beams. The effect of this is to produce colours according to the thickness of the specimen and the difference between the refractive indices (this difference is termed birefringence). Hence, crystals or ordered structures will often appear bright or coloured against a dark background. This is clearly useful for studying sugar and fats in particular, but also plant cell structures, meat striations and fibrous foreign bodies.

Polarised light microscopy can be extended to differential interference contrast (DIC) by the use of prisms to separate the polarised light into two beams with a phase shift between the two. In this way, features differing in refractive index can be imaged quite precisely, and not just birefringent features. The most common commercial form of this technique is known as Nomarski interference. It is used for similar specimens as phase contrast, but does not suffer from the formation of a halo at boundaries between features.

Figure 1.3 shows a sample imaged using a variety of the above contrast techniques in a compound microscope.

Fluorescence microscopy

Some materials fluoresce, that is, they can absorb light with a short wavelength and re-emit light at a longer wavelength. This can be used as a contrasting technique for light microscopy. Hence, if a specimen is illuminated with green, blue or ultraviolet light and re-emits yellow, orange or red light, it is possible to filter out the illuminating wavelengths and, thus, image only that light which has been re-emitted. This often gives high contrast, though not always
very bright images. More than the other contrast techniques, it has relied on developments in lighting to be fully applied. Mercury vapour or xenon lamps are rich in the blue–UV end of the spectrum, and provide very bright, small sources of light.

Initially, fluorescence microscopy was performed using transmitted light and fairly dark filters to remove the illuminating wavelengths; this gave severe problems in obtaining sufficiently bright images, particularly since many materials fade as they are illuminated with high-intensity short-wavelength light. A major improvement came with the development of the dichroic mirror, which allows incident light to be used to excite the fluorescence. The dichroic mirror reflects short-wavelength light, but allows the longer wavelengths to pass through. The need for additional filtering to remove the illuminating wavelengths is, therefore, reduced for two reasons – using an incident light source means that any light not absorbed by the specimen is largely lost to the imaging lenses, and only a small amount of light needs to be removed; this can be achieved by the dichroic mirror itself, which reflects the shorter wavelengths back towards the lamp and away from the imaging lenses.

Some specimens fluoresce naturally (auto-fluorescence) but, for the most part, fluorescent stains are used as described below. Fluorescence microscopy is most useful for specific location where fluorescent dyes can be attached to molecular probes to locate specific components. Also, the most useful confocal microscopy is best achieved in food materials using fluorescence mode.

**Staining**

Where contrast cannot be introduced optically, or where more specific identification of material is required, staining can be applied to samples for examination in the light microscope.
The basis of staining specimens relies on a number of different mechanisms. Thus, acidic dyes will bind to basic groups and vice versa. In other cases, differential solubility will cause dyes to accumulate according to polarity. It may be possible to carry out a chemical or enzymatic reaction on the microscope slide to stain a particular chemical entity, and some stains can be coupled to antibodies, lectins or nuclear probes to identify specific proteins, sugar residues or genetic material, respectively.

The stains themselves may be coloured dyes, opaque metals or salts, fluorescent compounds or even radioactive labels that can be visualised by coating the specimen with a thin film of photographic emulsion. At one end of the spectrum, staining techniques can be simply including a dye into the mounting media and, at the other end, it can be a most highly skilled and specialised technique. Potentially, staining techniques and light microscopy can be used to locate almost any material although, in some cases, other methods may be easier or indeed the cost of the most specialised techniques may exceed the benefit of the investigation.

Some examples of the simpler staining techniques that are most useful for light microscopy are described below.

A solution of iodine in potassium iodide will stain starch dark blue and, if the stain is diluted, then waxy starch (depleted in amylose) will stain pink-brown, while normal (amylose-rich) starch will stain blue. In more concentrated form or when used as a vapour stain, the iodine will partition itself preferentially into less-polar regions, such as lipids, and produce a brown coloration.

In aqueous solution, eosin is an acidic dye, and will bind to basic groups, notably amino groups in proteins, giving a pink colour. This makes it a useful counterstain to iodine in starch–protein mixtures. It is also fluorescent, which makes it a handy stain for simple fluorescence microscopy or for confocal microscopy (see section 1.2.2 and Chapter 2). Eosin is also soluble in ethanol, which allows its use as a stain for samples containing water-soluble components, such as sugar crystals. A particular use of ethanolic eosin is in milk chocolate where milk proteins can be visualised alongside the sugar crystals.

Toluidine blue is a basic dye, which will stain nucleic acids, carboxylic acid groups in proteins and pectin and other mucopolysaccharides. It is also a metachromatic dye, which gives different colours according to the spacing of reactive sites. As well as being a good general histology stain (particularly for resin sections), it also specifically highlights textured vegetable protein by its differential colouring. Acridine orange is another metachromatic nuclear stain, and it also shows textured vegetable protein in fluorescence microscopy. Acridine orange can also be used to distinguish between live and dead micro-organisms due to its metachromatic staining properties at certain pH values.

Lipids can be quite difficult to stain; osmium tetroxide stains lipids brown-black, and can be used in vapour form to avoid contact with water or solvents. However, it is a very hazardous material with an appreciable vapour pressure, and can lead to blindness or death by vapour exposure. It has a nasty habit of desensitising the sense of smell after a fairly short exposure. For this reason, it is only recommended for laboratories where proper safety precautions are possible. It has the advantage of being fairly specific – staining unsaturated fatty acids – and also of fixing the lipid as well as staining it. Some dyes will partition between lipids and a less-polar solvent. In this way, Sudan black B dissolved in ethylene glycol can be used to stain lipids blue, but it tends to be a ‘dirty’ stain, which can precipitate on the slide. Oil
Red O dissolved in propylene glycol is an alternative stain that colours lipids red, while Nile blue A can act as a fluorescent stain for lipids. Congo red can also stain lipids red and impart fluorescence, but is tricky to get the conditions right; it is, however, a most useful stain for showing damaged starch and, when used in fluorescence mode, is particularly sensitive.

An example of a specific chemical reaction being used for staining is seen in the periodic acid–Schiff reagent (PAS) stain. In this case, periodic acid is used to oxidise 1:2 glycol groups (found in sugar residues); afterwards, the Schiff reagent reacts with the newly formed carbonyl groups to produce a magenta colour. This is a good method for locating carbohydrates in food systems; the method can be made more specific by blocking free aldehyde groups (e.g. with dimedone) prior to staining. The method has been adapted for electron microscopy by replacing the Schiff reagent with thiocarbohydrazide (TCH) or thiosemicarbazide (TSC), which bridge the newly formed carbonyl groups and provide a sulphur moiety that can be further linked to silver or osmium.

1.2.2 Confocal scanning light microscopy (CSLM)

The confocal principle (as described in Chapter 2) was understood in the 1940s, but was difficult to apply until laser technology allowed bright coherent light sources to be used, and computing technology became sufficiently fast to allow data capture and image construction to be carried in a reasonable timescale. Once these technologies developed, CSLM became a viable technique and, literally, added a new dimension to food microscopy. Confocal microscopy offers many advantages in terms of allowing more precise localisation of specific substances, but its ability to recreate three-dimensional views of food structure is where it has advanced food microscopy most. It is difficult to illustrate the application of these three-dimensional techniques in a printed monochrome, and Chapter 9 illustrates some applications to ice cream. Confocal microscopy has tended to be a relatively expensive technique, but reductions in the price of computing power and the availability of cheaper and more varied laser sources should make it more accessible and with wider applicability.

1.2.3 Specimen preparation

For acceptable transmitted light microscopy in the compound light microscope, the specimen has to meet certain criteria. First, the specimen needs to be mounted in a suitable medium, and it must be of a refractive index that is slightly but not too different from the specimen. It must not swell or dissolve the features that are being studied. Solubility can be used to advantage in identifying components, e.g. sugar and fat crystals can be distinguished by their solubility in aqueous and non-aqueous mountants. If stained specimens are used, the mountant must not extract the stain from the specimen, and it must be able to ‘wet’ the specimen and not trap air. If the specimen has very fine particles, then it may be useful, if the mountant sets or has a high enough viscosity, to prevent Brownian movement from blurring the particles. In some cases, the mountant can be used to ‘clear’ the samples, particularly where dry powders are being examined; chloral hydrate and lactophenol can be used as clearing agents. For general purposes, aqueous glycerol can be used as semi-permanent mountant, and liquid paraffin can be used where water-soluble components are being examined. Commercial mountants are available that gel or set on standing; these may be aqueous or non-aqueous based.
The second criterion for samples is that they must be thin; the ideal thickness varies from about 20 \( \mu \text{m} \) for low magnifications to 1 \( \mu \text{m} \) for high magnification. Practical reality may dictate other thicknesses! In particular, if a specimen with 40- \( \mu \text{m} \)-diameter particles is being examined, then these may dictate the specimen thickness if they need to be examined intact; similarly, some samples may just not be able to be prepared at the optimum thickness.

At the most basic level, a sample may be prepared by dispersion or simple whole mount in a suitable mountant. Studying the fat globule size in milk or an emulsion may be best achieved by simply mounting on a microscope slide with a coverslip or possibly by mixing with a suitable mountant; similarly, milk powders may be examined by mounting in liquid paraffin. However, most complex products require sectioning of some sort. Sectioning techniques are reviewed in Chapter 2; mostly they either require the sample to be frozen and sectioned while frozen or require the sample to be embedded in a wax or resin. Before embedding, the sample will need to be fixed and dehydrated. Many procedures exist for fixing and dehydrating biological samples, but these often need to be modified for complex foods with high fat, sugar or air inclusions.

Proteins are fixed to prevent extraction or alteration by dehydration and embedding procedures, and formaldehyde and glutaraldehyde are commonly used for this purpose. Both compounds can solubilise partially gelled or modified starches, and should be used with caution if starch and proteins are being studied simultaneously. Fixing in glutaraldehyde in ethanol can help prevent extraction of sugars and more complex carbohydrates, but may equally cause precipitation. Where preservation of fine protein structure is not required, simple fixation in ethanol may allow polysaccharides to be retained. Unfortunately, there is not a great deal of scope for retaining lipids during dehydration and embedding other than fixation with osmium tetroxide. Long-time fixation (perhaps several weeks) will retain unsaturated fat – fixation can be through the vapour phase where contact with aqueous fixatives would not be advised. As previously mentioned, stringent precautions are required for using osmium tetroxide.

Generally, resin embedding will allow thinner sections to be obtained than wax although, for routine examination of a large number of samples, wax embedding may be the method of choice. Section thickness is governed by the requirements of the investigation; thinner sections will give better resolution of fine detail but, at lower magnifications, thicker sections will produce better contrast and may be easier to stain.

Where frozen samples require examination, as may be the case with frozen desserts, the frozen sections can be transferred to a cold stage and examined in the microscope while still frozen. More details on the procedure are given in Chapter 2, and examples of this application are given in Chapter 9.

1.3 Electron microscopy

Resolution in the light microscope is limited by the wavelength of light. In order to obtain better resolution than about 0.2 \( \mu \text{m} \), shorter wavelength irradiation is required, and the main candidates are electrons and X-rays. X-rays are difficult to focus, and so X-ray microscopy techniques are mostly limited to direct techniques where magnification is determined by the distance between the specimen and the detector. It is possible to achieve some focusing of
X-rays with Fresnel-type lenses, and a few microscopes have been made which either use these lenses in the same manner as a compound microscope or use the scanning principle. At present, X-ray microscopy is little used for food studies, but it has several potential benefits including being able to operate on thick specimens, producing three-dimensional representations and having the potential for elemental identification. It remains to be seen whether the technical difficulties of this approach will allow its potential to be tested.

Electrons have a shorter wavelength than X-rays and, consequently, can achieve resolutions down to about 0.2 nm or about one-thousandth that of the light microscope. Clearly, this gain in resolution is worthwhile, but it comes at a cost in terms of specimen preparation. Broadly there are two classes of electron microscope: (1) those that work on the principle of a beam being scanned in a raster over the sample, and the derived samples are used to generate the image; and (2) those that operate in a similar manner to the compound microscope where electromagnetic lenses magnify a transmitted image of the sample. Chapter 2 deals with scanning and transmission microscopes with some attention to the instrumentation.

### 1.3.1 Scanning electron microscopy

When an electron beam interacts with a specimen, a number of derived radiations are emitted as illustrated in Fig. 1.4. Any of the signals can be used to generate an image, and each signal provides different information about the sample.

The standard scanning electron microscope (SEM) operates with secondary electrons – this approach provides a topographical view of the specimen. At higher accelerating voltages in the SEM, the signal is a mixture of surface properties and, from a small volume under the surface as the accelerating voltage is decreased, the signal becomes more related to surface

![Fig. 1.4 Electron beam–specimen interactions: (1) secondary electrons – topological information; (2) backscattered electrons – information on atomic number; (3) Augier electrons – specific elemental information; (4) cathodoluminescence – used for electronic components; (5) bremsstrahlung X-rays – little specific information; (6) energy/wavelength-specific X-rays – give information on elemental composition; (7) specimen current – mostly used for semiconductors; (8) elastically scattered electrons – contributes to contrast in conventional transmission electron microscopy; (9) unaltered electron beam; (10) inelastically scattered electrons – information on specific elemental composition.](attachment:image.png)
properties. While the sample generally needs to be coated with a conducting layer to prevent charging, it is possible to examine uncoated samples at an appropriately low accelerating voltage. However, at lower accelerating voltages fewer secondary electrons are generated, and this can lead to difficulties in imaging. Typically, this form of SEM is most useful for examining powder particles and, more generally, with a cryo-stage to allow representation of a range of dairy products.

Backscattered electrons provide information about the atomic number distribution in the specimen with areas of higher atomic number appearing brighter than those with lower atomic number. In functionality studies, this could be applied to distinguish calcium-rich areas in an organic matrix; in trouble-shooting applications, it could show up mineral elements as contaminants.

Augier electrons are energy specific and can be used for elemental localisation in specimens. However, X-ray microanalysis techniques have been developed more extensively, and most elemental localisation would use X-ray emission. Cathodoluminescence and specimen current are most useful for mineral and electronic studies.

Comparatively recently, the environmental SEM has developed. This has the advantage of allowing the specimen to be examined at a relatively high pressure so that the sample does not need to be completely dry for examination. The detection system measures the level of ionisation in the specimen chamber and produces images similar to that obtained with secondary electrons. The ability to examine comparatively wet samples obviously represents a significant opportunity for dairy microscopy.

1.3.2 Transmission electron microscopy (TEM)

Scanning electron microscopy has some advantages over light microscopy in terms of improving the resolution by about twenty times, and by producing images that are more lifelike but, to gain full advantage of the resolving power of electrons, requires the transmission electron microscope. This approach uses electrons that pass through the specimen. Image formation, in a similar way to the compound light microscope, is based on scattered electrons, and it is possible to use dark field TEM to obtain high contrast. Specimens for examination by TEM need to be very thin to obtain good resolution. Around 60–120 nm is the most useful range of thickness, and specimens can be prepared in three basic ways.

Thin sectioning

Cutting sections at 100 nm or so thick is not straightforward, and the normal procedure is to embed in a resin. As with light microscopy sectioning, the specimen must be fixed to preserve the components, dehydrated (normally with ethanol or acetone), embedded in the prepolymerised resin, cured and sectioned. Sections are cut on an ultramicrotome and stained with heavy metal salts (normally uranyl acetate and lead citrate) before examination. Energy filtering can be applied to the electron beam to produce images based on the specific energy level electrons. This approach is fully described in Chapter 2; it allows unstained and thicker sections to be examined, and provides information on the composition of parts of the specimen.
Typically, acrylic and epoxy resins have been used for embedding. Acrylic resins are generally easier to embed and section, tend to be miscible with ethanol, are less hazardous and give better staining characteristics. However, they tend to be less stable in the electron beam than epoxy-based resins. This approach is probably the ‘routine’ electron microscopy approach, and it has been applied to a wide range of dairy products with good results. As with light microscopy, fixation of fats is a problem, and long-time osmium fixation is the only way to retain some fatty structures for examination. It is generally wise to prepare two sets of samples for fatty products – one where the fat is retained by osmium fixation, and one where the fat is allowed to be extracted during embedding. Osmium fixation can disrupt protein structures, while partial retention of fat can interfere with the polymerisation of the resin, and result in samples that are difficult to section. As with all microscopy methods, the final specimen structure is a combination of the intrinsic nature of the sample and the process used to prepare it for examination but, providing that the approach is consistent and controlled, then differences between specimens can be interpreted and related to functionality with good effect. As described in Chapters 2 and 9, fixation and dehydration can be carried out at freezer temperatures using alcoholic fixatives to provide information on frozen materials (freeze substitution).

**Replica techniques**

An alternative to cutting sections is to prepare a carbon–platinum replica of a surface. The replica is prepared by evaporating platinum at an angle of about 20° to the surface (under vacuum either by resistance heating, joint evaporation in a carbon arc or by ion bombardment of a platinum target), and a carbon layer is then evaporated (either carbon arc or resistive heating of carbon fibre) from directly above the surface. The carbon forms a coherent layer, while the platinum forms a pattern depending on the undulations in the surface. The specimen can be dissolved away from the replica (sometimes easier said than done), and the replica examined with TEM. The replica production technique does tend to produce heat and so, in its normal form, is only appropriate for solid, heat-stable specimens. Heat-labile samples can be replicated by freezing and keeping frozen during the replication process. This can be coupled with freeze-fracture and etching as described in Chapter 2.

Replica techniques provide a useful alternative to thin sectioning, and can avoid chemical fixation and alcoholic dehydration. Thus, this approach can be used to check critical findings from thin sectioning and to help interpret the significance of thin-sectioned images.

**Dispersions**

Some samples, e.g. viruses and isolate proteins, are small enough for direct observation by TEM. The requirement for imaging, however, is to obtain sufficient contrast. Negative staining, where the particles are effectively surrounded by a ‘glass’ of heavy metal, or shadowing with a heavy metal such as platinum, are the two common approaches. This approach can be used to demonstrate flagellae and fimbriae on bacteria, but these may also be demonstrated by specific antibodies adsorbed to the surface of colloidal gold. Figure 1.5 shows *Salmonella* spp. stained with specifically labelled colloidal gold.
1.4 Other techniques

During the first half of the twentieth century there were relatively few developments in microscopy but, during the second half, great developments occurred and food microscopy became exciting as developments allowed new insights into food behaviour to be gained. In this period, the electron microscope became a commercial possibility, and improved light sources allowed more routine use of contrast techniques. Towards the end of the century, improvements in computing allowed real-time image processing, which enabled the confocal microscope to reveal three-dimensional structures, and allowed X-ray microanalysis to become a regular feature of electron microscopes. Computer developments also allowed the realisation of other imaging methods – scanning probe microscopy (SPM), nuclear magnetic resonance (NMR) microscopy, Fourier transform infra-red (FTIR) microscopy and ultrasonic microscopy, for example. The full practical potential of many of these newer imaging modes in explaining food functionality remains to be seen. The principles and technical details of these methods are given in Chapter 2. These new methods offer some potential to food studies, but there are some problems to be solved in order to fully exploit their benefits.

SPM provides the highest available resolution of the direct imaging microscopy techniques, and can be used in time-lapse mode to follow change, for example, in food gelling systems. It provides information that is impossible to recreate in visual form by other techniques. As with much microscopy, the problem is in specimen preparation – generally, the samples need to be produced as thin films for best effect. Even so, impressive videos of gelation can be obtained and it is hoped that, as techniques evolve, more use can be made of this family of microscope techniques.

NMR microscopy has the possibility of localising food constituents, particularly fat and water in samples that have been relatively little altered in specimen preparation. However, the problem lies in resolution; at best, the resolution is about ten times worse than the light microscope and, more normally, is about a hundred times worse. To obtain the better resolutions requires smaller samples to fit into the poles of the magnet and, consequently, preparing...
samples without deforming the distributions can be a problem. In time, may be methods can be developed to produce reliable high magnetic fields inside larger specimens but, for the time being, this technique is useful at a relatively macro scale. Nonetheless, it is a unique and useful tool for solving many distribution and migration issues in food science.

FTIR offers the potential of obtaining diagnostic infra-red spectra from microscopical areas of a specimen. Compared with the relatively indirect methods of staining, this offers the potential of a much more definitive localisation of components. Thus far, the approach has found applications in the ‘forensic’ aspects of microscopy. Ideally, a system that uses confocal FTIR would be invaluable for food microscopists, and perhaps technical developments will allow this to happen.

1.5 Conclusions

The various techniques that constitute food microscopy represent a valuable and fascinating toolbox for understanding food behaviour. The composition of foods, while related mostly to biological samples, is unusual in that they often contain high levels of fat, sugar, salt and air, may exist at pH values beyond the normal biological range and may be quite heterogeneous in composition. Consequently, food and dairy microscopy offers both challenges and rewards. The food microscopist also functions in a scientific world that appreciates numerical results above visual ones, and where microscopy is often seen as providing pictures to support a preformed hypothesis.

A food microscopist’s lot can be challenging. The requirements are a sound knowledge of microscopy techniques and, particularly, of the effects of preparation techniques on foodstuffs. This microscopical expertise has to be coupled with an understanding of food science and technology, both to understand the significance of the microscopical observations and to explain them in terms that are comprehensible to food technologists who have the task of implementing those findings. The partnership between the microscopist and the technologist is crucial to the application of microscopy and, without application, food microscopy loses its value.

There is a vast array of techniques available but, in the real world, there are restraints on the application of resources and the microscopist has to make judgements on the most effective techniques to apply in any study. While it is unwise to make generalisations on method selection, a preliminary light microscope examination will often give a good general impression of changes in the samples, and give guidance on which more sophisticated techniques may be of most use in the study.

Bibliography

General microscopy

Molecular Expressions Optical Microscopy Primer (http://micro.magnet.fsu.edu/primer/); this is a comprehensive and interactive website covering most aspects of light microscopy and represents a good starting point for obtaining a general understanding of light microscopy; similar sites are sponsored by Nikon (http://www.microscopyu.com/sitemap.html) or Olympus (http://www.olympusmicro.com/index.html).

Wide range of microscopy subjects

The Royal Microscopical Society has published a Microscopy Handbooks Series (i.e. each volume is written by someone with hands-on knowledge of the subject area); these booklets are authoritative and practical; volumes 1–26 were published by Oxford University Press, Oxford and, from volume 27 onwards, the publisher is BIOS Scientific Publishing, Oxford; these booklets were published in conjunction with the Royal Microscopical Society under the series editorship of S. Bradbury. The list is not alphabetic but arranged by volume number.


**Photomicrography**


**Stains**


Other techniques

2 Instrumental Techniques for Sample Preparation

D.G. Pechak and A.K. Smith

2.1 Introduction

Instrumental techniques continue to be developed and refined to explore the structure of dairy products. Some earlier reviews covering the structure of dairy products include those of Kaláb (1981, 1993), Muniraju et al. (1991) and Schmidt & Büchheim (1992). The objective in all methods is to preserve the sample in its original state such that the system and its components can be assessed with confidence. This is a lofty goal because milk and dairy products are emulsions, comprised largely of fat, protein, water and air, making it difficult to preserve ultrastructural components and the products as a whole.

Traditional techniques to prepare emulsions for imaging range from using fresh material for light microscopy to chemical fixation or freeze-fracture replication for electron microscopy. Traditional wide-field light microscopy techniques on fresh material have their own limitation caused by the coverslip constraints, lack of depth of field and difficulty identifying the constituents. Samples for electron microscopy need to be dry to facilitate imaging within the vacuum system. This necessity can be accomplished by removing the water or, alternatively, immobilising it through freezing. Because of the gap in resolution capabilities between light and electron microscopy, it is difficult to assess artefacts caused by intensive preparation methods.

Confocal scanning laser microscopy (CSLM) has the advantage of imaging through a depth of sample such that the features can be reconstructed in the third dimension. Deconvolution wide-field light microscopy can edit out the out-of-focus features to achieve a similar result. Surface probe techniques, atomic force microscopy (AFM) and near-field scanning optical microscopy (NSOM) provide surface imaging on a nanometre scale. Newer wet cell AFM allows in-situ imaging and measurement of the electrochemical character of surfaces and interfaces.

This chapter will cover the more traditional technologies of microscopy (conventional light microscopy, conventional scanning and transmission electron microscopy) as they pertain to dairy research, and will also review literature on newer microscopy techniques, such as confocal scanning laser, environmental/variable pressure SEM, energy filtering (EF) TEM, and AFM. A wider interpretation of ‘structure of dairy products’ results in the addition of non-microscopy techniques, such as rheology, laser light scattering, NMR and diffusing wave spectroscopy. Other methods are included which, although not directly related to structure, are considered important ancillary techniques, such as digital imaging and image analysis or required subjects, such as laboratory safety. References are included when techniques are covered in detail or reviewed in existing literature.
2.2 Light microscopy techniques

2.2.1 Wide-field light microscopy

Transmitted light microscopy is a powerful technique for imaging dairy systems provided that the elements of the microscope are properly aligned (Köhler illumination) to make the best use of imaging capability (Delly, 1980). Because the limit of resolution of a standard light microscope is 0.2 \( \mu \text{m} \), even some casein micelles are visible (although not recognisable). The average size of native milk fat globules is 3.4 \( \mu \text{m} \) (Walstra & Jenness, 1984); 75% of the milk fat is in globules < 1.0 \( \mu \text{m} \). The light microscope is effective for exploring the distribution of the fat globules and other components of dairy products including air bubbles and ice crystals. These features can be used to assess instabilities in dairy systems. Cryo-sectioning of dairy emulsions provides uniform section thickness while retaining the fat. Image contrast can be enhanced by controlling the properties of the light by such techniques as dark field, phase contrast, polarised light and Nomarski (differential interface contrast) imaging (Delly, 1980). A brief description of the characteristics of each wide-field light mode are summarised in Table 2.1, and Table 2.2 lists the commonly used stains for identification of dairy constituents.

2.2.2 Fluorescence light microscopy

Fluorescence staining is a more sensitive tool for identifying proteins, carbohydrates and fats in dairy emulsions. Fluorescent stains interact at specific sites on the macromolecules and leave the rest unstained. Very low concentrations of fluorochromes can be detected through the microscope because the wavelength of fluorescence emission is longer than the wavelength of excitation radiation. The fluorescence light microscope uses epi-illumination that passes through an exciter filter to supply only the radiation required to excite the specific fluorochrome. After the fluorochrome has been excited, the emitted light is passed through a barrier filter, which isolates the spectral range from the emission spectrum. Table 2.3 lists the commonly used fluorescence stains in dairy research. Fluorescence staining is the basis for many CSLM investigations.

2.2.3 Confocal scanning laser microscopy (CSLM)

CSLM is a specialised light microscopy technique that uses a point source of light focused at a fixed depth in the sample to generate specimen information. Signal collection, collected into a pinhole in the front of the detector, is synchronous with the scanning of the zone in the specimen. Confocality is achieved because the light source, which is focused in the specimen, and the pinhole of the detector, are both in the focal plane of the imaging lens (Blonk & van Aalst, 1993). These authors give a good, very basic introduction to the principal of confocal microscopy as well as its application in food systems including fat spreads, butter, margarine and various low-fat spreads including cheese of high and low fat content.

The image is built up, line by line in the computer, as the beam scans the specimen. Magnification is the ratio between the area scanned and the area displayed. Resolution is
Table 2.1  Some characteristics of wide-field light microscopy.

<table>
<thead>
<tr>
<th>Mode of light</th>
<th>Comments</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark field</td>
<td>Only light that is scattered or reflected can enter the microscope objective. The apex of a hollow cone of light is focused on the plane of the specimen. The objective is inside the dark base of the hollow cone so that if no specimen is present no illumination occurs. When a specimen is present, the structures in the specimen cause the light to deviate, forming a bright image on a black background.</td>
<td>No staining is required. The system is capable of detecting small particles because of the high contrast provided.</td>
<td>No colour is produced. A special dark-field condenser is required to use higher-resolution oil immersion lenses. Trapped air bubbles produce a lot of flare.</td>
<td>A dark field stop is inserted below the condenser of the conventional light microscope for low or medium power dry objectives.</td>
</tr>
<tr>
<td>Phase contrast</td>
<td>The light beam is split, and one ray is retarded by ¼ wavelength. When each beam is passed through the specimen, it encounters different refractive index and specimen thickness. Interference occurs because of specimen features (refraction and retardation), and the difference between the specimen and the background (diffraction).</td>
<td>Creates contrast without staining. Can have better resolution than the dark field method. Suitable for black and white photography.</td>
<td>Better at higher magnification.</td>
<td>Annular source with ¼ wave ring. Mounting medium should have a different refractive index from the sample. Green filter enhances contrast.</td>
</tr>
<tr>
<td>Polarised</td>
<td>A polarising element (optical slit) transmits 32% of the heterogeneous light beam as plane-polarised light. Material that is isotropic does not react to polarised light and the image is black. Anisotropic substances have 2 or 3 principle refractive indices and so appear lighted and coloured at some orientation between the 2 polarisers. Interference colours, also called birefringence, are related to the numerical difference between the refractive indices. Birefringence is affected by the thickness of the sample and the degree of anisotropism.</td>
<td>The sample does not require staining. Can identify the presence of crystals in the sample, e.g. fat.</td>
<td>Only useful for anisotropic specimens.</td>
<td>A polariser is situated below the sample, usually on the field diaphragm. An analyser (another polariser) is placed at the back of the objective. The two elements are oriented so that their vibrational directions are crossed and the background appears black.</td>
</tr>
<tr>
<td>Nomarski (i.e. differential interface contrast)</td>
<td>The optical system produces two interfering beams: the object beam (provides the image), and the reference beam (provides the background). Interference is created by the two different beams rather than arising from the specimen as in phase contrast. The beams are separated by a shear system; they are in focus at the specimen plane, but laterally displaced. The two beams recombine below the eyepiece.</td>
<td>Contrast without staining. Resolves more of the fine structure than phase contrast. Suitable for all powers of objective lenses. Permits use of all light from the condenser.</td>
<td>Gives an artificial impression of depth in the sample.</td>
<td>Wollaston prisms.</td>
</tr>
<tr>
<td>Stains</td>
<td>Another contrast enhancement approach for light microscopy imaging is the use of staining techniques. Specific stains can be used to identify components in addition to providing contrast (Carson, 1997). A few commonly used stains are listed in Table 2.2.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
dependent on the volume of interaction as determined by magnification and the numerical aperture (NA) of the lens, and may be as much as 1.4 times that of a conventional light microscope. Lateral resolution is improved due to the exclusion of the out-of-focus light, which detracts from light microscope image quality. The ability to control the depth from which the signal is generated depends on the size of the pinhole and the NA. When the pinhole is fully open, the microscope is no longer confocal.

Optical sections, generated by controlling the $z$-direction, can be stacked to provide three-dimensional reconstruction and lead to three-dimensional animation as the sections are projected at various angles. A shadow is added to provide a greater impression of three dimensions (Blonk & van Aalst, 1993). CSLM is a useful technique for food structure investigations because it is not limited to thin sections. The spatial arrangement of structural components can be assessed and dynamic processes such as coalescence, aggregation and phase separation can be followed (Dürrenburger et al., 2001), and these processes can be correlated to rheological changes.

While not covering dairy products in particular, Vodovotz et al. (1996) reviewed the benefits foreseen in the application of confocal microscopy to food research. Their paper

Table 2.2  Common light microscopy stains for identification of dairy constituents.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stain</th>
<th>Colour reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>Oil red O</td>
<td>Red</td>
</tr>
<tr>
<td></td>
<td>Sudan III</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td>Sudan IV</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td>Sudan black B</td>
<td>Black</td>
</tr>
<tr>
<td>Protein</td>
<td>Amido black 10B</td>
<td>Black</td>
</tr>
<tr>
<td></td>
<td>Methylene blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Alcian blue</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td>Periodic acid–Schiff</td>
<td>Pink</td>
</tr>
</tbody>
</table>

Table 2.3  Some fluorescence stains for light microscopic identification of dairy constituents

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Stain</th>
<th>Excitation-emission maxima (nm)</th>
<th>Fluorescence colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>Nile red</td>
<td>549/628</td>
<td>Red</td>
</tr>
<tr>
<td>Protein</td>
<td>Acridine orange</td>
<td>500/526 (DNA)</td>
<td>Green/red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>460/650 (RNA)</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Rhodamine isothiocyanate</td>
<td>535/610</td>
<td>Red</td>
</tr>
</tbody>
</table>

makes interesting reading as it points out some of the technical challenges of the technique in the early days of its application. One of the earliest publications on confocal microscopy techniques applied to dairy products was by Heertje et al. (1987), who briefly described the structure of margarine, butter, Gouda cheese as well as mayonnaise and dough using Nile blue, 1-anilino-8-naphthalene sulfonic acid (ANS) or fluorescein isothiocyanate as the fluorescent markers for fat or protein. While only supporting existing observations of dairy products, the same researchers clearly indicate the advantage of the technique in resolving fine structural detail below the surface of non-deformed samples, and the usefulness of the confocal microscope to document dynamic events while being observed. In a later paper (Heertje et al., 1990), the ability of the confocal microscope to document the displacement of a fluorescently labelled sodium caseinate by monoacylglycerols and proteins was demonstrated. These rather early papers on confocal imaging elegantly demonstrate the power of the technique to deliver more than static images. Other early works (e.g. Brakenhoff et al., 1988) demonstrate the combination of confocal imaging and computers to deliver three-dimensional imaging in a number of samples including food (mayonnaise). Modern instruments commonly have these functions built into the software that comes with the workstation. The advantages of confocal microscopy in food research were reported by Auty et al. (1999), and they are:

- Fixation and/or dehydration are usually unnecessary.
- Optical sectioning below the surface enables three-dimensional reconstruction of the undisturbed microstructure.
- Fluorescent probes can be used to identify specific food components (Heertje et al., 1987).
- Food microstructure can be continuously monitored.

A very simple ‘environmental cell’ is explained, which allowed the observation of milk protein gelation over a period of up to 3 hours. In a separate experiment, cheese melting on a Linkam C102 warm stage was observed while capturing confocal images, thus demonstrating the dynamic capabilities of the instrumentation.

In a later paper (Auty et al., 2001), a number of fluorescent probes and probe mixtures to be used with either cheese, dairy powders or chocolate are listed along with concentrations, solvents and excitation wavelengths. In both papers the objective was to preferentially label fat and proteins in situ for these products. Figure 2.1 illustrates the dual labelling of fat and protein in processed cheese. Vasbinder et al. (2004) studied gelation in whey protein isolate–casein systems, and correlating gel hardness using a texture analyser and microstructure as imaged by CSLM. Auty et al. (2005) used dynamic CSLM to quantify microstructural changes in micellar casein gels during acidification. Many authors have studied various aspects of cheese structure, which were reported by Buffa et al. (2001), Joshi et al. (2004) and Rowney et al. (2004).

While they do not deal specifically with dairy products, the reader may find the following references valuable for background information before pursuing research involving confocal microscopy (Paddock, 2000; Hollows, 2001; Diaspro, 2001; Matsumoto & Adams, 2002; Hibbs, 2004; Pawley, 2006).
2.2.4 Sectioned material for light microscopy

An alternative approach to imaging fresh mounts is to embed the sample in paraffin or resin, and then section the material. Sections can be stained while they are affixed to the slides. This approach offers several advantages, such as: (1) the depth of field is uniform so that the whole section is in focus; (2) because the thickness is uniform, the density of staining can be used to quantify constituents; and (3) sections are secured to the slide to better withstand the staining procedure, which also facilitates multiple staining. For example, Regand & Goff (2002) stained proteins and polysaccharides in the same section with Amido black 10B and acid-leucobasic fuchsin (Schiff reagent), respectively, to explore the use of biopolymer interactions as a means of retarding ice crystal growth in ice cream.

2.2.5 Cold-stage light microscopy

Cold-stage light microscopy has been used since 1915 to study ice cream, as described by Arbuckle (1960). Ice crystal size distribution and morphology were assessed to relate these features to textural attributes. Early investigators prepared 10-μm hand-sections of ice cream at –23 to –26°C, and photographed the ice cream through a light microscope in the hardening room (Reid & Hales, 1934; Arbuckle, 1940). The latter author quantified ice cream constituents by making measurements, from photographs, of the size and distances between air bubbles and the size and distance between ice crystals. To improve the clarity of emulsion constituents, sections were immersed in solvents with a specific refractive index. A mountant with the same refractive index as one of the ingredients renders that structure optically invisible. Table 2.4 illustrates some examples of refractive indices.

Improvements on the basic technique were made by varying the mountant composition, by cutting sections at lower temperature, and by using polarised light (Berger & White, 1979; Sztehlo, 1994). An example of low-magnification cold-stage light microscope imaging of a squash mount of ice cream showing ice crystals and air bubbles is shown in Fig. 2.2. Characterising ice crystals and air bubbles through light microscopy meant that the effect
of composition, physical properties and processing conditions on ice cream structure and 
texture could be measured (Arbuckle, 1960; see also Chapter 9).

Modifications to the basic method were made by Berger & White (1979), who prepared 
squash mounts of ice cream mounted in the amyl alcohol–kerosene mixture (50 : 50, \( n_\omega = 1.430 \)) at –14.4°C, to measure the ice crystal size. Pictures were taken of sections imaged 
through a Kofler cold stage using polarised light. More recently, a refrigerated glove box 
was designed for the light microscope to image squash mounts of ice cream (Donhowe et 
al., 1991; Chang & Hartel, 2002a,b). Temperatures inside the glove box could go down to 
–20°C and be ramped up or down at 1°C min\(^{-1}\). Controlling the temperature to measure the 
air bubbles was an improvement over those earlier methods that required that the sample 
be warmed up in a well on the light microscope slide (Reid & Hales, 1934) or sectioning 
methods that distorted air bubble morphology (Arbuckle, 1960).

Using a cold-stage light microscope system, the mechanism by which the stabiliser 
effects ice re-crystallisation in ice cream was explored by Regand & Goff (2002). Sections 
40 \( \mu \)m thick from cycled and non-cycled samples were cut at –24°C, and then dispersed in 
iso-amyl butanol on a microscope slide. The slide was transferred to the cold stage (–24°C) 
for bright field imaging. Subsequent computer-assisted analysis of ice crystal morphology 
was conducted on acquired images.

### Table 2.4 Refractive index of ice cream components and solvents for imaging.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Refractive index of constituent</th>
<th>Solvent for imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air bubbles</td>
<td>( n_\omega = 1.000 )</td>
<td></td>
</tr>
<tr>
<td>Ice crystals</td>
<td>( n_\omega = 1.309 )</td>
<td>10 : 15 amyl alcohol and kerosene ( (n_\omega = 1.446) )</td>
</tr>
<tr>
<td>Fat globules</td>
<td>( n_\omega = 1.430 – 1.456 )</td>
<td>Acetone ( (n_\omega = 1.357) )</td>
</tr>
<tr>
<td>Unfrozen phase</td>
<td>( n_\omega = 1.460 )</td>
<td>Ethyl acetate ( (n_\omega = 1.379) )</td>
</tr>
</tbody>
</table>

After Berger & White (1979).

Fig. 2.2 Cold-stage light microscopy of ice cream showing ice crystals (a) and air bubbles (b). Iso-amyl 
alcohol was used to match the refractive index of the serum phase and accentuate the appearance of the 
ice crystals. Data by permission of A. Regand (personal communication) and University of Guelph, Ontario, 
Canada.
2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) has been used to image a wide variety of dairy products including spray-dried milk powders, cheese, yoghurt and kefir following chemical fixation and drying, and products such as whipped cream and ice cream after freezing for imaging on a cold stage.

The electron gun of the scanning electron microscopy will provide a high, stable current in a narrow electron beam in order to generate an image with higher resolution, depth of field and signal-to-noise ratio than is obtainable using a light microscope. Electron sources currently available include tungsten hairpin, lanthanum hexaboride (LaB₆), cold field-emission and Schottky emitters. The selection of the electron source will be determined by the application, i.e. the resolution required. The differences in electron sources can be compared based on brightness, source size and energy spread as detailed in Goldstein et al. (1992).

2.3.1 Electron guns

The most common electron source for SEM is the tungsten filament cathode or tungsten hairpin, and it emits electrons when heated to 2700 K, i.e. the escape potential for tungsten. The Wehnelt cylinder surrounds the filament and is maintained at a potential slightly more negative than the filament. The Wehnelt controls the number of electrons leaving the gun, and focuses the electrons to a crossover point between the Wehnelt and the anode. The anode accelerates the electrons based on the selected voltage (500 eV to 30 kV) from the high negative potential at the source to ground.

The lanthanum hexaboride (LaB₆) electron gun has the same basic cathode/Wehnelt/anode design as the tungsten filament gun. The single crystal of LaB₆, ~ 1 mm in diameter, is polished to a point and then a small polished flat is added to the tip to define the area of emission. The crystal tip is heated to 1700–2100 K, and the brightness of this source is greater than that of a tungsten filament; however, a better vacuum is required.

In the cold field-emission gun a wire of single-crystal tungsten with a sharp point (100 nm) is welded to a tungsten hairpin. Because the point is so fine, the electric field can be concentrated to the extent that, when the filament is negatively biased (3–5 kV) relative to the first anode, the electrons can tunnel through the energy barrier without heat. The result is an electron beam with a high current per solid angle and, therefore, a high brightness. A second anode sets the operating voltage. This gun requires a higher vacuum (< 10⁻⁸ Pa) to support stable emission. Gas atoms collecting at the tip surface impede emission, and the filament must be ‘flashed’ or momentarily heated to 2500 K to clean off adsorbed gas molecules. The advantages of field emission are that the source is so small that minimal demagnification is required to create a 1–2 nm spot and, because the energy spread of the beam is low, performance under low voltage conditions is enhanced.

The Schottky emitter is a field-assisted heated source. Tungsten with its reservoir of ZrO is heated to 1800 K, and the ZrO flows down the tip and reduces the work function from 4.5 eV to 2.8 eV. The high temperature keeps the tip clean, and the flat emitting area provides emission stability. Because the source size is larger than cold field emission, greater demagnification is required to achieve a 1–2 nm spot size.
2.3.2 Cathode comparison parameters

In brief, there are three parameters in comparing cathodes.

1. The brightness (current density per solid angle) is the most important measure of gun performance, and the beam brightness is constant over the length of the column disregarding lens aberrations. Therefore, the level produced by the gun is maintained and, if brightness is lost at the gun, it cannot be regained.

2. The source size is the area from which the electrons are emitted, and is not necessarily the size of the tip. The smallest source produces a beam that requires less demagnification and, therefore, fewer lenses and less chance of aberration. Image resolution is improved by decreasing the spot size without reducing the current, and this is accomplished by increasing the brightness at the source. Field-emission sources are 1000 times brighter than tungsten hairpins and 100 times brighter than LaB$_6$ guns.

3. The electron beam energy spread is the range of electron energies leaving the filament. The cathode design determines the energy spread, which is large from the tungsten source (1–3 eV), and has the greatest effects at low-voltage imaging. A narrow spread in energy enables the production of high-resolution and/or low-energy images.

There is no ultimate best choice in gun cathode design (Goldstein et al., 1992). The choice depends on the user’s requirements and resources available. Recent micrographs of casein micelles imaged through the filtering energy (FE)-scanning electron microscope (Hitachi S-4800) and published by Dalgleish et al. (2004b) illustrate the superior resolution of cold FE-SEM, and the advancements offered to dairy researchers.

2.3.3 Low-temperature scanning electron microscopy

The availability of cryo-sample preparation equipment and cold stage for low-temperature (LT) SEM has facilitated the preparation and imaging of dairy products that are high in water (ice), fat, or air with minimal distortion. The technique is ideal for emulsions including cheese and yoghurt, and foams such as whipped cream and ice cream. Because ice cream is consumed while frozen, it must be examined in the frozen state and, since the ice crystals are already present, the artefact of creating ice crystals and rearranging solutes during the freezing process is less important. For samples prepared from fresh, the freezing rate is extremely important. Ideally, the water in the sample should be vitreous so that no rearrangement of solutes occurs but, as long as the ice crystals cannot be resolved, the freezing rate may be considered sufficient for microscopic imaging. There are many freezing methods available, but probably the most used method for SEM cryo-preparation is plunge-freezing. It is important to know that freezing in liquid nitrogen (−196°C) or liquid nitrogen slush (−207°C) is slow compared to the freezing rate in liquid propane (−189.6°C) or ethane (−183.6°C) when these cryogens are held at their melting point with liquid nitrogen (Robards & Sleytr, 1985). The sample dimension, composition and mass are factors that have an impact on the freezing rate. A fresh sample can be frozen, stored in liquid nitrogen, and then clamped into frozen holders for LT-SEM (Caldwell et al., 1992a) to exclude the
holder from the sample mass during freezing. The use of flammable solvents as cryogens requires the use of a flameproof fume hood.

LT-SEM protocol requires that the sample (2 mm³) be mounted on a copper holder and quenched in liquid nitrogen (−196°C). The sample is kept under vacuum throughout the preparation and imaging to prevent frost on the surface. The sample may be fractured to provide a fresh surface whose topography is dictated by the composition. Frozen samples can be imaged in the fully hydrated condition, because the water is immobilised as ice, or sublimated (usually at −80°C) to remove some ice and further elucidate the composition and structure at the surface of the sample. Figure 2.3 illustrates the effect of sublimation on a fracture surface of ice cream during preparation for LT-SEM. The fracture face is sputter-coated with gold, while frozen, and then examined on the cold stage in the microscope at a temperature below −30°C, at which temperature solutes are no longer mobile.

LT-SEM has provided images of microstructure to study the effect of ingredients and processing on dairy products. The effect of polysaccharide stabilisers on the microstructure of ice cream was qualitatively assessed from micrographs; freezing rate and storage time and temperature were quantified through computer-assisted image analysis by measured changes in ice crystal size (Caldwell et al., 1992b). Brooker (1993) used LT-SEM to study the importance of fat for the stabilisation of the air bubbles in food systems, and suggested that the protein–fat interactions were responsible for the incorporation of air and the stability of the air bubbles. Pelan et al. (1997) studied milk protein emulsions and determined that smaller air bubbles and greater fat adsorption were indicative of stable foam.

The effect of stabilisers on ice crystal sizes and their control over recrystallisation in ice cream were explored using ice crystal size distributions measured from LT-SEM images (Flores & Goff, 1999a,b). Goff et al. (1999) varied the emulsifier content and freezing regimes during the preparation of ice cream to test the effect on fat destabilisation and the composition of the air bubble interface using both LT-SEM and freeze-substituted samples for TEM.

![Fig. 2.3](image)

**Fig. 2.3** Low-temperature scanning electron microscopy of 35 g 100 g⁻¹ fat whipped cream following sublimation at −80°C for 1 hour. Note the fat globules (f) at the air bubble interface (A). Bar = 30 μm.
Chang & Hartel (2002a) compared LT-SEM to light microscopy as a measurement tool for the size distribution of air bubbles. The authors questioned the error introduced by fracturing the sample for LT-SEM because of the assumption that it occurs through the middle of the bubble. If this does not occur, the implication is that a smaller mean size will be calculated. However, it was found that measurement from both techniques agreed ($p = 0.1$), suggesting that either the error is small or that the fracture tends to be through the middle of the air bubbles. LT-SEM was required to image bubbles after storage because the channels that formed could not be preserved with light microscopy (Chang & Hartel, 2002a).

The stability of air bubbles during hardening and storage of ice cream (Chang & Hartel, 2002a) and air bubble development in the batch freezer (Chang & Hartel, 2002b) were measured using both low-temperature light microscopy and LT-SEM. Chang & Hartel (2002b) used the term ‘ripening’ to describe air bubble size development during processing. The term takes into account coalescence, disproportionation and drainage as mechanisms affecting air bubble size; the air bubbles under controlled conditions on a light microscope slide will not directly reflect the changes that occur in the bulk sample because of the surface effects imposed on the air bubbles by the glass slide. A correction factor for bubble distortion was introduced for calculation of the volume of air in foam. In the early part of the study, air bubble measurements from light microscopy and LT-SEM agreed ($p < 0.05$) but, as storage time increased, so did the size of the air bubbles and the discrepancy in measurement between the imaging techniques. Artefacts arose during imaging at –6°C on the light microscope because air bubbles rounded up and because partially coalesced bubbles separated. LT-SEM was found to be a better method for imaging air bubbles in ice cream after two months of storage (Chang & Hartel, 2002a).

### 2.3.4 Environmental/variable pressure scanning electron microscopy

Cryogenic environmental SEM (cryo-ESEM) was used by Fletcher (1997) to examine the structure of ice cream in its ‘native’ state to evaluate artefacts created during preparation for light microscopy and TEM. The chamber of the ESEM can be maintained at a much lower pressure (1–20 torr) than a conventional SEM (10⁻⁵ torr). The hydration state of the sample is controlled by manipulating the partial pressure of water vapour in the ESEM chamber. The problem of ‘charging’, caused by build-up of electrons in the surface of naturally insulating biological samples, is prevented by an interaction between primary beam electron and gaseous molecules, which results in the production of sufficient positive ions to neutralise charge. Ice cream was pre-cooled to –80°C with solid carbon dioxide, fractured and then mounted on copper holders, plunged into liquid nitrogen and transferred onto the microscope stage. This stage was held initially at 0°C to prevent frost build-up while the sample was inserted and then was cooled to –80°C. The stage temperature was raised to –70°C for sublimation. A strict pumping regime must be followed, and the gaseous environment must be carefully controlled; the temperature must be maintained at or below –30°C to prevent rupture of the air bubbles (Fletcher, 1997). Cryo-ESEM made it possible to study the microstructure of ice cream without sputter coating, but beam damage was a problem, and the resolution was less than that with LT-SEM.
2.4 Transmission electron microscopy

2.4.1 Embedded material for transmission electron microscopy

Imaging thin sections (70–90 nm) of resin-embedded dairy products allows ultrastructure to be examined at the magnification and resolution offered by the TEM; images are based on the impedance of the beam by the electron density of the specimen through a mechanism similar to light microscopy. The resolution is determined by the wavelength of the electrons and the beam current. However, the sample must be thin, dry and electron dense and, therefore, requires intensive preparation. Chemical fixation, dehydration and embedding in resin are standard steps to prepare samples for TEM. Resin-embedded samples are a uniform texture that can be sectioned. Ultramicrotomed sections (70–90 nm) provide a basis for imaging, which allows ultrastructure to be scrutinised. Numerous artefacts of sample preparation have been identified particularly because of inadequate fixation of the fat or structural changes caused by the processing (Schmidt, 1982; Liboff et al., 1988; Wilson, 1989; Schmidt & Büchheim, 1992).

2.4.2 Negative staining transmission electron microscopy

Negative staining is a direct approach to imaging dairy proteins or carbohydrates on a solid surface, such as mica or a Formvar-coated EM grid, for TEM (McMahon & McManus, 1998). A typical preparation procedure (Kavanagh et al., 2000) is to place a drop (5 μL) of very dilute solution on the grid and allow the particles to settle. Excess solution is wicked off the grid with filter paper and a drop of stain is applied. Typical stains used include uranyl acetate and phosphotungstic acid (PTA) to increase electron density (Hayat, 2000a). The stain is wicked off and particle features are imaged.

2.4.3 Low-temperature transmission electron microscopy

Freeze-fracture replication

Freeze-fracture (freeze-etch) replication techniques, pioneered in the 1950s, were the first approach to using low-temperature methods to preserve the structure of dairy products for TEM (Büchheim, 1982). A replica of the surface of casein micelles (McMahon & McManus, 1998), and dairy emulsions (Büchheim, 1982; Heerjte et al., 1985) can be studied at TEM magnification and resolution, as demonstrated in Fig. 2.4. This freeze-fracture replication image of ice cream shows an air bubble (Fig. 2.4a) and ice crystals separated by serum phase (Fig. 2.4b) (Berger & White, 1971).

Replica preparation in this example was conducted using a Balzers freeze-etch unit. The steps were conducted with the sample frozen and under vacuum as follows: (1) the sample was frozen at an estimated 100°C s⁻¹, fractured (−100°C), and sublimated for 1.5–2.0 min to remove surface ice; (2) the prepared surface was shadowed with platinum-carbon (Pt-C) at a 25° angle, and then sputtered with carbon at a 90° angle to create the replica; (3) once the replica was made, it was cleaned by numerous acid and alcohol washes; (4) the cleaned replica was lifted onto an EM grid and solvent was evaporated (Berger & White, 1971).
The image in TEM is based on the direction and angle of shadowing. Because the heavy metal is not deposited in the lee of the protruding features, a shadow is created, which appears white in the micrograph. It is important that the shadowing should reflect the surface features faithfully; too much shadowing will give too high a contrast, which will obscure detail, while insufficient shadowing will result in poor contrast and resolution (Robards & Sleytr, 1985).

Many artefacts are inherent in the freeze-fracture replication procedure with most occurring during the initial fracturing step, which results in plastic deformation (Büchheim, 1982) or relocation of solutes and, therefore, structures may be misinterpreted (Heerjte et al., 1985). Condensation of material on the fracture surface can obscure surface details. Etching of the frozen surface by means of sublimation may cause collapse or aggregation of structure (Büchheim, 1982). Replicas can be hard to clean and delicate to handle. Metals shadows may not precisely reflect surface topography.

Low-temperature preparation methods

Whipped cream was suspended in platinum loops and exposed to formaldehyde vapour, and then osmium vapour at 4°C in a low-temperature approach to preserve the foam structure for TEM (Graf & Muller, 1965; Brooker et al., 1986; Anderson et al., 1987). Foams were dehydrated, embedded and polymerised through a conventional temperature regime. Brooker (1985) followed a similar approach to study the ultrastructure of interfacial layers in milk foams, while Schmidt (1982) and Schmidt & Büchheim (1992), in order to minimise artefacts resulting from dehydration and embedding, fixed casein micelles at room temperature, but then dehydrated through decreasing temperature as the concentration of ethanol increased. The procedure was taken further at low temperature by embedding and polymerising the resin at low temperature under UV light (Horisberger & Vautey, 1984;
Horisberger & Rouvet-Vautey, 1984). Structural and functional integrity, as exhibited by the subsequent success of immunohistochemistry of casein micelles in sectioned material, attested to the benefits of low-temperature methods.

A different approach involves temperature fixation/preservation and cryo-ultramicrotomy. This typically involves the very rapid removal of heat, sufficient to preserve the physical structures (and chemistry) of the samples in ways that potentially can be superior to chemical fixation. According to Reid (1975) the first attempt to use cryo-ultramicrotomy on biological material was made by Fernández-Morán (1952), and the early successful cryo-ultramicrotomy involved placing ultramicrotromes into refrigerated cabinets. This was followed by cryo-chambers being manufactured to fit around the sample and knife-holders of commercially available ultramicrotomes. This approach allowed for lower temperatures to be reached and the cryo-chamber units could be easily put on or removed from the ultramicrotome. The promise of fast, convenient and potentially superior preservation of the samples, both chemically as well as morphologically, led to optimism in the wide use of cryo-ultramicrotomy and cryo-TEM in the mid-1970s (Reid, 1975; Glauert, 1975).

A more exotic and costly method employs high-pressure freezing, which is discussed by Studer et al. (1989). For an in-depth review of various cryo-preservation methods, see Gilkey & Staehelin (1986). As with other cryo-techniques applied to high-resolution imaging methods, the first and most significant step is the need to preserve the structure and chemistry by quick freezing. Once adequately frozen, the sample needs to be maintained at cold temperatures during the sectioning process and during the observation and image recording stages in TEM. The most current methodologies have been reported by Reid & Beesley (1991), Bozzola & Russell (1999a) and Hayat (2000b).

Although the technique of cryo-sectioning has been utilised in selected laboratories over the past 30 years, it has not been widely employed for dairy product studies, and it appears to be used mostly for preserving chemical information and the application of energy-dispersive X-ray analysis (Zierold, 1984, 1988, 2002; Wroblewski et al., 1990).

**Freeze-substitution for transmission electron microscopy**

The freeze-substitution and low-temperature embedding protocol has been developed to fix and embed cryo-fixed samples entirely at low temperature. The protocol begins with the freezing step, the rate of which is critical to eliminate the rearrangement of solutes when ice crystals are formed. The ideal result would be vitreous ice but, unless the sample is a monolaye, this is impossible to achieve, a fact that is further complicated by the presence of fat, sugar and air, which slow the freezing rate. The most common approach to cryo-fixation is plunge-freezing in ethane or propane (in a flameproof fume hood). High-pressure freezing may be a better alternative (Robards & Sleytr, 1985; Echlin, 1992). High-pressure freezing takes advantage of the fact that at 212.77 MPa the melting point of water changes from 0°C to –22°C because the water becomes 1500 times more viscous than at atmospheric pressure. The increase in viscosity reduces the ice crystal growth rate such that samples up to 1 mm³ can be vitrified (a depth of 0.5 mm). It should be noted that high-pressure freezing does not increase the freezing rate (Echlin, 1992); a freezing rate of 1000°C s⁻¹ is actually sufficient to control ice crystal size. The high-pressure freezer must apply pressure a millisecond before
the cooling process takes place to ensure that the tissue is not damaged by the application of pressure; the timing is critical (Robards & Sleytr, 1985).

Once frozen, the sample is immersed in a solvent–fixative mixture, and maintained below the recrystallisation temperature of water (Robards & Sleytr, 1985). Traditional fixatives, namely glutaraldehyde, osmium tetroxide and uranyl acetate, may be dissolved in the solvent. These fixatives are carried to the ice–solvent interface to stabilise constituents, and enhance electron density after the solvent substitutes the ice and the system is warming up (−40 to −20°C). After sufficient fixation, the solvent–fixative solution is replaced with a low-temperature resin, which is low in viscosity, can tolerate some water in the sample, and may be polymerised at temperatures as low as −80°C (e.g. Lowicryl HM23). Freeze-substitution and low-temperature embedding protects the structure of dairy constituents making it possible to image dairy emulsions and foams such as whipped cream (Smith et al., 2000a,b) and ice cream by TEM (Goff et al., 1999). The procedure provides a substrate for specific staining, including immuno-gold labelling, for identification of β-lactoglobulin and κ-casein in ice cream.

Freeze-substituted samples prepared for electron microscopy can also be sectioned and stained for light microscopy. For example, Regand & Goff (2002) used the freeze-substitution method to study the effect of biopolymers on ice recrystallisation. Thin sections of ice cream (90 nm) were stained with silver proteinate or uranyl acetate–lead citrate. Sections for light microscopy (0.5 μm) were stained using periodic acid–leucobasic fuchsin and Amido black 10B to differentially stain the polysaccharides and proteins, respectively. Glutaraldehyde (3 mL 100 mL−1; 6 mL of 50% stock – the concentration of the original glutaraldehyde is important in order to limit the amount of water) in 100 mL methanol was employed as the substitution solution to avoid the loss of binding sites to osmium tetroxide, leaving them available for subsequent staining. The resin used for low-temperature embedding was LR Gold plus an initiator, benzil (0.1 g 100 g−1). This resin proved to be less brittle than Lowicryl HM23 and less hydrophilic and, therefore, better suited to post-staining techniques.

Cryo-transmission electron microscopy

Direct imaging of frozen samples by cryo-TEM is the ultimate approach to the study of very small and fragile dairy-based structures. Waninge et al. (2003, 2004) studied milk membrane lipid vesicle structures and fat globule membrane (FGM) structure using cryo-TEM. A thin film of liquid (20 to 200 nm) was prepared on a carbon film supported by a copper grid and was frozen by plunging into liquid ethane. The preparations were stored in liquid nitrogen and transferred to the cryo-stage (−180°C) of the transmission electron microscope. The microscope was equipped with a post-column energy filter to provide contrast since no contrast-enhancing chemicals are used in the preparation. The authors explored interactions between milk proteins and membrane lipid vesicles. Casein micelles and aggregated β-lactoglobulin are visible, but adsorbed layers of low-density proteins may be present and cannot be observed because of the lack of contrast. The authors point out that the only possibility of staining for cryo-TEM is immuno-gold labelling (Waninge et al., 2003).
2.4.4 Energy-filtering transmission electron microscopy techniques

In conventional TEM (CTEM) mode, one is most interested in the ability to separate an elastically scattered electron signal from the unscattered electrons as a means of achieving maximum contrast in the resulting image. This is typically achieved by enhancing the sample’s ability to scatter electrons by the introduction of heavy metals (Hayat, 2000c), and then selecting a suitable objective lens aperture (smaller objective for increased contrast). The result is the removal of scattered electrons from the image signal (dark areas) and the inclusion of the unscattered electrons in the image signal (bright areas).

There is a third class of interactions between the primary electron beam and the sample, referred to as inelastic electrons. This class represents primary beam electrons that have interacted with electrons indigenous to the atoms of the sample. Unlike the elastically scattered electrons, the inelastically scattered electrons are only slightly diverted from their path through the sample and do contribute to the final image signal. In CTEM, the inelastic component of the image signal results in additional noise and loss of image clarity. The EF-transmission electron microscope is equipped with energy-filtering capabilities in order to take advantage of the situation by its ability to select which class of electron interactions to not only image but additionally record as a spectrum. When the primary beam electrons are inelastically scattered due to their interaction with a specimen electron, they lose a specific amount of energy to the specimen electron, which is specific to the atom and electron shell involved in the exchange. This means each inelastic scattered electron carries specific information relative to the specimen atoms. EF-transmission electron microscopes are equipped with electron energy loss spectrometers either built into the optical column (Zeiss/Libra®) or as attachments below the viewing screen (Gatan/GIF or Tridiem/GIF 2000). The spectrometers separate the electron signal based on electron energy and, thus, allow new information to be acquired above and beyond that with CTEM. The early studies on energy filtering are referenced by Reimer et al. (1990) in an article describing the capabilities of an in-column EF-transmission electron microscope.

Global imaging

In global imaging mode, the EF-transmission electron microscope functions in much the same manner as a conventional instrument, as both unscattered and inelastically scattered electrons are used to create the final image. In this mode, the instrument is capable of doing everything the conventional instrument can do.

Contrast enhancement

In simplest terms, the EF-transmission electron microscope utilises a spectrometer to separate the electron signal based on electron energy and a slit aperture, in the projector lens system, to define which electrons’ energies are to be used for imaging. By removing the image-deteriorating inelastically scattered electrons, and selectively utilising the unscattered electrons, a high-contrast image is produced. An example of contrast enhancement is shown in Fig. 2.5.

While such contrast enhancement is an appreciated benefit when observing conventional sectioned and stained sections, its true strength is demonstrated when working with unstained
immuno-labelled material. Here the simultaneous need to easily discern small gold particles while still being able to visualise underlying morphological structures is often problematic. With EF-TEM and selective signal selection, the gold particles can be viewed by unscattered electron imaging and the morphology viewed by selecting the inelastic electrons associated with the carbon-specific absorption edge ($\delta E = 284$ eV).

**Thick section imaging**

While much of the investigation in dairy microscopy (biological research) is performed on essentially two-dimensional material, i.e. sections for both light microscopy and TEM, there are times when a three-dimensional image is beneficial. Due to the limitations inherent in CTEM, extraordinary efforts are typically required to obtain the three-dimensional information. Either the tedious exercise of serial sectioning and reconstruction of individual images into a ‘complete’ image is required, or access to a high-voltage transmission electron microscope is needed in order to penetrate and view thick sections. The EF-transmission electron microscope is capable of viewing thick sections at high resolution and contrast by again selecting only the electron signal which fulfils this need.

Typically a thick section results in extensive numbers of inelastically scattered electrons in the image signal, resulting in low contrast and compromised resolution. With EF-TEM, a portion of the inelastic signal, which produces the desired contrast, can be selected to produce the final image. The process is called ‘contrast tuning’ and, once high-quality imaging of thick sections is possible, then stereo pair imaging can reveal a true three-dimensional image of the samples. Figure 2.6 demonstrates stereo pair imaging of a cheese spread.

**Frozen hydrated specimens**

There are a number of benefits to utilising frozen hydrated sample preparation techniques for some dairy products. Observing milk or milk components by CTEM requires the typical fixation, dehydration, embedment, sectioning and staining regimen common to everyone
utilising conventional methods (Glauert & Lewis, 1998; Hayat, 2000d). These methods have some significant drawbacks when processing materials with high water content. Retaining the original structures and their orientation to one another is very difficult if not impossible using the conventional methodology. A number of techniques have been described in the literature in attempts to minimise the displacement of components during processing (Henstra & Schmidt, 1970; Allan-Wojtas & Kaláb, 1984; Veliky & Kaláb, 1990). A comparison between some of the earlier techniques and the use of microcubes was described by Alleyne et al. (1993). Each of the techniques has its advantages and disadvantages relative to ease of use and reproducibility. Kaláb & Larocque (1996) demonstrated that the original distribution of casein micelles and fat globules are not suitably preserved with gel encapsulation methods, and they suggested the only methods at that time suitable for milk and cream was cryo-fixation followed by freeze-fracture and replication.

When the interest is in inherently small components such as casein micelles, whey proteins, gums and/or liposomes, the use of frozen films should be considered. The technique has the advantage of being relatively quick (once mastered) and preserves the native state of the material in ways not possible with chemical preservation. The technique has been used more extensively in the area of virus and nanoparticle studies based on techniques first described by Adrian et al. (1984) and, more recently, a published study by Lam et al. (2004). In order to be successful, the technique requires accessories that make it possible to quickly freeze small amounts of sample directly onto TEM grids as very thin films. These grids are best prepared specifically for cryo-film purposes when the windows of the grid are covered by a thin perforated film. Methods for producing such films are described by Fukami & Adachi (1965) and Jahn (1995). The best imaging is achieved over the holes of the film and in regions of thin vitrified liquid.

Fig. 2.6 Stereo pair energy images of thick section of soy protein-based cheese spread imaged with a Zeiss 912AB EF-TEM operating at 120 kV. Bar = 0.5 μm.
Cryo-preparation instruments are available from vendors, such as Zeiss SMT AG and Leica Inc., and typically employ an apparatus for holding a TEM grid and plunging it forcibly into a cryogen. Once adequately frozen, the grid containing the thin film is transferred to the microscope while maintaining temperatures at –140°C to –170°C utilising a specially designed ‘cryo-rod’. The instrument itself must be equipped to handle frozen samples and maintain the minimum temperatures while exposing the sample to an electron beam. Microscope software can help by utilising low-dose imaging techniques and allowing for ‘off field’ focusing. Major complications in high-quality imaging result from the fact that biological materials, with their low atomic weights, produce very few elastically scattered electrons (there is no post-staining in cryo-prepared films) and, due to the high water content in frozen films, the vitreous ice produces excessive numbers of inelastically scattered electrons. The resulting image quality can be significantly improved by the use of energy filtering techniques primarily designed to give maximum image contrast by removal of inelastically scattered electrons. Less dependence on under-focused imaging results in fewer artefacts in the final image. The energy filtering capabilities of the EF-transmission electron microscope allows sufficient contrast of the unstained biological material as well as permitting image focus and capture with low-dose exposure by the electron beam. Figure 2.7 demonstrates the use of EF-TEM to image intact frozen casein micelles in skimmed milk.

**Electron spectroscopic imaging**

In addition to permitting the selection of electron energies to be used in image quality improvement, the EF-transmission electron microscope additionally lends itself to producing spectrographs of the energies. In a process known as electron spectroscopic imaging (ESI) a spectrum is collected of the energy loss electrons, which are specific to the atoms and chemical bonds resident in the sample. From the spectrum, an element of interest can be selected, and the

![Fig. 2.7](image)

**Fig. 2.7** Casein micelles in a frozen film of skimmed milk obtained in high-contrast image mode with a Zeiss 912AB EF-TEM. Bar = 200 nm.
region of the spectrum specific to the element of interest can be captured, background subtracted and redisplayed over the ‘morphological’ image. The result is an elemental map at very high spatial resolution measured in nanometres. Although the technique of ESI has been published in material science (Du Chesne et al., 1994) publications in dairy-related studies appear to be lacking. An example of the technique of ESI is seen in a model cheese in Fig. 2.8.

2.5 X-ray microanalysis

X-ray microanalysis is one of the older methods of accessing chemical information from what is commonly thought of as an imaging instrument in the biological disciplines. Founded on SEM, TEM and scanning transmission electron microscopy (STEM), the combination of imaging and chemical analysis is a powerful tool. In its simplest description, one uses the electron beam to acquire a high-resolution image and to act as an energy source capable
of generating characteristic X-rays of the region of interest. Although more complex in the
details of correctly performing this type of analysis, it is possible to detect calcium at a
concentration of less than 0.1 g 100 g⁻¹ in bulk samples using SEM (Postek et al., 1980).

There are a number of resources which give a general description of X-ray microanalysis
(Postek et al., 1980; Bozzola & Russell, 1999b) as well as more complete descriptions
(Garratt-Reed & Bell, 1993; Warley, 1997). A very practical but quite complete treatment
of the subject based on the Lehigh University (Pennsylvania) microscopy course on SEM
and X-ray analysis can be found in Goldstein et al. (2003).

Here, we cover only the most general aspects of the technique. A number of significant
events occur when an electron beam interacts with a sample. The production of secondary
electrons is commonly used for imaging in SEM; the selective deflection of the primary
beam electrons is commonly used for imaging in TEM and STEM. However, some of the
primary beam electrons interact with the atoms of the sample, which results in the dislodg-
ing of sample electrons. The electron shell, which is now devoid of an electron, is quickly
repopulated by an electron from a higher-level (more energetic) shell of the same atom. In
the process, the excess energy is released as an X-ray of a specific energy that is character-
istic of the difference between the vacated shell electron and the replacement shell electron.
By measuring the energy of the generated X-rays, it is possible to determine the elemental
composition of the region being irradiated by the primary electron beam. The method can
be both quantitative as well as qualitative, and can be performed quickly on bulk samples
for determining general elemental composition or can be used to create elemental maps,
which may be combined with morphological images. For robust samples, the technique is
considered non-destructive.

In addition to the above-mentioned microscopes used to generate X-rays, the technique
requires an X-ray spectrometer/detector mounted on the column of the instrument in
close proximity to the sample, and an analyser for quantification and displaying the data.
Typically, but not always, a liquid nitrogen dewar is associated with the detector in order to
maximise signal-to-noise ratio and to maintain stability of the detector crystal. A number
of well-defined rules set the lower and upper limits of the system to detect elements and
another set of physical/geometric guidelines help to maximise the count rate or efficiency
to collect adequate X-rays. Sample preparation is critical for controlled experiments when
quantitative or even semi-quantitative analysis is the goal.

2.6 Rheology

A thorough understanding of rheology as an important quality attribute is essential to the
development of dairy products. Texture is a leading factor in consumer preference, and is
directly related to the structure and microstructure of the product. Although the rheology of
cheese (Tunick, 2000; Lucey et al., 2003) has been measured most extensively at all stages
of production, a range of dairy emulsions and foams such as whipped cream (Smith et al.,
2000a,b), ice cream (Briggs et al., 1996; Granger et al., 2004; Vega & Goff, 2005) and yoghurt
(Özer, 1998; van Marle et al., 1999; Tunick, 2000) have also been widely studied.

Rheology is the measure of deformation or flow of materials in response to shear; solids
deform and liquids flow. One end of the rheological continuum is defined by the ideal solid
(Hookean solid), which exhibits ideal elastic behaviour. When an ideal solid is subjected to stress (force area\(^{-1}\)), it exhibits proportional deformation (strain). This response occurs when an elastic solid is deformed by low strain. A non-ideal solid is characterised using large deformation, such that stress is not proportional to strain as defined by the *apparent modulus* (equation 2.1). Large deformation measurements are more appropriate for the study of rheological properties of solid foods.

\[
\text{Stress} = \text{modulus} \times \text{strain}
\]

\[
\tau = G \times \gamma
\]

At the other end of the scale is the ideal liquid (Newtonian liquid); Newtonian liquids exhibit a linear relationship between stress (\(\tau\)) and shear rate (\(\frac{dv}{dy}\) or \(\dot{\gamma}\)), i.e. viscosity is independent of shear rate as shown by equation 2.2 (http://www-unix.oit.umass.edu/~mcclemen/581Rheology.html).

\[
\text{Shear stress} = \text{viscosity} \times \text{strain rate}
\]

\[
\tau = \eta \times \dot{\gamma}
\]

The viscosity constant \(\eta\) is a measure of the friction between the layers of the liquid that shift according to applied force (http://www-unix.oit.umass.edu/~mcclemen/581rheology.html). The force is generated by the rotation of a top plate such that a decrease in resistance to force causes the top plate to rotate faster. Therefore, the change in shear stress is determined by the rate of strain (\(\dot{\gamma}\)). Non-Newtonian fluids exhibit a non-linear response to shear stress and the viscosity constant is referred to as apparent viscosity.

Dairy products are viscoelastic materials including both solid properties (elastic) and liquid properties (viscous). Instruments for measuring viscoelastic properties vary depending on whether the product is best suited to compression or elongation test methods or if the product is subjected to shear (dynamic tests) to measure its rheological properties. Compression/elongation methods are conducted on solid or semi-solid foods, for example, cheese (Boutrou *et al.*, 2002) and ice cream (Sofjan & Hartel, 2004). For these measurements, a Universal Testing Machine (Instron) has replaced early empirical test equipment, including compressors, penetrometers and curd tension meters, which were used in such a way that experimental variables were not consistent and, therefore, results were not comparable between laboratories (Tunick, 2000). The Universal Testing Machine applies force with a moving probe to a sample mounted on a fixed plate. Measurement variables include sample size, temperature, design of the probe (plate, blade, spike, etc.), and the magnitude, speed and duration of the force. Using this equipment, the relationship between stress at applied strain produces a graph where the slope equals the elastic modulus (equation 2.1). If force is applied until the sample ruptures, a yield stress can be measured or, if force is applied repeatedly, sample response can mimic first bite–second bite deformation of the food (texture profile analysis) (Tunick, 2000). Stress or strain versus time can be measured to study the time required for relaxation of structure following a given deformation (stress relaxation) or change in strain with time under constant stress (creep). As long as the fundamental parameters are carefully set, results are comparable with those from other laboratories.
The rheological properties of dairy products that exhibit a more liquid (viscous) behaviour are commonly measured by dynamic experiments during which a sinusoidal stress is applied, and strain responses recorded using a controlled stress rheometer (or strain is applied and stress recorded in a controlled strain rheometer). During small amplitude oscillatory testing stress is applied at maximum amplitude (τ₀) and angular frequency (ω). The amplitude of the test must be small enough that the material is unaltered by the applied force, which means that the test is conducted in the linear viscoelastic range (LVR). The standard approach to finding the LVR is to run a torque sweep and identify the linear area on the curve. Once the LVR is established a frequency sweep can be run using a force that will not break the structure. In the LVR the frequency of the strain will equal that of the stress but the material will alter the phase (δ). The phase change of the strain at maximum amplitude is a measure of viscoelastic properties as defined by the complex shear modulus G where \( G^* = G' + iG'' \). The parameter \( G' \) represents the energy stored by the material (elastic modulus); for perfectly elastic material \( δ = 0° \). The viscous or loss modulus \( G'' \) represents energy lost per cycle (viscous modulus); for perfectly viscous material \( δ = 90° \). The equation (Gunasekaran & Mehmet, 2000) defining the stress response to sinusoidal strain of a material measured in its LVR is:

\[
σ(t) = γ_o G'(ω)\sin(ωt) + γ_o G''(ω)\cos(ωt)
\]  

(2.3)

where \( G' \) and \( G'' \) are frequency-dependent functions.

Complex viscosity (\( η^* \)), the fundamental parameter of flow, calculated from \( G^* \) (complex modulus)/frequency (\( ω \)), and tan \( δ(ω) \), which represents the balance between elastic and viscous components, is measured by \( G''/G' \). It is important to be able to separate the elastic and viscous parameters in viscoelastic dairy products to be able to better assess product development variables.

The shear properties of materials ranging from ingredients to the final product can be tested in a number of configurations to determine response as a function of time, temperature, shear rate or frequency (http://www-unix.oit.umass.edu/~mcclemen/581Rheology.html). Configurations include the concentric cylinder, parallel plate, and cone and plate. The concentric cylinder design accommodates fluid samples by placing the liquid in the gap between two cylinders and applying a constant torque (angular frequency). The viscosity of the liquid affects the rate of rotation of the inner cylinder (rate of strain). The torque can be ramped so that viscosity or elastic modulus can be measured in response to shear stress (http://www-unix.oit.umass.edu/~mcclemen/581Rheology.html). An experiment run with the parallel plate design requires that the sample be placed in the gap between two plates; stress is applied by angular rotation of the top plate. The cone and plate design is similar except that the top plate is a cone designed to provide uniform shear across the gap. The parallel plate accommodates a larger volume, but not uniform shear.

Rheological properties of dairy materials can be measured by low-amplitude oscillatory shear following creep, flow, or stress/relaxation protocols (Steffe, 1996).

To measure creep recovery, constant stress is applied and strain is measured over time. The result is a measure of compliance \( J \) where \( J = \text{strain/stress} \). Creep recovery (change in strain) can be measured when stress is removed. Constant force, applied to a viscoelastic material resembling a solid, results in a maximum value for \( J \) after extended time. When the force is removed, compliance approaches zero. If the viscoelastic material has greater
viscous properties, compliance continues to increase under constant force and the material does not recover when the force is removed.

To measure stress-relaxation (step strain), the sample is subjected to instantaneous strain, which deforms the sample. The stress required to maintain deformation is measured as a function of time. Relaxation extremes are represented by ideal elastic materials, which do not recover, and ideal viscous materials relax immediately when strain is removed. Viscoelastic solids relax to an equilibrium point determined by the microstructure of the material, while viscoelastic liquids will continue to a zero stress point. Since each elastic component may have a different relaxation time, the measurement is an overview of the properties of the product. It is difficult with an unstable or non-homogeneous material to establish equilibrium, and also to remain within the LVR under large deformation conditions (Steffe, 1996). The stress-relaxation modulus is determined by:

$$G = f(t) = \frac{\sigma}{\gamma_{\text{constant}}}$$ (2.4)

To measure start-up flow (stress overshoot), a standing viscoelastic fluid is subjected to shear rate, which causes the material to overshoot the equilibrium value, emphasising the elastic response. The material returns to a steady state with time. Flow tests serve to simulate fluid thickness perception by humans to the material in the mouth (Steffe, 1996).

It is important to monitor the rheological character of dairy products during product development and storage and correlate results to changes in microstructure.

### 2.7 Light scattering

#### 2.7.1 Laser light scattering

The diameter of milk fat globules, proteins and protein-polysaccharide aggregates are examples of dairy product constituents that can be measured by laser light scattering (LLS). The ability to measure constituents is integral to understanding the dynamics of change in dairy products and contributes to the design of new functional ingredients.

Light-scattering instruments use a laser beam which emits electromagnetic radiation of given wavelength with a defined momentum and energy. These parameters gain or lose energy or momentum when scattered by particles in the light path (Dalgleish & Hallett, 1995). The extent of motion affects the change in energy or frequency but, because frequency shifts caused by Brownian motion are small, any energy difference between the incident and scattered light can be ignored. The change in momentum of photons is significant and provides a momentum transfer vector or scattering vector ($Q$), which is the value of the difference between scattered ($k_s$) and incident ($k_0$) wave vectors (Dalgleish & Hallett, 1995) as expressed by:

$$Q = k_s - k_0$$ (2.5)

The most common scattering angle is 90°, and therefore, the magnitude of the scattering vector is:

$$|Q| = \frac{4\pi n}{\lambda} \sin \left( \frac{\theta}{2} \right)$$ (2.6)

where $n$ = refractive index of the medium, and $\theta$ = scattering angle.
$Q$ has the dimensions of inverse length and sets the distance scale that will be probed by the light scattered at $\theta$. Because light scattered at a low angle results in a relatively large value for $Q^{-1}$ (1 μm), this value provides information about larger-scale structural and dynamic properties of particles in the micrometre range. When light is scattered at a high angle, the value for $Q^{-1}$ is smaller ($\leq 0.1 \times 10^{-6}$ m). From this value, smaller-scale properties of the particles are revealed by their effect on the scatter of the light (Dalgleish \& Hallett, 1995).

Static light scattering (i.e. integrated light scattering), which is possibly the most widely used light-scattering technique, is low-angle diffraction using equipment such as the Malvern Mastersizer X (Malvern Instruments Ltd). This instrument measures particle size by integrated laser light scattering based on the fact that particles in suspension scatter light. The scattering pattern is dependent on the intensity of the scattered light as a function of the scattering angle ($\theta$) between the incident and the scattered beam. The intensity of scattered light depends on the size of the particles, the index of refraction and the wavelength ($\lambda$) of the incident light (Corredig, 1998). The technique has been advanced by the availability of helium-neon lasers.

The result of light-scattering measurements can be presented as a size histogram, as shown in Fig. 2.9, and to give an intensity-weighted distribution, such that amplitudes represent the amount of light scattered at each particle size. Furthermore, amplitudes are related to the number of particles in each size category. Using a reverse Fourier optic configuration, particle sizes down to 0.1 μm can be measured. The sample must be dilute enough that an incident photon be scattered by each particle only once.

A value calculated to characterise the changes in average fat globule diameter is $d_{3,2}$, called the ‘volume-surface diameter’. This value is calculated by dividing the average particle volume by the average particle surface area. The distribution of particle sizes represented by the histogram is mathematically reduced to a single parameter to represent the population.

![Fig. 2.9 Fat globule size distribution in whipping cream presented as a histogram and cumulative fat content (background) measured by integrated light scattering using the Malvern Mastersizer X.](image-url)
Small particles measured at the end of the range (close to 0.1 μm) may actually be outside the sensitivity of the equipment and it is possible that a greater proportion fall into this size range than are reflected by the data. It is assumed that there is no interaction between the particles and that the particles are spherical. The equipment cannot be used to quantify the contribution of membrane proteins to the volume of globules because of a lack of sensitivity.

2.7.2 Dynamic light scattering

The dynamic light scattering (DLS) technique is also known as photon correlation spectroscopy, and uses a laser beam, of given frequency, to measure the size of particles in Brownian motion. DLS relies on the fact that particles are in motion when they scatter the light; particles will scatter light differently with time because smaller particles move with higher velocity and cause a greater shift in light-scattering frequency than larger particles. The difference in frequency is called a Doppler shift. A measured change in frequency can be used to determine differences in particle size for particle ranging from a few nanometres to a few micrometres. Because DLS is more sensitive than LLS, the technique can be used to measure smaller particles in milk-based systems, such as casein micelles (50–500 nm). Changes in micelle size, caused by the use of rennet, have been used to characterise the interaction of micelles with κ-casein.

The DLS equipment can be configured in two ways: homodyne or heterodyne. The homodyne detection configuration only allows light from the sample to reach the detector. Particles moving toward the detector give positive Doppler shifts and those moving away give negative Doppler shifts. In the heterodyne configuration, the light from the laser light source is mixed with light shifted by the particles. The source light is reflected to the detector and serves as a baseline for the frequency-shifted light. The interference between incident and shifted light removes the high-optical frequency component, leaving the lower values that can be related to particle size (Dalgleish & Hallett, 1995).

Light-scattering techniques, including integrated light scattering (ILS) and DLS, require clean, dilute, transparent samples and long experimental runs. The best results require single scattering events, such that each particle scatters the light once. It is not possible using this technique to study processes, for example, gelation or interactions of biopolymers; further, dilution of a food system to make it suitable for testing can change the physical properties of the particles (Dalgleish & Hallett, 1995). Light scattering is useful for quick, non-invasive measurement of dilute food systems to determine particle size distribution (see diffusing wave spectroscopy, section 2.11.2).

2.8 Nuclear magnetic resonance spectroscopy

In its simplest form, the technique of nuclear magnetic resonance (NMR) spectroscopy involves placing a sample in a magnetic field, exciting the atoms of the sample to a higher energetic state, then recording the signal created when the atoms return to their more stable state. For a complete explanation of the technique and the various forms of NMR spec-
troscopy, the reader is referred to Eads (1998). NMR measures the magnetic properties of atomic nuclear spins. As stated by Eads (1998),

‘This magnetic behaviour is determined by molecular and ionic structure, motion and interactions. These in turn are determined by chemical composition, distribution of mass among different phases (solid, viscous, liquid), molecular mobility (rotational and translational diffusion), and chemical and physical changes in food material.’

Since radio frequencies are used to probe these properties, the food material need not be transparent and indeed requires little manipulation before analysis.

Colquhoun (1993) provides the following list of NMR active nuclei of interest in foods: hydrogen, deuterium, carbon-13, nitrogen-14, nitrogen-15, oxygen-17 and phosphorus-31. Examples of the use of NMR in food research can be found in the collections of presentations of the International Conference on Applications of Magnetic Resonance in Food Science (Belton et al., 1995, 2003; Webb et al., 2001; Engelsen et al., 2006). The application of NMR techniques to dairy products has been reviewed by Wahlgren & Drakenberg (1995) and Belloque & Ramos (1999). According to Belloque & Ramos (1999) the nuclei most commonly used for dairy research are hydrogen, carbon-13 and phosphorus-31, with hydrogen and carbon-13 commonly being used for milk fat, and phosphorus-31 for phosphorylated compounds such as phospholipids, phosphorylated carbohydrates and phosphoserine. The authors further reference literature which shows the benefit of using NMR for the study of in-vivo reactions (incubation of micro-organisms), physical state of water in rehydration of caseins, characterisation of carbohydrates such as oligosaccharides and even the study of rather large molecules such as β-lactoglobulin in whey proteins.

NMR is a unique analytical tool for non-destructively characterising not only the structure of specific molecules, but the molecule’s relationship to its environment; thus providing insight into food structure/function relationships at atomic resolution.

### 2.9 Digital imaging and image analysis

Although not strictly a microscopy technique or restricted to dairy research, image analysis has become an important part of scientific research, and a small section on the topic seems appropriate for this techniques chapter. It is probably a safe assumption that most modern researchers in microscopy as well as numerous other disciplines have long ago made other uses of the previous darkroom facilities and replaced this function with digital cameras, software and computers. Indeed, digital imaging has not only replaced conventional darkroom functions, but also opened up new capabilities for acquiring data, manipulating images and blurring the lines between traditional microscopy and chemical analysis.

For the purposes of this chapter, only a brief explanation of image processing will be undertaken, and the reader is encouraged to use the references to acquire the more in-depth aspects of the topic as needed for their work. An excellent introduction to the basics as applied to SEM and TEM is provided by Bozzola & Russell (1999c).

As stated by Russ (1995) in the introduction to his well-received book on the topic, ‘image processing is used for two somewhat different purposes: (a) improving the visual appearance of images to a human viewer, and (b) preparing images for measurement of the features and structures present.’ While both of these statements could be true for traditional
photographic and darkroom operations, the following section will be directed to digital systems and computer-assisted image processing.

### 2.9.1 Hardware

There are a number of ways to incorporate computer-assisted microscopy into the research laboratory. In fact, it is unlikely that one starts out by going totally ‘digital’ in a single event. A not uncommon practice might be to use traditional image capture and printing methods, followed by scanning such images to reproduce the images in a digital format for purposes of storage, or publication. This is a very inexpensive first step since the investment in cameras and darkrooms has already been made. Converting light microscopes to accommodate digital cameras then brings up considerations of camera specifications, computer and software choices and, at some point, the need to archive, organise and retrieve images either at a stand-alone workstation or in a networked arrangement. The process of buying new instruments for SEM or TEM today will necessitate the introduction to digital data requirements, as would the purchase of many of the other microscopy/imaging tools covered in this chapter such as confocal, atomic force and spectroscopy instruments.

Digital cameras are designed to fill specific needs, such that one would not expect a camera suited for bright-field optics and colour to also work for TEM applications. But even in the realm of light microscopy, there are some cameras designed for bright-field colour imaging that would be unsatisfactory for fluorescence imaging or for high-speed video imaging. It is best to discuss one’s needs and expectations with a number of camera vendors before making a choice. In some situations, the camera and associated software need to be thought of as a package, while other cameras can be accommodated by a variety of software packages; again a discussion with the vendor is warranted.

### 2.9.2 Software

As mentioned, the choice of digital camera and software may go hand-in-hand, while at other times they can be obtained separately. A major consideration in imaging software selection is the desired endpoint: capture images only, capture and archive, or a more fully featured software package that will capture, archive, process and perform sophisticated image analysis functions. A free image analysis software program (NIH Image) has been available for some time for Macintosh users and is downloadable from the internet (http://rsb.info.nih.gov/nih-image/). A similar program (ImageJ) is also available from the same website for Windows and Linux users. If familiarity with commercially available software such as Photoshop® already exists, then plug-in software for imaging functions may be a logical choice, such as Fovea Pro by Reindeer Graphics Inc. If a stand-alone software package is desired, then programs such as ImagePro® by Media Cybernetics Inc., AnalySIS® by Soft Imaging Systems GmbH or MetaMorph® by Molecular Devices Corp. may be appropriate.

Some benefits and consequences are:

- Once the initial awkwardness of the using digital imaging versus photographic imaging passes, most would agree there are some significant advantages.
• Speed and ease of inserting images into manuscripts or e-mails.
• Convenience; ease of storing, retrieving, copying of images.
• Costs: after the initial investment in hardware and software, there are no chemicals or papers to purchase.
• Health: chemical disposal, contact dermatitis.

However, other considerations are: (1) costs associated with keeping up with changes in computer operating systems, software upgrades and camera obsolescence; (2) the advancement in these new systems occurs in months or relatively short years; (3) for laboratories with several digital workstations, the cost of maintaining upgraded systems should be factored into the budget.

For the basic steps in image analysis, the increase in computer speed and sophistication of the software make it now relatively easy to perform analyses, which in the past would have been considered sizable undertakings in time and effort. Having stated that it is relatively easy to perform image analysis with modern systems, it is by no means a turnkey operation. There are a number of excellent books that cover a wide range of detailed considerations for applying image analysis, of which a few are listed here:

• An excellent introduction to the concept of using images in combination with computers for the purpose of measurement (Russ, 1990).
• One of the best recognised texts covering the topic in a very readable format. The major steps in image analysis are introduced and sufficient depth is available for those interested in greater detail on any specific analysis technique (Russ, 2002).
• In a book specifically written with the food scientist in mind, the major steps of image analysis are covered by using examples of a broad range of food materials. Also, the book would be of significant benefit for food scientists faced with addressing questions covering a broad range of food types (Russ, 2005).

2.9.3 Major steps in applying image analysis

Image acquisition

Acquire/input refers to any of a number of functions resulting in a digital file representing an image being stored in the computer’s memory. The acquisition could be from an existing image or photograph being scanned by a flatbed scanner or it might be an image digitised directly from a light microscope, by SEM, TEM, or by any other imaging tool. One typically thinks of the acquired image as the ‘true’ representation of the subject of interest. It could be in colour or grey scales.

Calibration

Calibration refers to the process of defining a size or colour scale that can be correlated back to the image for purposes of quantifying distance, area, brightness or colour value. Without a calibration step, the following image analysis is often restricted to qualitative relationships; a notable exception would be counting objects.
Image enhancement

Image enhancement commonly involves modifying the original image to help define the regions of interest. A number of adjustments, such as contrast, sharpness, background correction and edge enhancement, are typical of this step. While the aim of image enhancement is to increase the ‘visibility’ of the objects of interest, it likewise necessitates the reduction or loss of information from other regions of the image. It is routine practice to perform this and subsequent steps on a copy, not the original image file.

Segmentation or thresholding involves a number of steps that help define the objects of interest and separate them from all other regions of the image (background). In its ultimate form, this results in a black and white (binary) image. Most commonly the objects of interest are black and the background is white. It is not uncommon to get to this point in the process and realise the original image acquisition was not optimised as much as possible to allow for a clean thresholding process. There are a number of steps that can be performed on the binary image to further prepare the image for the next step of counting or measuring, such as erosion, dilation or skeletonisation.

Count/measure is the process of acquiring numbers based on the desired measurements, which can appear to be a rather simple step in the process but must be well thought-out even before the initial step of image acquisition. In at least one popular commercial imaging program, one can choose from over 50 measurement parameters. At this point the difference between quantity of data and value of data becomes critical. While the most common measurements might be diameter, area and size distribution, numerous other options are possible such as roundness, nearest neighbour, feret and fractal dimension.

Analyse: Once the data are collected in the count/measure step, it is often useful if not critical to manipulate the data in order to summarise or visualise the relevant aspects. Some image-processing software has functions built into the program for this purpose while others allow for easy exporting of the raw data directly to other programs such as Excel or in a format that is recognisable by other programmes via dynamic data exchange.

2.10 Laboratory safety

The importance of good laboratory practices cannot be overstated in reference to the areas discussed in this chapter. While the uninformed may assume that a laboratory containing microscopes must be a relatively neutral territory and safety may have more to do with protection of the instrumentation, this section is to state otherwise. It is eminently important that those performing the techniques discussed in this chapter know and abide by specific safety guidelines. A number of publications already exist covering this topic (Mahn, 1991; Barber & Mascorro, 1994; Clarke, 1995), and we do not intend covering general laboratory safety here, but rather the specific hazards associated with the techniques discussed in this chapter.
2.10.1 Light microscopy

While the modern-day light microscope may appear more intimidating in operation than presenting issues relative to personal safety, there are some precautions to consider nonetheless.

- Modern halogen lamps produce exceptionally bright illumination which, if uncontrolled, creates a potential for eye injury. Rheostats and filters are meant to control the amount of light used for observation.
- Fluorescence microscope operation requires extra care due to the harmful effects of high-energy wavelength light which is meant to be filtered before observation by the user.
- For the person responsible for maintaining such instruments, the task of exchanging bulbs, either halogen or fluorescent mercury or xenon lamps, presents the potential for implosion. All manufacturers’ safety precautions should be followed. Such lamps also produce significant heat during operation, which must be considered when handling the lamps and their housings.
- Hazards related to sample preparation abound in both the physical sense as well as chemical hazards.
- Razors, knives, microtome blades, pins, needles and probes deserve respect for their potential to cut, puncture and transmit harmful material to unintended targets.
- Numerous chemical hazards exist and only a partial list is included here. MSDS (material safety data sheets) for all chemicals should be readily available in the laboratory. One of many well-documented websites dealing with MSDS topics is the physical and theoretical chemistry laboratory safety website of Oxford University (http://www.physchem.ox.ac.uk/MSDS).
- Fixatives, such as formaldehyde, glutaraldehyde, osmium tetroxide.
- Dehydrating agents, such as ethanol and acetone.
- Stains and buffers commonly carry a number of hidden dangers associated with skin contact and inhalation. Some of these involve long periods of low-level exposure.

2.10.2 Scanning electron microscopy/transmission electron microscopy

In addition to the precautions mentioned under light microscopy, the SEM and TEM techniques include:

- Full understanding of hazards associated with the SEM and TEM instrumentation itself in association with high voltages, vacuum systems, potential electrical shock (for those who insist on removing panels).
- Although not restricted to SEM and TEM techniques, the common use of liquid nitrogen must be accompanied by a number of safety concerns, such as freezing damage to body parts, eye protection, and asphyxiation.
- Safety instructions should be clearly understood and followed for the specialised equipment used in preparing samples for SEM and TEM operations, such as critical point dryers, sputter coaters, high-vacuum evaporators and freeze-substitution accessories. Of
particular concern is the use of propane and other explosive cryogens in quick-freezing preparations for freeze substitution.

2.10.3 Systems using lasers

While modern instrument manufacturers go to great lengths to prevent accidental exposure to direct laser beams or their reflections, operators of such instruments, including confocal microscopes and spectrophotometers, are advised to know the hazards of laser generators.

2.11 Future techniques in dairy product structure

2.11.1 Scanning probe microscopy

Scanning probe microscopy (SPM) describes a range of surface imaging techniques based on the invention of the scanning tunnelling microscope by Binning et al. (1982). These microscopy techniques use a sharp tip to scan a specimen surface. Measurement of the interaction between the tip and the surface provides three-dimensional topographic information on a sub-nanometre scale (Elofsson et al., 1997).

The atomic force microscope (AFM) uses the SPM technique to record force interaction between a cantilever tip and the specimen surface. Repulsive forces on the surface deflect the cantilever. This action is detected by a laser beam and then reflected into a photodetector (Elofsson et al., 1997). Topographic information may be generated through constant contact with the surface. Contact mode AFM most often refers to constant force imaging such that the repulsive force exerted by the sample is kept constant by means of a feedback circuit and vertical height is recorded. Alternatively, contact mode may refer to constant height imaging during which the tip is held at a constant height and the deflection of the cantilever is measured. Because shear force, generated in contact mode, can damage the sample, non-contact methods have evolved. In non-contact mode AFM, the cantilever oscillates at a frequency equivalent to resonant frequency. The amplitude of the oscillation changes as the tip responds to a change in height on the surface. A feedback mechanism responds to keep the amplitude constant; these measurements are used to map the topography. In true non-contact AFM, the tip oscillates above the surface and van der Waals forces damp the oscillation; higher elevations exert greater force causing a greater response. The other non-contact mode is tapping mode; here the tip oscillates more strongly so that it touches the surface on the end of its swing. Contact with the surface results in a decrease in amplitude as damping occurs.

The AFM must be free of external vibration and the sample must be well supported on a substrate to provide high resolution of surfaces. Attachment must be firm because samples may be shifted during contact with the tip. However, some freedom of movement should still be possible because molecules must have an opportunity to change conformation in response to their environment (Uricanu et al., 2004). The substrates of choice for dairy proteins are mica or glass (Elofssen et al., 1997). Because these substrates are negatively charged, it may be necessary to improve or control attachment by means of a chemical linker. Protein binding may be facilitated with the use of self-assembled monolayer (SAM) and covalent coupling
to attach protein groups to the SAM. Uricanu et al. (2004) used carbodiimide chemistry to selectively attach casein micelles to the substrate via their alkylamine groups.

Casein micelles were imaged (Uricanu et al., 2004) in a wet cell. The micelles were maintained in a hydrated state. The AFM was operated in constant-force mode to produce a height profile and then in the force-distance mode to measure deformation of micelles in response to the AFM tip.

Whey proteins, specifically, β-lactoglobulin, whey protein concentrate (WPC) and heat modified and cold-gelling WPC were imaged with the AFM in tapping mode to observe their aggregation states (Elofsson et al., 1997). Protein solutions were air-dried onto freshly cleaved mica sheets. When proteins were at or below monolayer, coverage aggregation states could be imaged and were found to be different. The authors reported that the technique was ‘sufficiently simple to be of value even for routine testing’.

2.11.2 **Diffusing wave and ultrasonic spectroscopy**

Estimates of mean particle size and degree of mobility in undiluted milk or other food emulsions have been made possible by diffusing wave spectroscopy (DWS). Each particle in the dispersion will refract the light and, because the particles are moving, the intensity of the scattered light will change with time. In a concentrated solution, a high number of multiple scattering events occur until incident photons undergo a random walk in the sample (Dalgleish & Hallett, 1995). Individual scattering events also occur. The contribution of individual events is characterised by a measure of the average. It is assumed that all photons are scattered as the light passes through the sample and that there is no interaction between reflected and incident light. DWS is designed to conduct both incident and backscattered light by fibre optics back to the detector and, before any measurements can be made, it is essential to know the photon transport mean free path length ($l^*$), a measure of the turbidity of the system. The quantity $l^*$ is related to $l$, the mean free path length of the photon (distance travelled without scattering) by:

$$l^* = l/(1 - \cos \theta)$$  \hspace{1cm} (2.7)

where $\theta$ is the scattering angle and $(1 - \cos \theta)$ represents system average of multiple scattering events.

For non-interacting particles the factor $l^*$ depends on particle size, concentration and the refractive index of the medium. In more concentrated solutions, the position and correlation between the particles affects the angular distribution of scattered light. The degree of mobility of individual particles is measured as a change in the dynamics of the system and will be characterised by time-averaged autocorrelation function.

The two basic configurations of DWS are the backscattering mode and the transmission mode (Alexander & Dalgleish, 2004). The backscattering mode places the detector on the same side as the source. This design requires a less powerful laser because the light does not have to traverse the entire sample to get to the detector. The technique is useful for the study of gelation during which particle size and interaction between particles changes during the time scale of measurement. Transmission mode DWS requires a more powerful laser because the light must cross the length of the sample, and provides information not only about the change in size of the particles, but also on the ordering of the system (Alexander
& Dalgleish, 2004). The same authors used transmission DWS to follow the gelation of milk by acidification or use of rennet. A more technical explanation of the technique is presented by Weitz & Pine (1993).

Ultrasonic spectroscopy involves the measurement of velocity and attenuation of ultrasound as it is passed through a liquid sample. Ultrasound frequencies are selectable depending on the sample being analysed. Other parameters of importance during ultrasonic studies are temperature control and minimising bubble formation (Dalgleish et al., 2004a). More technical explanations of ultrasonic spectroscopy can be found in papers by Chanamai et al. (1998) and Buckin & Smyth (1999).

The techniques of DWS and ultrasound spectroscopy were used by Dalgleish et al. (2004a) to study the acid gelation of skimmed milk. The authors demonstrated that the process of aggregation occurs at pH values considerably higher than previously reported for heated skim milk. The techniques are sensitive enough to capture the slight decrease in the apparent radius of casein micelles just prior to the formation of the acid gel. Ultrasound data were shown to correlate with changes in micellar structure via the dissolution of the micellar calcium phosphate.

The benefit of DWS over more traditional methods of rheology, microscopy and dynamic light scattering for the study of aggregation and gelation processes was demonstrated by Alexander et al. (2006). In addition to being a non-intrusive technique, DWS is suitable for turbid suspensions. While studying milk gelation and oil-in-water emulsions employing pectin, the authors suggest that the technique of ‘transmission DWS and its turbidity parameter $I^*$ contains information that has yet to be fully exploited’. This is due to its relationship to both the scattering properties of a particle as well as the interparticle correlations. The technique has advantages over conventional physical analysis, and is predicted to provide new insight into interpretive parameters. A second example of the application of both DWS and ultrasonic spectroscopy comes in a study of flocculation of whey protein-stabilised emulsions (Gancz et al., 2006). Using these novel techniques, the authors demonstrated the differences between depletion flocculation and bridging flocculation, again taking advantage of the non-invasive nature of the testing.

While studying the effects of glucono-δ-lactone and calcium chloride on sodium caseinate emulsions, Eliote et al. (2005) used DWS in combination with rheological parameters and confocal microscopy. The authors acknowledge that the ‘zero-stress’ technique of DWS has advantages over the finite stress applied during a small-deformation rheological experiment, but point out the difficulty in defining the onset of gelation based on the three techniques and suggest that the rheology and DWS are sensitive to different aspects of the structural and dynamical changes taking place.

2.11.3 Microwave techniques in microscopy

According to Hayat (2000e), the use of microwave energy for tissue preservation dates back to a paper by Mayers (1970). Although it has not found wide use as an aid to tissue processing, it is covered here as a method that should be considered in special situations in the study of dairy products. In the 4th edition of his classic book covering electron microscopy techniques, Hayat (2000e) devoted a new chapter to the ‘application of microwave heating for microscopy’. In its simplest form, Hayat explains the uniqueness thus: ‘microwave
energy essentially induces di-electric fields, causing dipolar molecules to rapidly oscillate; this rapid kinetics movement causes accelerated chemical reactions, and produces instead heat as a result of disruption of chemical bonds in the tissue'. Further detail is available in Giberson & Demaree (2001).

Hayat (2000e) further describes the use of microwaves in several aspects of tissue preparation, as summarised below.

**Fixation**: Although initially used alone for preserving tissue, it is common practice now to use microwave energy in combination with more conventional chemical fixatives, such as glutaraldehyde, formaldehyde and osmium tetroxide. Consideration must be given to the correct combination of microwave energy, concentration of fixative, temperature and time. Once the correct combination is determined, the fixation times can be measured in seconds or minutes.

**Resin embedding**: Hayat (2000e) summarised the work of several papers on the topic (Giammara, 1993; Login & Dvorak, 1994a,b), and described the use of microwave energy for resin embedment as follows:

- Use silicone flat embedding moulds.
- Polymerisation is carried out in 15 minutes at 50% power.
- Place a container of water in the microwave to avoid overheating and specimen damage.
- Vent the microwave to the outside or into a fume hood.
- Cool moulds at room temperature before removing samples.
- Calibration of the microwave is critical in order to prevent uneven polymerisation.
- Avoid polyethylene capsules as they melt in the microwave.

**Staining**: While referencing primarily histological work, Hayat (2000e) mentions the benefit of decreased staining times when combined with microwave energy. The authors are not aware of the technique being applied to dairy research, but it is worth keeping in mind for laboratories that already employ microwaves ovens.

**Epitope retrieval**: One of the more unique applications of microwave energy in the area of microscopy is its usefulness at retrieving epitope activity after fixation and embedding. Again there appears to be no application of this technique in the dairy science area, but the reader is referred to the section of Hayat (2000e) which covers the topic and includes more specific references.

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3 Microstructure of Milk Components

A.K. Smith and B.E. Campbell

3.1 Introduction

Milk is a precursor for many food products. Its value has been enhanced by an enormous amount of research, especially over the past 50 years, to support the development and commercialisation of dairy-based products with an increasing variety of flavour, texture and shelf-life. Research efforts have been progressing with increasingly sophisticated approaches that now include genomics and proteomics. As a result, new questions can be addressed concerning the regulation of milk components’ production and assembly prior to secretion. The emergence of additional structural details regarding various milk components will be accompanied by new options for future dairy product innovation.

The colloidal nature of cow’s milk is a crucial structural feature that affects final product quality as well as its processing behaviour. The colloidal system can be divided into two compositional domains, the casein micelle and the milk fat globule. These colloidal domains comprise nearly 80% of the approximate 12.7 g total solids 100 g⁻¹ in milk. Therefore, the structure and interactions of these colloidal particles continue to be important areas in milk research.

Following a brief structural and compositional review of cow’s milk, it is the purpose of this chapter to emphasise some of the latest features and approaches towards a more detailed understanding of the molecular structures influencing milk colloidal behaviour. Primary attention is given to the known structural features for the milk fat globules and casein micelles. Although the non-colloidal milk constituents such as soluble proteins, lactose and minerals can influence milk fat globule and casein micelle structure, many of these interactions are process-dependent. Therefore, references to these interactions will be limited.

3.2 Cow’s milk composition

A ‘colloidal dispersion of liquid droplets in another liquid phase’ (Dickinson & Stainsby, 1988) is an apt description for milk in which the milk fat is stabilised as globules and dispersed in the aqueous phase. Of the major milk components shown in Table 3.1, the components with the most structural impact on fluid milk are fat and protein, specifically, the caseins. However, the whey proteins, lactose and salts participate in a variety of structural transformations by their ability to aggregate and form bonds or bridges as well as controlling the pH and ionic strength of the emulsion (Kaláb & Carić, 1990).
Cow’s milk fat is approximately 3.9 g 100 g\(^{-1}\) of whole raw milk (Walstra & Jenness, 1984), and partitioned into globules ranging in size from 0.2 to 15 µm (average 4 µm) (Michalski et al., 2004). The milk fat globule (MFG) is structurally and compositionally complex. The complexity arises, in part, from the molecular variety of triglycerides enclosed by the structurally complex interfacial layer known as the milk fat globule membrane (MFGM). Seasonal dietary changes, breed and the lactation cycle modify the milk fat globule triacylglyceride composition. Furthermore, the MFG structure continually evolves after its secretion into the udder as well as after milking. A scanning electron micrograph, shown in Fig. 3.1, illustrates the complexity of the MFGM. This image is just one of many possible views because environmental conditions and processing parameters affect the composition and arrangement of MFGM constituents. Therefore, the evolving chemical composition and resulting milk fat globule physical properties will dictate its range of structural features and behaviour.

### Table 3.1  Chemical composition (g 100 g\(^{-1}\)) of cow’s milk.

<table>
<thead>
<tr>
<th>Component</th>
<th>Average content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.3</td>
</tr>
<tr>
<td>Fat</td>
<td>3.9</td>
</tr>
<tr>
<td>Protein</td>
<td>3.25</td>
</tr>
<tr>
<td>Casein</td>
<td>2.6</td>
</tr>
<tr>
<td>Serum proteins</td>
<td>0.65</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.14</td>
</tr>
</tbody>
</table>


**3.2.1 Milk fat globule**

**Fig. 3.1** Scanning electron micrograph of fat globules from raw milk. The milk fat globule membrane contains both casein micelles (c) and whey proteins. Bar = 2 µm.
Milk fat

Of the lipid within the milk fat globule 98% is triacylglycerol, with a substantial amount of short-chain fatty acids (C4 to C10) and only a small amount of long-chain polyunsaturated fatty acids (Jensen et al., 1991). Stereochemically there are more than 200 triglyceride isomers (Lopez, 2005), and their relative proportions in the fat globules impart unique thermal and structural properties. All of the fat prior to milking is in the liquid state and will increase its proportion of crystallinity to 100% when the temperature decreases below –40°C. The broad melting range has provided opportunities to fractionate milk fat into low, medium and high temperature melting fractions (Mulder and Walstra, 1974; Bystrom & Hartel, 1994; Schmelzer & Hartel, 2001). For example, there is a coexistence of crystalline and liquid fat between –40°C, where it is completely solid, and 40°C, where it is completely liquid (Pilhofer et al., 1994). The ratio of solid to liquid fat depends on triacylglyceride composition, temperature history and state of dispersion. The major crystal forms are classified as: α, β’ or β, listed in order of increasing stability and melting point. The major impact of fat polymorphism upon dairy product performance has led to detailed investigations of crystalline milk fat.

Coupled with high-sensitivity differential scanning calorimetry (DSC), Lopez et al. (2002) studied the crystallisation behaviour of triacylglycerols within MFGs in cream using time-resolved synchrotron X-ray diffraction (XRDT), at both small and wide angles. Cooling milk fat from 60°C to –10°C, at rates of 3 and 1°C min⁻¹, produced three identifiable α-type lamellar structures, i.e. two double-chain length stackings of 47 and 42 Å and one triple-chain length stacking of 71 Å. When the fat was subsequently heated at 2°C min⁻¹, it was apparent that the crystal forms were unstable and that they tended to go through an α to β’ monotropic transition. Depending on the cooling rate, a total of five crystalline species were identified at wide angle XRDT, and six lamellar stackings at small angle were observed. MFG size and size distribution affects the formation of different fat crystal morphologies and is sensitive to thermal history. For example, smaller crystals formed within smaller MFG with faster cooling rates, while slower cooling rates favoured formation of layered crystals in small globules, and led to more of the small globules undergoing the crystallisation process.

Temperature-controlled monitoring of the crystallisation process in MFGs by polarised light microscopy provides insights regarding the cooling rate dependence on the development (size, shape and location) of crystal birefringence. At a slow cooling rate (0.5°C min⁻¹) needle-shaped crystals, up to 10 μm depending on globule size, plus a mixture of layered and needle-shaped crystals, occurred in the largest MFGs. In small globules, smaller crystals, spherulites (radially organised crystals) and birefringent globules were observed (Lopez et al., 2002). When the MFGs were rapidly quenched (< 5 s from 60°C to –8°C), the randomly oriented crystals in large globules had an average size of 1 μm without any evidence of globule ‘shell crystallisation’. Hence, it was possible to infer the cooling rate history based on crystal morphology (Lopez et al., 2002).

Higher-resolution images of the fat crystals can be achieved by freeze-fracture replication techniques (Schmidt & Büchheim, 1992) or carbon fixation method (Heerjte & Leunis, 1997) for transmission electron microscopy (TEM). Traditional fixation and embedding techniques extract a considerable amount of the fat and, therefore, cryo-techniques must be used to prepare fat samples for electron microscopy (Schmidt & Buchheim, 1992). Heerjte
& Leunis (1997) compared two methods for the preparation of fat crystals including a detergent washing procedure, and attachment of the crystals to a carbon support film. Both techniques were found to be equally useful for providing images of the fat crystals that could be used to observe morphology and measure the size. However, the carbon film technique was better suited to the preparation of individual crystal clusters.

Freeze-substitution followed by low-temperature embedding to study the effect on whipping on the fat globules in whipping cream was another approach to examining milk fat using TEM (Smith et al., 2004). Fixation and embedding of the fat globules in a frozen state followed by sectioning at room temperature protected the fat structure for high-resolution imaging that can be achieved from sectioned material. The crystals in the fat can be easily distinguished, as shown in Fig. 3.2.

*Milk fat globule membrane*

The milk fat naturally occurs as MFG. The presence of the MFGM limits the impact of de-destabilisation phenomena, such as flocculation, coalescence and creaming (Mulder & Walstra, 1974) while protecting the milk fat from lipolysis (Lopez, 2005). The term ‘membrane’ was adopted because the globule acquires a coat of the plasma membrane during secretion (Keenan et al., 1983); however, it is not considered a true biological membrane, i.e. a phospholipid bilayer. The strength and elasticity of the MFGM, with its ability to reduce interfacial tension, contributes to emulsion stability. The MFGM surrounds the apolar fat globule with a trilayer structure through association with the triglycerides in the liquid portion of the fat (Keenan & Mather, 2002). This association makes it hard to identify the internal boundary of the membrane. The membrane has been reported to be approximately 10 nm

![Fig. 3.2](image-url) Transmission electron micrograph of a coalesced fat globule from whipped cream. The foam was freeze-substituted and low-temperature-embedded to preserve the integrity of the fat including fat crystals (FC), proteins in the fat globule membrane (arrows) and casein micelles (C) in the serum phase are clearly visible. Bar = 1.5 μm. Source: Smith et al. (2000) *Food Research International*, 33, 697–706 by permission of the authors and Elsevier Academic Press, Oxford, UK.
thick, and it is comprised mostly of proteins, glycoproteins, enzymes, phospholipids, neutral glycerides, water, cerebrosides and cholesterol (Mulder & Walstra, 1974; Lopez, 2005).

The protein in the MFGM represents approximately 1 g 100 g\(^{-1}\) of the total protein in the milk (Needs & Huitson, 1991). The identities, characteristics and sequences of these proteins is the subject of an extensive review (Mather, 2000), which included recent results of biochemical approaches, molecular cloning techniques and computer-assisted sequence analysis. Variation in thickness of the membrane on one MFG and between MFGs (Walstra & Jenness, 1984) is suggestive of differences in surface activity. Raw milk direct from the cow is rarely available to the modern-day consumer. Instead, the milk is homogenised and heat-treated (pasteurised or sterilised) to create a more stable emulsion and eliminate microbiological risk. Homogenisation can dramatically increase the MFG total surface area from 4 to 8 times, as measured by laser light scattering (Lopez, 2005). These smaller MFGs are stabilised by a ‘synthetic’ interfacial layer composed of native MFGM (about 10%) as well as adsorbed proteins from the serum including casein (70%) and whey proteins, primarily \(\beta\)-lactoglobulin (Cano-Ruiz & Richter, 1997). The actual composition and structure will be dictated by the milk processing history, and subject to differences based on the selected isolation methodology (Fox & McSweeney, 1998).

Polar lipids in the native MFGM have been studied by cryo-TEM (Waninge et al., 2003, 2004). Polar lipids, cholesterol, sphingolipids and phospholipids appear to form a monolayer on the surface of the newly formed MFG. An aqueous layer, containing various proteins, separates the monolayer from the outer lipid bilayer. When milk is processed, changes occur in the MFGM beginning with a 20% loss of polar lipids during initial cooling. Additional processing increases the loss of phospholipids and results in a loss of the triple layer membrane, and in the production of lipid-protein vesicles and fat droplets surrounded by a monolayer membrane (Waninge et al., 2004). By means of cryo-TEM, without contrast enhancement (staining), vesicles prepared from model MFGM mixtures were imaged. Although it was possible to image aggregated \(\beta\)-lactoglobulin and casein micelles (because they have enough natural density), it was not possible to study the interaction of individual proteins with the vesicles due to lack of contrast (Waninge et al., 2003). Interactions between vesicle surface and proteins could be explored through immuno-gold labelling techniques as described by Škalko et al. (1998). When the cryo-TEM method was applied to vesicles recreated from dairy cream, it was found that vesicles form from loss of the outer bilayer membrane or through coalescence; these vesicles are non-aggregating (Waninge et al., 2004).

The availability of cryo-SEM, cryo-TEM and immuno-gold labelling to identify the presence and interactions between lipids and proteins in the MFGM will enhance the understanding of the functionality of milk fat globules in milk and dairy products.

### 3.2.2 Colloidal milk proteins

In addition to the milk fat globules’ colloidal properties, milk structure is strongly influenced by the colloidal nature of its protein components. Representing 80% of the total milk protein, the major caseins are \(\alpha_s\), \(\alpha^\prime\), \(\beta\)- and \(\kappa\)-caseins, and they are in a ratio of 1 : 4 : 4 : 1, respectively. Most of the casein, 95%, is in the form of colloidal particles known as micelles with diameters ranging from 50 to 500 nm, and averaging 120 nm (Fox & McSweeney, 1998). The remaining 20% of the protein content is whey protein, primarily a 2 : 1 molar ratio of \(\beta\)-lactoglobulin.
and α-lactalbumin (Walstra & Jenness, 1984). All proteins exhibit colloidal behaviour in which their interactions are governed by a combination of electrostatic, van der Waals and steric repulsion forces. However, the casein fraction consists of a unique set of proteins that are co-assembled with calcium phosphate clusters to form super-aggregates known as casein micelles. Furthermore, the casein micelles do not have a unique size, but rather a size distribution with no strict stoichiometry of calcium phosphate nor casein subunits. Casein micelle structural heterogeneity in addition to conformational flexibility has conspired to make them unlikely ever to be crystallised for direct structural determination. As a result of this limitation, several approaches have been pursued to develop a structural model to interrelate compositional details, physical dimensions and colloidal behaviour.

**Microstructure of casein micelles**

Each of the casein polypeptides has been extensively investigated in terms of amino acid sequence including genetic variants and post-translational modifications, most notably, phosphorylation of αs1, αs2, and β-casein and the glycosylation of κ-casein (Holt, 1992). Furthermore, the casein polypeptide primary sequences with an elevated frequency of proline residues predict an extended conformation with minimal secondary and tertiary structure. Establishing a molecular basis for casein micelle integrity and stability has remained elusive and demands an increasingly sophisticated and broad set of spectroscopic, molecular modelling and microstructural tools to further refine the current structural models that have become the subjects of intense debate.

Two of the most persistent models proposed for casein micellar structure are the core-coat and sub-micelle models (Holt, 1992; Dalgleish et al., 2004). The core-coat model describes sub-micelles in terms of spatially-dependent κ-casein content in which there is a κ-casein deficit at the core and a κ-casein-rich layer on the surface. In this model, the hydrophilic C-terminal of the κ-casein extends 5–10 nm past the surface to provide a ‘hairy layer’. The hairy layer imparts steric stabilisation, which adds significantly to its low zeta potential (–20 mV) (Fox & McSweeney, 1998).

The sub-micelle model evolved from electron microscopy studies suggested that the micelle is constructed from sub-micelles ranging from 5 to 25 nm. It has been hypothesised that these sub-micelles are linked together by calcium-sensitive caseins (Slatterly & Evard, 1973) through calcium phosphate (Schmidt, 1982) or by both calcium phosphate and hydrophobic bonding (Rollema, 1992). Subsequently, Walstra (1999) isolated calcium phosphate, in independent units, and identified it as a linker for sub-micelles. However, Horne (1998) has placed less importance on the sub-micelle model by emphasising the role of hydrophobic and ionic interactions in the formation of micelles, while also taking into account individual casein structure and calcium phosphate salt bridging. Although Horne’s model is less dependent on self-assembled casein polypeptides as the main structural micelle unit, one could still consider it a variation of the sub-micelle model because strongly bound casein complexes are held together by weak hydrophobic interactions (de Kruif & Holt, 2003).

Support for the sub-micelle model has been further eroded on the basis that various electron microscopy sample preparation procedures produce misleading artefacts (McMahon & McManus, 1998; Holt et al., 2003; Dalgleish et al., 2004). Shrinkage occurs from the fixation and dehydration procedures used for both SEM and TEM. Sputter coating for SEM
reduces surface resolution and, therefore, the opportunity to image delicate protein structure including the κ-casein hairy layer. Recently, images of casein micelles have been acquired at high resolution, without sputter coating, using a field-emission scanning electron microscope (FE-SEM) (Dalgleish et al., 2004). As shown in Fig. 3.3 it is possible to see the structural details on the surface of the micelle. However, evidence of shrinkage caused by steps in preparation make the information suspect. The significance of artefacts upon the elucidation of casein micelle structure has been elegantly reviewed by McMahon & McManus (1998). In an attempt to overcome these problems, the same authors attach native casein micelles to a Parlodion-coated grid, stained briefly with uranyl oxalate, rapidly freezing the sample in Freon 13, and freeze-dried prior to imaging with TEM. Cryo-preparation minimises deformation of the micelles and preserves their fine structure.

Direct imaging of casein micelles by cryo-TEM following freezing in a controlled environment vitrification system with ethane produces a superior image (Waninge et al., 2004) because samples remain frozen and fully hydrated during imaging, while the post-column energy filter provides contrast for unstained micelles. Images obtained by cryo-TEM were similar to those obtained through freeze-dried preparation for TEM, and reach the same conclusion; there is no evidence to support the presence of sub-micelles as a distinct subunit structure. While there is general agreement that calcium phosphate plays an important role in casein micelle integrity, there is no direct structural data to indicate which sequence-specific amino acid moieties are being bridged.

The micellar distribution of calcium phosphate may not be a reflection of proteinaceous sub-micellar structures. Holt et al. (2003) favoured a calcium phosphate nano-cluster model following small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) experiments. These authors attributed an inflexion point \((Q = 0.35 \text{ nm}^{-1})\) to inter-particle interference caused by scattering of calcium phosphate. The model was based on the presence and distribution of calcium phosphate in the micelle, corroborated by intrinsic viscosity measurements for the weight fraction of calcium phosphate and the core mass of

![Fig. 3.3](image-url) Field-emission scanning electron micrograph of casein micelles. Surface shows features of unknown composition. Bar = 200 nm. Source: Dalgleish et al. (2004) International Dairy Journal, 14, 1025–1031 by permission of the authors and Elsevier Academic Press, Oxford, UK.
model calcium phosphate nano-clusters (Holt et al., 2003). The nano-cluster model defines a fairly homogeneous protein matrix held together by a random distribution of calcium phosphate.

*Molecular structure of casein micelles*

The structural features emerging through prudent interpretation of microstructural evidence indicate that the casein micelle has no uniform surface structure, and is devoid of discrete subunit organisation. Therefore, the interactions among the casein polypeptide are not predicted to occur with specific stoichiometry or even orientation. The nature of casein polypeptide interactions impacting casein micelle structure has been probed by a number of physicochemical approaches, such as nuclear magnetic resonance (NMR), fluorescence, circular dichroism (CD), Fourier transform infra-red spectroscopy (FTIR), Raman, SAXS and SANS in addition to molecular modelling simulations (Kumosinski et al., 1994; Malin et al., 2005) in several casein systems.

The non-globular and much extended nature of casein polypeptides in solution provides a situation conducive to a large number of possible inter-polypeptide interactions and, therefore, structures. The multiplicity of possible inter-polypeptide interactions effectively produces a distribution of structures possessing a broad size distribution rather than a single discrete multimeric entity typical for multimeric enzymes, hemoglobins or viral capsid proteins. However, structural data show that the casein micelle size distributions have a finite range and micellar growth is thought to be limited by κ-casein adsorption to the outer micelle surfaces. Therefore, there must be predominant molecular interactions that limit casein micelle size as well as physicochemical properties such that κ-casein can interact with the varied surfaces of α-casein/β-casein complexes. Direct measurements of selected casein peptides in terms of their solution structures in conjunction with molecular dynamic simulations provide a compelling basis for identifying the most important structural features underlying α_{st}-casein structure (Malin et al., 2001, 2005). With the use of bifunctional crosslinking reagents, Livney et al. (2004) were able to identify pairwise sets of lysine residues on β-casein that could be crosslinked with a series of bifunctional reagents of varying end-to-end lengths. Using the bifunctional crosslinking reagents as rulers on the Ångstrom scale, their results showed the lysine pairs were largely consistent with the modelling predictions, and demonstrated the inherent molecular flexibility within the β-casein polypeptide. Taken together, these studies show that the extended nature of the casein polypeptides with their high degree of mobility permits a broad range of molecular structures that self-assemble to accommodate both ionic and hydrophobic interactions. As a result, the casein micelle retains its open structure while stabilising calcium phosphate nano-clusters.

*Interactions and stabilisation of casein micelles*

Application of the fundamental theories regarding colloidal dispersion stability provides the rigorous context needed to explain casein micelle behaviour. Early work by Payens (1966), Walstra & Jenness (1984) and, more recently, by Tuinier & de Kruif (2002) concluded that electrostatic interactions are insufficient to stabilise the casein micelles and, therefore, inhibit micelle flocculation. The absence of flocculation in milk demonstrates the importance of
steric repulsion. The existence of steric repulsion was subsequently explained by the pre-
ponderance of κ-casein on the casein micelle surface. The unique nature of the κ-casein
confers steric repulsion through its ability to extend its polypeptides several nanometres
beyond the micelle surface.

Casein has two primary functions, the transport of calcium from mother to young and
self-association of calcium phosphate into colloidal form (Farrell et al., 2003). Farrell et
al. (2003) have concentrated their efforts on determining molecular structure as a basis to
explain self-association reactions (function). Do caseins have persistent structures and would
this state allow them to be flexible as function indicates or are they constantly altering and
reforming until aggregates are produced, as suggested in the rheomorphic model put forth
by Holt & Sawyer (1993)? The applicability of the rheomorphic model for caseins was also
shown in the synucleins and tau proteins using Raman spectroscopy by Syme et al. (2002).
The amino acid sequence for the proteins in the micelle and information obtained from
CD experiments to determine secondary structure was a starting point for the authors to
develop a three-dimensional molecular model to explain the structure–function relationships
of casein. Structural data collected from several laboratories has been selected to provide
clues to micelle structure. Physical data from experiments which include CD, FTIR, NMR,
SAXS, electron microscopy (EM) and light scattering under various conditions has been
integrated to build a computerised rendition of the micelle to explain how it can both be
rigid and flexible, and also hold a large amount of water (Farrell et al., 2003).

In spite of five decades of structural and behavioural research directed towards the struc-
tural constraints underlying micelle formation and behaviour, the structural model remains
hotly debated. Therefore, the challenge of relating colloidal behaviour with advances in
structural determinations remains a promising and active research area.

3.2.3 Whey proteins

Whey proteins, or serum proteins, remain in solution when milk is adjusted to pH 4.6, the
point at which caseins precipitate (Walstra & Jenness, 1984). Whey proteins are comprised
primarily of β-lactoglobulin (50%) and α-lactalbumin (20%), listed in order of heat stabil-
ity. A significant feature of β-lactoglobulin structure is the five cysteine residues per mole;
four of these residues are involved in two intra-polypeptide disulphide bonds. The single
free thiol group is of consequence during the heating of milk because it reacts with other
β-lactoglobulin proteins, κ-casein and α-lactalbumin. α-lactalbumin is a globular protein
containing four disulphide bonds, which play an important role in reversal of conformational
changes caused by heating. Two atoms of calcium are strongly bound to α-lactalbumin
(Walstra & Jenness, 1984); the removal of calcium results in conformational changes in
α-lactalbumin, which further reduces its heat stability.

The degree of serum protein denaturation is dictated by the extent of the heat treatment.
Denaturation rate is temperature-dependent at < 90°C, but much less temperature-dependent
above 100°C (Burton, 1984). Denaturation occurs extensively at temperatures above 70°C
(Dalgleish & Banks, 1991). Denatured whey proteins are less soluble and more sensitive
to calcium ion concentration than native whey proteins, and denatured β-lactoglobulin
associates with κ-casein of the casein micelle and serves to increase the micelle stability
(Walstra, 1977).
Dalgleish & Banks (1991) studied the quantity and composition of proteins in newly created MFGMs following different levels of heat treatment. The amount of interfacial protein reached a maximum by 4 min of heating at 85°C. The newly bound proteins were serum proteins regardless of the fact that they were only 50% denatured at this point in the heat treatment. Both β-lactoglobulin and α-lactalbumin increased with time at temperatures above 80°C. Below 80°C α-lactalbumin was absent, and below 65°C β-lactoglobulin was absent. No increase in the amount of casein was reported. Any caseins found on the MFGM were present before heat treatment, and may even have been displaced by serum proteins. The monomer/dimer configuration of denatured serum proteins enhanced their mobility to the MFG surface. Intermolecular disulphide bonds may facilitate binding to membrane proteins. Heat treatment resulted in a new, stronger MFGM.

Kaláb & Carič (1990) reported that, during pasteurisation of the milk, casein micelles, which are smooth in raw milk, develop a ragged appearance when heated to 85°C due to adsorption of aggregated β-lactoglobulin to form a β-lactoglobulin–κ-casein complex. Davies et al. (1978) investigated the role of intermolecular disulphide bonds in the formation of the β-lactoglobulin–colloidal calcium phosphate (CCP)–casein complex, and reported that complex development was inhibited by sulphydryl-blocking agents. β-lactoglobulin adsorption occurred preferentially at pH 6.4, and resulted in an increased hydrophobicity at the micelle surface, thus stabilising the micelle against aggregation. At low pH (5.5), loss of the hydration layer on the micelle will facilitate aggregation and gel formation can occur. Although the CCP bonds between sub-micelles are at their lowest value, electrostatic attraction maintains micellar structure (Walstra, 1989). At pH 7 the β-lactoglobulin–κ-casein complex will be dispersed in the milk plasma resulting in an increase in hydrophobicity, which will also allow for aggregation (Kaláb & Carič, 1990). Therefore, at lower and higher pH, the stability of casein micelles against aggregation is reduced. Calcium activity has control over κ-casein depletion from the micelle. The loss of κ-casein reduces the stability of the micelles due to the loss of the hairy layer and the steric repulsion it provides.

Other protein complexes formed because of heat treatment include α-lactalbumin reacting with β-lactoglobulin at temperatures above 90°C (Baer et al., 1976), and β-lactoglobulin with αs2-casein at ultra high temperature (UHT), i.e. 140°C. A change in the location of the calcium phosphate on the micelles may cause the micelles to enlarge (Creamer, 1991). This increase in size is accompanied by the presence of many tiny particles, which may be denatured serum proteins that have not associated with the micelle, or they may be indicative of the formation of soluble casein as a result of the heat treatment (Burton, 1984).

3.2.4 Lactose

On a molar basis, lactose (β-D-galactose-(1→4)-α-D-glucose) is the most abundant solid in cow’s milk at nearly 0.1 M. As the main energy source for neonates, it provides controlled glucose delivery since its release is dependent on β-glucosidase activity. As a milk solution component, lactose imparts only a slight increase in solution viscosity without any noticeable effect on macromolecular solution behaviour. Its solution structure has been intensively investigated with NMR using the nuclear overhauser effect (NOE) as well as residual dipolar couplings. These approaches have shown that lactose primarily adopts one conformation with the glycosidic dihedral angles, syn-Φ/syn-Ψ, of 49°/12.8° (Martin-Pastor et al., 2005).
With regard to its reactivity, lactose is a reducing sugar and will react with amines (lysines and N-terminal amines) to form Schiff base adducts, which subsequently undergo a series of non-enzymatic reactions leading to browning and caramelisation. Lactosylation also reduces the pI of protein as a result of amine substitution which, in turn, influences the electrostatic contribution of the protein–protein interactions.

### 3.3 Concluding remarks

Although the major milk structural characteristics discussed in this chapter are primarily applicable to cow’s milk, there are many similarities to other mammalian milk systems. As an essential neonatal dietary component, each milk system will have compositional differences reflecting the neonatal growth rate requirements. Essential to its nutritional functionality are the molecular and higher-order structures that fine tune nutrient bioavailability. Therefore, a detailed understanding of the molecular structures and the interactions that control their assembly and utilisation will provide opportunities for dairy food design and processing.

### References


4 Microstructure of Dairy Fat Products

S. Martini and A.G. Marangoni

4.1 Introduction

In countries where dairying has been long established, eating patterns include a substantial contribution from milk and milk products. It would be difficult for such populations to sustain an appropriate balance of nutrients such as calcium and riboflavin should dairy products be removed from the diet. Milk contains a good balance of protein, fat, minerals and carbohydrate. Indeed, consumer surveys have shown that consumption of dairy products contributes up to 20% of daily energy intake as well as up to 60% of calcium intake in these populations (Miller, 1989). Figure 4.1 shows the world per capita consumption in kg for the year 2000. Ireland is the highest consumer of milk, while France and Italy are the countries with highest consumption values of cheese, followed by Sweden, Australia and Canada. On the other hand, butter consumption is highest in France and Italy followed by Ireland.

Milk fat contains a number of bioactive components that have anticancer potential, including conjugated linoleic acid (CLA), sphingomyelin, butyric acid, ether lipids, β-carotene, and vitamins A and D. Calcium is quantitatively the most important mineral supplied in milk but significant contributions are also made to daily intakes of magnesium, zinc and potassium, a point that could be stressed more by the dairy industry. Although milk contains saturated fatty acids and trans fatty acids, which are associated with atherosclerosis and coronary heart disease, it also contains oleic acid which is negatively correlated with these diseases. Furthermore, milk fat contains the essential fatty acid linoleic acid, and small amounts of the ω-3 linolenic acid, which have many diverse functions in human metabolism and may control a variety of biochemical and physiological processes.

Butyric acid has recently been the subject of intensive research due to its effects against colon cancer. Although the intake of butyric acid from milk may not be high, its effects are enhanced several-fold due to synergy with other anti-carcinogenic components of milk fat, e.g. vitamins A and D₃.

In milk, the fat is present in the form of small globules ranging from less than 0.2 μm to about 15 μm in diameter. These fat droplets are formed within the lactating cells of the mammary gland. During secretion they are enveloped in the fat globule membrane, which plays an important role in attaining high emulsion stability of the fat phase in milk. The fat in the globules (milk fat) consists almost entirely of triacylglycerides (TAGs), whereas the membranes are composed of phospholipids protein complexes (Precht, 1988), and the milk nutritional components can be consumed as different typical dairy products, such as cheese and butter.
Shortening is a 100% fat product formulated with animal and/or vegetable oil that has been processed for baking functionality. It is used as an ingredient in bakery products such as bread, cakes, cookies, short pastries, fillings and icings and it is also used in frying. In order to formulate these byproducts the milk fat has to be isolated from the milk before processing. Anhydrous milk fat (AMF) is obtained from butter or directly from milk cream or fresh cream. Traditionally butter and lard have been the fats used in bakery products, but due to economic and nutritional considerations consumption patterns have shifted away from traditional animal-based fats to vegetable oils and fats. Nowadays there is a tendency to re-incorporate milk fat into formulations in order to replace high-trans fats, from hydrogenated vegetable oils, with the low-trans milk fat. However, the physicochemical and sensory characteristics should be the same between products formulated with milk and vegetable fat. To achieve this goal, several researchers have focused on the study of physicochemical

![Fig. 4.1](image-url)

**Fig. 4.1** World consumption (kg per capita) of dairy products for the year 2000: (a) milk, (b) cheese and (c) butter.
characteristics of milk fat, its fractions and blends with vegetable oils. The structural characteristics of a material (microstructure) affect its macroscopic behaviour such as rheology, flavour and texture. The objective of this chapter is to describe recent research on crystallisation behaviour of milk fat, milk fat fractions and their blends with vegetable oil, focusing on the microstructure and rheological properties of the resulting material.

4.2 Microstructure of cream and butter

4.2.1 Background

Cream is defined as ‘the yellowish fatty component of unhomogenised milk that tends to accumulate at the surface’. Cream is raw material for the manufacture of butter. Apart from the higher amount of proteins and the small amount of fat that has passed into the skimmed milk during the separation process, cream differs from milk primarily in fat globule concentration. The fat content of cream is not uniform. This may be partly because the minimum standard set by law differs from country to country, or because the fat content has been adapted to the prevailing technological and internal conditions in the creamery, or due to variations in fat composition which may be attributed to feeding conditions.

Butter or whipped cream is produced from cream. During this ripening phase, crystallisation processes take place in the fat globules, depending on the temperature. This results in the formation of different crystal structures within the fat globules, and induces destabilisation processes by damaging the fat globule membrane. The various crystal formations in the fat globules can have a considerable effect on the consistency of butter or on the physical properties of whipped cream (e.g. its stability) (Fig. 4.2).

![Schematic structure of butter](image)

**Fig. 4.2** Schematic structure of butter.
4.2.2 Cream

As described by Precht (1988), electron microscopy studies showed that crystallisation of high-melting fat fractions during cooling of milk and cream often starts at the globule boundary. Supercooling phenomena certainly play a role in the variation of fat crystallisation in different fat globules. In a cream emulsion, 1 mg of fat is dispersed into some $10^8$ fat globules. So, at least one nucleus must be formed in every globule to achieve full crystallisation. It follows that the supercooling temperature of fat within the cream fat globules is lower than that of pure non-emulsified milk fat. Further, supercooling is very dependent on the size of the fat globule: the smaller the size, the lower the degree of supercooling. In addition, it is assumed that monoglycerides molecules act as catalytic impurities for the crystallisation of fats (Walstra & Jenness, 1984).

4.2.3 Whipped cream

It is well known that a stable foam is produced by beating air into stored cream. During this process larger air bubbles are formed at first, which, on prolonged beating, increase considerably in number (Precht, 1988). Many of the larger bubbles collapse immediately, while others are disrupted into smaller ones and coalesce. The electron microscopic examinations performed by Schmidt & van Hooydonk (1980) demonstrate that, in pasteurised cream, the average diameter of the initially large air cells decreases rapidly during whipping. This technique was also used to show that foams are stabilised by dense embedding of the fat globules in the air cell surface with mutual interlacing by released liquid fat and partial coalescence of the globules (Schmidt & van Hooydonk, 1980). Figure 4.3 shows a schematic of whipped cream structure. The thickness of the fat globule layer corresponds primarily to the diameter of the existing fat globule or fat globule clusters. In such cases, however, free fat globules were only rarely observed, so that practically all fat globules participate in stabilisation. Each fat globule, containing crystalline fat and coming into contact with the air cell, is adsorbed irreversibly onto the surface of the bubble. The globule then spreads rapidly to reach its equilibrium position, as defined by the balance of interfacial forces.

Fig. 4.3  Schematic structure of whipped cream: (a) partially crystallised fat globules in the air surface; (b) partially coalesced fat globules forming a structure around air cells.
4.2.4  Butter

Precht (1988) describes the submicroscopic structure of butter crystals using freeze-fracture micrographs. He shows several fat globules apparently still largely intact along with isolated butterfat crystals and agglomeration of platelet-shaped crystalline fat aggregates, but also with fragments of more or less destroyed fat globules. He also describes the effect of continuous buttermaking machines; the marked shearing forces of this equipment, compared with the traditional process, resulted in a higher degree of destruction of the fat globules.

All fat globules in butter possess an outer crystal shell with a thickness of about 0.1–0.5 μm, consisting of concentrically deformed, superposed monomolecular triglyceride layers about 4–5 nm thick. On heating a butter sample to about 25°C, the greater part of this crystal shell remains unchanged, while in the interior the fat is exclusively present in the liquid form. The formation of the crystal shell, consisting of high-melting triglycerides associated with numerous crystal fat aggregates in the interior of this globule type, has apparently caused an increase in the inner firmness to such an extent that these globules are able to withstand the strong shearing forces during churning.

4.3 Milk fat

4.3.1 Composition

Milk can be described as a dilute oil-in-water emulsion. The fat in milk is present in fat globules which are dispersed in the aqueous plasma phase. The plasma is a colloidal mixture of casein micelles, serum proteins, sugars and minerals (Walstra & Jennes, 1984). Triacylglycerols constitute the bulk of the lipid mass in natural fats and oils, including milk fat. The fatty acids in milk fat result in a group of TAGs with a very wide compositional range. More than 200 species of even-numbered TAGs alone have been quantified in milk fat (Gresti et al., 1993). This wide variety of constituent fatty acids has been identified in cow’s milk fat (Jensen & Newburg, 1995; Hartel & Kaylegian, 2001), although only 13 of those are present at concentrations exceeding 1 g 100 g⁻¹ (Fig. 4.4). Fats from ruminant species are characterised by a relatively high proportion of short-chain fatty acids (C4–C10) and trans unsaturated acids. In addition, the distribution of fatty acids within milk fat TAGs is not random (Jensen et al., 1991). The acyltransferase enzymes involved in milk fat synthesis preferentially synthesise TAGs with short-chain fatty acids (C4–C10) in the sn-3 position and longer chain fatty acids in the sn-1 and -2 positions. As much as 85% of the butyric acid and 58% of the caproic acid in milk fat are found at sn-3 (Dimick et al., 1970). These short-chain fatty acids have an important role in dairy flavour, and also affect the crystallisation of milk fat by influencing the arrangement of milk fat TAG in the crystal lattice (Hartel & Kaylegian, 2001).

However, variation in milk fat composition occurs also as a result of season, region, diet and stage of lactation. Winter butter, for example, typically has a higher palmitic acid content and lower oleic content than summer butter and, overall, a higher level of saturation which makes it notoriously harder than summer butter (Prentice, 1972). The iodine value for winter butter, about 30, is lower than for summer butter which is typically 36. A change in iodine value of 3 g 100 g⁻¹ can effect a 50% change in firmness (Hayakawa et al., 1986).
Seasonal variations are related to the fact that the polyunsaturated content of fresh green fodder consumed by ruminants during the summer is higher than that of winter feed which has undergone extensive oxidation during storage.

Although milk fat contains hundreds of TAG species, milk fat composition is often discussed in terms of groups or fractions of TAGs, which are chemically and physically distinct (Timms, 1980; Bornaz et al., 1993; Marangoni & Lencki, 1998). Typically such fractions are distinguished from each other on the basis of their melting behaviour.

### 4.3.2 Fractionation

Milk fat is isolated from the globules after several steps. First, cream is separated from the milk and then churned to obtain butter. Finally, butter is melted to enable the separation of the water phase. After cooling the system at approximately 3°C the milk fat can be separated from the water. Fractionation is a common processing technology used to enhance the value of natural fats. It involves separation of components of a natural fat, based on some physical or chemical property. By separating a natural fat into components with specific chemical composition and physical properties, value-added ingredients can be produced from a commodity with lower value. The potential for milk fat fractionation has been reviewed by Kaylegian & Lindsay (1995), and more recent advances in this topic have been described by Hartel & Kaylegian (2001).

Basically, fractionation of milk fat results in three main fractions – high-melting fractions (HMF), medium-melting fractions (MMF) and low-melting fractions (LMF) – depending on their melting points (Marangoni & Lencki, 1998; Wright et al., 2000). In reality, however, numerous fractions of unique composition can be obtained by fractional crystallisation depending on the processing conditions and primarily crystallisation temperature (van Aken et al., 1999). Cooling rate, agitation, solvent properties and the presence of additives also influence crystallisation and the fractions obtained.
There are basically two different types of fractionation (dry and solvent fractionation). Dry fractionation can be accomplished by crystallising melted milk fat and separating the crystals from the liquid oil. Solvent fractionation is a more efficient process, improving the separation of TAG during the crystallisation and allowing for washing of the crystals to minimise entrainment. Solvent fractionation has some disadvantages, however: large quantities of solvent requiring disposal or recycling are produced and the nature of organic solvents requires extra safety precautions and lends an unnatural image to the products obtained. Therefore, a more friendly solvent-based approach would be attractive for milk fat fractionation. Wright et al. (2005) explored the potential of fractionating AMF into value-added partially crystalline fractions using canola oil as a solvent.

4.4 Microstructure

The microstructure of fat crystal networks includes elements in the length range between 0.5 μm and 500 μm. At the lower range of this microstructure, crystallites may be encountered, while in the upper ranges aggregates of microstructures (clusters of crystallites) are observed. This level of structure has an enormous influence on the macroscopic rheological properties of the network, noted by de Man & Beers (1987). Other researchers have also noted the importance of the microstructure for the rheological properties of the network, and the fact that the microstructure is altered with processing conditions of crystallisation (Heertje et al., 1987, 1988; Herrera & Hartel, 2000c; Campos et al., 2002; Martini et al., 2002b), as well as with interesterification (Marangoni & Rousseau, 1996, 1998a,b,c; Rousseau et al., 1996a,b,c, 1998; Rousseau & Marangoni, 1998a,b,c).

When a fat system is placed at a temperature below its melting point, it crystallises. The crystallisation kinetics induced by this process depend on several factors, such as the chemical composition of the sample. Some other factors that influence the crystallisation process include processing conditions such as cooling rate, crystallisation temperature and agitation. The presence of emulsifiers and/or impurities also affects the crystallisation of fats (Litwinenko et al., 2004). Of course, milk fat is not an exception and, therefore, several researchers have focused on the effect of all these variables on the crystallisation behaviour of milk fat, its fractions and some blends with different vegetable oils. The crystallisation process described before generates a crystal network at the microscopic scale. The study of this network and its correlation with some macroscopic characteristic is extremely important to re-introduce the use of milk fat in confectioneries and food products.

4.4.1 Effect of minor components

Together with the wide variety of TAGs that are present in milk fat, some minor components can be found when the chemical composition is analysed in detail. Milk fat is composed of 96–98 g 100 g⁻¹ of TAGs and 2–4 g 100 g⁻¹ of minor lipids (Christie, 1988; Bitman & Wood, 1990; National Dairy Council, 1993). These minor lipids are usually polar in nature and include compounds such as glycerides, free fatty acids, phospholipids and cholesterol. Table 4.1 shows the composition of the lipid species in cow’s milk fat.
A number of authors have studied the effect of minor components (monoacylglycerides and diacylglycerides) on the physicochemical characteristics of the milk fat (Herrera et al., 1999; Wright et al., 2001; Wright & Marangoni, 2002, 2003). Specifically, they found that the addition of minor components to the isolated milk fat TAGs delayed crystallisation. However, the structure and mechanical properties (yield force and storage modulus) of the crystallised material remained unchanged. On the other hand, as described by Tietz & Hartel (2000) for milk fat–cocoa butter blends, the removal of minor lipids resulted in the formation of irregular primary and secondary crystals with inclusions of liquid fat, whereas the crystals were spherical and uniform in shape in the presence of minor lipids. They also showed that minor lipids from milk fat, even at the low concentration typically found in nature, affected the crystallisation of milk fat–cocoa butter blends, impacted on the chocolate microstructure and affected bloom development in chocolate.

### 4.4.2 Processing conditions

#### Crystallisation temperature

The crystallisation of fat systems results in the formation of a microscopic network. In addition, different crystallisation conditions may affect these microstructures, as described by Wright & Marangoni (2003). One of the processing conditions that strongly affects the crystallisation behaviour and, therefore, the microstructure of fats is the crystallisation temperature. Research performed in our laboratory showed, in fact, the strong influence of crystallisation temperature on the crystal size and morphology of the system. In particular, when AMF was crystallised at 5°C and kept at this temperature for 24 h, a granular microstructure was observed with very small crystals and a narrow crystal size distribution (Fig. 4.5a). When the same sample was crystallised at 20°C (Fig. 4.5b), a combination of granular and spherulitic microstructures were observed resulting in a broader crystal size distribution. The higher the crystallisation temperature, the bigger the crystal clusters and more spherulitic-shaped crystals were observed. When the crystallisation temperature was as high as 27.5°C, only large spherulites were present after 24 h (Fig. 4.5c).

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<tr>
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<tr>
<td>Triacylglycerols</td>
<td>97.5</td>
<td>95.80</td>
<td>97–98</td>
</tr>
<tr>
<td>Minor, polar lipids</td>
<td>2.5</td>
<td>4.20</td>
<td>2.9</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>0.36</td>
<td>2.25</td>
<td>0.28–0.59</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>0.027</td>
<td>0.08</td>
<td>0.16–0.38</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>Trace</td>
<td>0.02</td>
<td>NR</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.31</td>
<td>0.46</td>
<td>0.419</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.596</td>
<td>1.11</td>
<td>0.2–1.0</td>
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<tr>
<td>Glycolipids</td>
<td>Trace</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Free fatty acids</td>
<td>NR</td>
<td>0.28</td>
<td>0.10–0.44</td>
</tr>
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NR = not reported.
Another important variable that should be taken into account when processing milk fat is the cooling rate. Samples crystallised at different cooling rates may result in very different microstructures and, therefore, different macroscopic properties that will in turn affect the quality of the final product. Figure 4.6 shows the microstructure of AMF crystallised to 5°C at different cooling rates; slow cooling rates (0.1°C min⁻¹, Fig. 4.6a) promote the formation of large crystals. In fact, they are not single crystals, but clusters that grow from agglomeration of small microstructures. These clusters are spherulite-shaped and are similar to the ones observed when samples are crystallised at high temperatures (see Fig. 4.5c). As the cooling rate increases from 0.1 to 5°C min⁻¹ (Figs 4.6b,c), the crystals are smaller, the spherulites tend to disappear and single crystals are more likely to form (Campos et al., 2002; Rye et al., 2005). When samples are cooled slowly, the system is closer to a thermodynamic equilibrium and the TAG molecules have more time to rearrange in clusters forming spherulites. When samples are cooled rapidly, the molecules do not have enough time to migrate and rearrange themselves into clusters and, therefore, more nuclei are formed resulting in smaller crystals (Martini et al., 2001).

Agitation

Usually, higher agitation rates promote crystallisation resulting in smaller crystals (Herrera & Hartel, 2000c). Martini et al. (2002b) showed that, for high-melting milk fat fractions—sunflower oil blends, different microstructures were obtained when crystallised under static and shear conditions. Samples crystallised statically resulted in more defined crystals or
clusters than when crystallised under agitation. Also, samples crystallised with agitation showed two populations of crystals. Large spherulites were observed together with smaller ones as shown by Rye (2003). The formation of smaller crystals was promoted by higher agitation rates. Also, this effect was more significant when samples were crystallised at slow cooling rates.

Mazzanti et al. (2003) used synchrotron X-ray diffraction to study the effect of shear on the crystallisation of different fat systems (cocoa butter, milk fat, stripped milk fat and palm oil). They showed that shear induced orientation of crystallites when milk fat was crystallised to 18°C at 3°C min⁻¹, resulting also in a reduction in phase transition times. The effect of shear on crystallising fats is of great importance for the industrial processes carried out in chocolate, dairy, margarine and shortening production, since temperature and shear treatments are the tools for tailoring the desired crystalline structures.

### 4.4.3 Chemical composition

Crystallisation behaviour and therefore microstructure of the fat network can be influenced by the chemical composition of the sample. As described before, even small amounts of minor components added to isolated TAGs can affect the rheological characteristics of a food product.

To extend the use of milk fat in food, pharmaceutical and cosmetic applications, fractionation may be performed to produce components with specific properties (e.g. melting points). Milk fat fractions are also blended to give a manufacturer greater flexibility to tailor milk fats as an ingredient to specific functional requirements than could be accomplished with
fractionation alone (Kaylegian & Lindsay, 1995). Milk fat fractions have distinct chemical and physical properties and rheological characteristics. They have application in various food products. For example, butter spreadability can be improved by fractionating milk fat and then recombining the fractions in various proportions (Kaylegian & Lindsay, 1992; Shukla et al., 1994; Illingworth, 2002). The fractions themselves have use in other foodstuffs. They function as high-melting shortenings in pastry making, as bloom inhibitors in chocolates, as butter-flavour-rich concentrates and as texture modifiers in reduced fat cheese curds (Bhaskar et al., 1998; Kaylegian, 1999; Rosenberg, 2000). Therefore, many researchers have studied the physicochemical characteristics of modified milk fat (interesterified) and different fractions melted with other fat sources, especially vegetable oils.

**Interesterification**

Previous work performed in our laboratory (Marangoni & Rousseau, 1996, 1998a,b,c; Rousseau & Marangoni, 1998a,b,c; Rousseau et al., 1996a,b,c, 1998) using polarised light microscopy (PLM) and scanning electron microscopy (SEM) also showed how the microstructure of milk fat can be modified by changing not only the crystallisation temperature but also the composition of the material. Butterfat, which was viewed with polarised light, revealed singular platelets measuring <5 μm (Fig. 4.7a). Replacement of 20 g 100 g–1 butter fat with canola oil altered the crystal morphology of butterfat crystals leading to gradual aggregation of the single crystals to form spherulites (Fig. 4.8a). Interesterification of butterfat led to aggregation, which was further promoted by the presence of canola oil in the interesterified blend. In general, the interesterified blends consisted of spherulites of varying densities measuring ~ 15 μm, with a lacy network of small crystals. The aggregates (flocs) of crystalline particles found in most samples generally had aspect ratios of ~ 1, indicating the circular aspects of the crystal aggregates (Figs 4.7, 4.8). They were structurally weak, i.e. slight agitation led to their dismemberment. Both chemical and enzymatic interesterification led to similar blend morphologies. PLM revealed substantial information on the crystalline nature of the fat crystals, but little concerning their interaction in a three-dimensional network. SEM results provided substantial topographical information on the fat crystal networks.

Figure 4.9 shows the surface structure of various samples examined by SEM. Figures 4.9a and 4.9b show a non-interesterified (NIE) butterfat and canola oil blend (90 : 10 g 100 g–1) after 1 and 8 days of tempering at 5°C; structure increased from day 1 to day 8. Figures 4.9c and 4.9d show NIE and interesterified (IE) 80 : 20 g 100 g–1 blends after 8 days of tempering, respectively; surface structure was different for each blend. In the NIE blend, a definite segregation of liquid and solid was visible that was not present in the IE equivalent. Figures 4.9e and 4.9f show NIE and IE 50 : 50 g 100 g–1 blends, respectively, after 1 day of tempering. Both samples appeared equally detailed but contained different structures. The NIE 50 : 50 g 100 g–1 blend showed a relatively smooth surface, whereas the IE 50 : 50 g 100 g–1 blend revealed segregation of spherulitic agglomerates.

**Blending anhydrous milk fat with vegetable oils**

When milk fat is blended with different amounts of vegetable oils, the chemical composition of the resulting blend is different from those of the original materials. Martini et al.
(2001) described, for example, the differences in TAG chemical composition of different blends of high-melting milk fat fraction (HMF) and sunflower oil (SFO) blends (Table 4.2). While SFO has high amounts of $C_{52}$ and $C_{54}$, HMF has high amounts of $C_{38}$, $C_{48}$, and $C_{50}$. Therefore, the addition of SFO to HMF resulted in a decrease of $C_{38}$, $C_{48}$ and $C_{50}$ values and an increase of both $C_{52}$ and $C_{54}$.

Wright et al. (2005) described the chemical composition of AMF and canola oil (CO) blends (Table 4.3). Similar to the SFO data, CO has high amounts of $C_{52}$ and $C_{54}$ while AMF has significant amounts of $C_{36-40}$ and $C_{48-52}$. Therefore, the addition of CO to AMF resulted in an increase of $C_{52}$ and $C_{54}$ TAGs and a decrease of $C_{36-40}$, $C_{48}$ and $C_{50}$ due to dilution effects.

Chemical composition can also be modified by blending two different fractions of milk fat, for example, low-melting milk fat fraction, LMF, with HMF as described by Herrera. 

Fig. 4.7  Polarised light microscopy of butterfat and butterfat–canola oil (g 100 g⁻¹ mixing ratio) samples tempered for 24 h at 5°C: (a) non-interesterified (NIE) butterfat; (b) interesterified (IE) butterfat; (c) NIE 90 : 10; (d) IE 90 : 10.
These changes in chemical composition will affect the microstructure of the crystal network obtained when cooling. For example, the addition of 40 g 100 g\textsuperscript{-1} of SFO in HMF resulted in bigger crystals when it was crystallised at 36°C compared to those obtained when HMF is cooled at the same temperature (Martini et al., 2001, 2002a). When AMF is blended with different proportions of CO and crystallised at 5°C, significant changes in the microstructure are observed (Fig. 4.10). AMF crystallised forming large spherulitic clusters and, as CO was added, these clusters were more uniform in size. Also, it is evident from Fig. 4.10 that a lower amount of solid is present when 30 g 100 g\textsuperscript{-1} of CO is added since the clusters are less bright and not very dense. These differences in crystal size are a consequence of the crystallisation kinetics of these systems. When CO is added to the AMF, the melting point of the resulting blends decreases as a result of the addition of unsaturated...

Fig. 4.8  Polarised light microscopy of butterfat–canola oil (g 100 g\textsuperscript{-1} mixing ratio) samples tempered for 24 h at 5°C: (a) non-interesterified (NIE) 80 : 20; (b) interesterified (IE) 80 : 20; (c) NIE 50 : 50; (d) IE 50 : 50.
fatty acids in the TAG molecules. Considering that all the samples were crystallised under the same conditions (same crystallisation temperature and time), the supercooling applied to the blends with CO addition is lower and therefore less material is crystallised. Also, differences in morphology could be attributed to different polymorphisms generated as a result of CO addition (Wright et al., 2005).

Fig. 4.9 Scanning electron micrographs of butterfat-canola oil (CO) blends (g 100 g⁻¹ mixing ratio) tempered for 1 or 8 days at 5°C: (a) non-interesterified (NIE) 90 : 10 for 1 day; (b) NIE 90 : 10 for 8 days; (c) NIE 80 : 20 for 8 days; (d) IE 80 : 20 for 8 days; (e) NIE 50 : 50 for 1 day; (f) IE 50 : 50 for 1 day.
### Table 4.2  Chemical composition (g 100 g⁻¹) of high-melting milk fat fraction (HMF) and sunflower oil (SFO).

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![Fig. 4.10](image-url)  Effect of canola oil (CO) addition (g 100 g⁻¹) on milk fat crystallised at 5°C at 0.1°C min⁻¹: (a) 0; (b) 10; (c) 20; (d) 30.
Blending different anhydrous milk fat fractions

As mentioned elsewhere, AMF can be fractionated into different components, which are characterised by different physicochemical parameters, such as melting point. Previous work in our laboratory demonstrated that blending different proportions of these fractions also affects the microstructure of the system. As an example, Fig. 4.11 shows the micrographs of HMF–MMF blends crystallised at 21°C and kept at this temperature for 24 h. When no HMF was added (Fig. 4.11a), MMF crystallised forming large, well-delimited clusters. These clusters were arranged as spherulites with needle-shaped crystals oriented radially. It can be also observed that these elements are very bright, which indicates that a large amount of material has crystallised. When only 10 g 100 g⁻¹ of HMF is added, spherulites become smaller and less dense. As the amount of HMF is increased, the microstructure of the crystals is changed. Clusters became less delimited and the needle-shaped crystals were more evident. When pure HMF was crystallised (Fig. 4.11k) a clear network of needle-shaped crystals was formed. Herrera & Hartel (2000c) also found significant differences in the microstructure of HMF–LMF blends when crystallised at 25°C and when different agitation and cooling rates were assayed.

### Table 4.3 Chemical composition (g 100 g⁻¹) of anhydrous milk fat (AMF) and canola oil (CO) and their blends.

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<th>Triacylglycerides (TAG)</th>
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ND = not detected.

**Microstructure of Dairy Fat Products**

87
In order to use milk fat blends in confectionery, milk fat fractions can also be blended with cocoa butter (CB). Figure 4.12 shows the effect of HMF addition to CB. When CB was crystallised without the addition of HMF (Fig. 4.12a), very small crystals forming a compact network were obtained. As HMF was added, the network was less compact and small needle-like crystals were observed. When 100 g 100 g\(^{-1}\) HMF was crystallised, it is clear (Fig. 4.12k) how the needles became bigger and arranged themselves into clusters.

**Fig. 4.11** Microstructure of high-melting fraction (HMF)–medium-melting fraction (MMF) blends (g 100 g\(^{-1}\)) crystallised at 21°C for 24 h: (a) 0 HMF; (b) 10 HMF; (c–k) successive increments by 10 of HMF in MMF.

*Blending anhydrous milk fat with cocoa butter*

In order to use milk fat blends in confectionery, milk fat fractions can also be blended with cocoa butter (CB). Figure 4.12 shows the effect of HMF addition to CB. When CB was crystallised without the addition of HMF (Fig. 4.12a), very small crystals forming a compact network were obtained. As HMF was added, the network was less compact and small needle-like crystals were observed. When 100 g 100 g\(^{-1}\) HMF was crystallised, it is clear (Fig. 4.12k) how the needles became bigger and arranged themselves into clusters.
4.4.4 Addition of emulsifiers to milk fat and its blends

In many food products, fat is present as an emulsion. That is, the fat is either inside droplets (oil-in-water emulsions, e.g. dressings) or as a continuous phase (water-in-oil emulsions, e.g. margarine and butter). During the formation of small dispersed droplets in an emulsion, the interfacial area between the two liquids becomes enormous. The resulting heterogeneous system is therefore thermodynamically unstable and thus emulsifiers are needed to stabilise the system. Considering the importance of the presence of emulsifiers in food products, several researchers have studied the effect of emulsifier addition on the crystallisation behaviour, and the microstructure of, milk fat, milk fat fractions and their blends. Different

![Microstructure of high-melting fraction (HMF)–cocoa butter (CB) blends (g 100 g⁻¹) crystallised at 21°C for 24 h: (a) 0 HMF; (b) 10 HMF; (c–k) successive increments by 10 of HMF in CB.](image)
emulsifiers of different chemical composition were studied and the effect on crystallisation behaviour was seen to be dependent not only on the emulsifier structure, but also on the chemical composition of the sample.

As described by Garti (1988) and Garti & Yano (2001), two mechanisms can be described to interpret the effect of emulsifiers on fat crystallisation in bulk fat systems. First, the emulsifier can act as heteronuclei accelerating nucleation through the catalytic actions of such impurities. During crystal growth, the emulsifiers are adsorbed at steps or kinks on the surface of growing fat crystals and, thereby, inhibit crystal growth and modify the crystal morphology. Second, fats and emulsifiers are able to co-crystallise because of their somewhat similar chemical structures. However, the structural dissimilarities between TAGs and emulsifier molecules can delay nucleation and inhibit growth. Therefore, this type of emulsifier would be a good inhibitor of crystallisation. In emulsion systems, another accelerating mechanism has also been observed. Hydrophobic emulsifiers can be absorbed at the oil–water interface and may accelerate the heterogeneous nucleation of the oil phase at the surface of the droplets when the oil-in-water emulsion is cooled.

Several researchers have studied the effect of emulsifier addition on both bulk and emulsified fats (Herrera & Marquez Rocha, 1996; Awad & Sato, 2001, 2002a,b; Awad et al., 2001). Specifically for milk fat, Martini et al. (2002c) studied the microstructure of HMF–SFO blends with the addition of sucrose ester emulsifiers of different hydrophobicity. They observed that the addition of SFO and lipophilic sucrose esters modified the microstructural properties of HMF. Also, Cerdeira et al. (2003) showed in detail how the addition of sucrose ester to HMF–SFO blends affects its crystallisation behaviour. In general, they observed that lipophilic sucrose esters delayed crystallisation of the blends and generated smaller microstructures.

Litwinenko et al. (2004) studied the effect of glycerol and Tween 60 addition on the physicochemical characteristics of a blend of triolein and HMF. They observed an increase in hardness induced by a small addition of Tween 60, which was attributed to decreases in crystal size at constant SFC and fractal dimension. Figure 4.13 shows the effect of Tween 60 addition on crystal sizes. For samples crystallised at 5°C, no effect on crystal morphology was observed (Fig. 4.13a–c). On the other hand, when samples were crystallised at 30°C, a significant increase in crystal size was observed, especially for high levels of Tween 60 addition (Fig. 4.13d–f). When only 0.1 g 100 g⁻¹ was added (Fig. 4.13b,e), the effect on crystal size was opposite, and smaller crystals were obtained when compared with the control samples (Fig. 4.13a,d). Therefore, depending on the product and whether a promotion or an inhibition of the crystallisation is required, a specific emulsifier should be chosen taking into account not only the emulsifier structure, but also its concentration and the chemical composition of the fat used and whether or not it is in an emulsified fat or a bulk system.

### 4.5 Rheology

As described before, the different microstructures obtained by changing processing conditions alter the macroscopic characteristic of the sample and, in turn, the final quality of the
food product. Therefore, in this section, the modification of rheological characteristics of fats by processing conditions will be briefly described. Campos et al. (2002) studied the crystallisation behaviour of milk fat and lard. These samples were crystallised at fast and slow cooling rates. When both samples were crystallised at slow cooling rates, bigger crystals were obtained, which is in accordance with the data described in Fig. 4.6. Also, the final SFC obtained when these samples were crystallised at 5°C was higher when the samples were crystallised at a fast cooling rate. In addition, the same researchers showed that the

Fig. 4.13  Polarised light micrographs of high-melting fraction (HMF)–triolein systems containing various levels of Tween 60 (g 100 g⁻¹) after 24 h at 5°C (A–C) and 15 min at 30°C (D–F). (A, D) control; (B, E) contain 0.1 Tween 60; (C, F) contain 0.5 Tween 60.
hardness of these samples was directly correlated with their SFC. Therefore, as stated before, the microstructure of a sample determines, in turn, the final appearance of the product. In this case, bigger crystals resulted in softer samples.

Many other examples can be found in the literature. Nor Aini (2001) showed that blends of 80 g 100 g⁻¹ of palm oil in AMF tempered at low temperatures (10°C for 2 days) were firmer with a high yield value compared with the same sample tempered at 23°C and 30°C. Also, when the amount of AMF in the blends was increased, the samples became softer, and a significant increase in the solid contents of the shortenings after 8 weeks of storage was observed. Litwinenko et al. (2004) showed that the addition of 0.1 g 100 g⁻¹ of glycerol or Tween 60 increased the yield force of a 70 : 30 g 100 g⁻¹ mixture of triolein and HMF at 5°C. Further increases in glycerol or Tween 60 caused a decrease in yield force. The increase in hardness induced by small additions of Tween 60 could be attributed to decreases in crystallite size at constant SFC and fractal dimension. The increase in hardness induced by small additions of glycerol could only be attributed to increases in the crystal melt interfacial tension, at constant SFC and fractal dimension, despite increases in crystallite size.

Puppo et al. (2002) studied the effect of sucrose esters on the crystallisation kinetics and rheological behaviour of HMF and SFO blends. They demonstrated that the addition of sucrose esters delayed crystallisation. When sucrose esters were added, a SFC greater than 10 g 100 g⁻¹ was necessary to give a stronger structure that could be noticed as a higher yield point.

As mentioned before, Rousseau and co-workers studied different blends of milk fat with canola oil (CO), and the effect of interesterification of these blends (Marangoni & Rousseau, 1996, 1998a,b,c; Rousseau et al., 1996a,b,c, 1998; Rousseau & Marangoni, 1998a,b,c). They found that both blending and chemical interesterification diminished the hardness of the blend in a non-linear fashion. In addition, hardness changes in IE blends were more pronounced than in NIE blends. They also showed that the addition of 30 g 100 g⁻¹ of CO led to significant changes in small deformation measurements, while the addition of 20 g 100 g⁻¹ of CO resulted in significant changes in large deformation measurements (Rousseau et al., 1996c).

Herrera & Hartel (2000a), on the other hand, studied the effect of processing conditions on rheological behaviour of three blends of high- and low-melting milk fat fractions. They showed that the storage modulus varied with all processing conditions and, even for the same solid fat content, different rheological properties were found. Moduli were higher for samples crystallised at a slow cooling rate, decreased with agitation rate, and were lower for the blend with lower amounts of HMF for all processing conditions. Storage moduli also increased with storage time.

In conclusion, processing conditions, chemical composition and the addition of emulsifiers affect the microstructure of the fat and, therefore, its texture which is a direct measurement of the product macroscopic characteristics and in turn of its quality.

### 4.6 Fat crystal networks and microstructure

A fat crystal network is the product of the aggregation process of molecules into particles, and of particles into larger clusters, until a space-filling three-dimensional network is formed. Studies by Heertje and co-workers originally demonstrated that fat crystal networks of fat spreads can display such structural hierarchy (Heertje et al., 1987; Juriaanse & Heertje, 1988;
Heertje, 1993). When TAGs are cooled from the melt to a temperature below their melting point, i.e. when they are supercooled, they undergo a liquid-to-solid transformation to form primary crystals with characteristic polymorphism. These primary crystals aggregate (or grow into each other) to form clusters, which further interact, resulting in the formation of a continuous three-dimensional network. The macroscopic rheological properties of networks formed by lipids are of great importance in food products that contain significant amounts of fats. Such products include margarine, butter, chocolate, peanut butter, many spreads such as cream cheese, and ice cream. Many sensory characteristics, such as spreadability, hardness, appearance and mouth-feel, are dependent on the mechanical strength of the underlying fat crystal network (Marangoni & Rousseau, 1996, 1998b; Narine & Marangoni, 1999a,b; Marangoni, 2002). Therefore, the firmness and solid-like properties of manufactured plastic fats, such as margarine, are established during the formation of a fat crystal network. In order to truly understand, and eventually predict, the macroscopic properties of soft materials, it is necessary to characterise and define the different levels of structure present in the material and their respective relationship to a macroscopic property. Knowledge of the relationships between molecular composition and phase behaviour, solid-state structure, growth mode, static structure and macroscopic properties will eventually allow for the rational design of specific macroscopic properties.

The formation of a fat crystal network is of key importance in the manufacture of plastic fats, because it provides firmness or solid-like properties (viscoelasticity). This network can be visualised as being built from aggregates rather than from straight chains of fat particles, and can be thought of as a colloidal aggregate, analogous to a protein gel. Each of the fat particles is, in turn, comprised of several aggregated fat crystals. The quantitative description of such a complex and ‘random’ system is difficult. Recently, fractal geometry has proven to be extremely helpful in the characterisation of these fractal objects. Two methods for characterising macrostructure in fat systems have been developed in our laboratory. These methods include small deformation rheology and microscopy techniques, employing a fractal approach (Marangoni & Rousseau, 1996; Narine & Marangoni, 1999b). The description of these methods exceeds the scope of this chapter, but detailed information can be found in several publications (Narine & Marangoni, 1999a,b, 2001; Marangoni, 2000, 2002, 2005; Marangoni & Narine, 2002).

Rousseau & Marangoni (1999) noted that the rheological properties of IE and NIE butterfat–CO blends did not seem to be strongly related to either solid fat content or crystal polymorphism, but rather to the microstructure of the fat crystal network. Chemical interesterification decreased the fractal dimension (D) of the fat crystal network from 2.46 to 2.15. They proposed that fat microstructure, as quantified by fractal dimensionality, could be modified to attain specific rheological properties. Fractal dimension values calculated using the microscopic method are associated both with the degree of fill of the image and with crystal size. The higher the fractal dimension value, the higher the degree of fill and smaller crystal size and therefore a larger amount of crystals.

When the fractal dimension of AMF with and without the addition of emulsifiers was studied by microscopy or rheology at 5°C, no differences were observed. There was, however, a strong temperature effect on D, increasing from 1.87 at 5°C to 1.98 at 22.5°C, which was in agreement with the different microstructures found at these temperatures (Wright & Marangoni, 2003).
4.7 Milk fat in emulsions

4.7.1 Introduction

Milk, which is the starting point of many food products, is a natural oil-in-water emulsion. Natural milk fat, mainly composed of about 97 g 100 g\(^{-1}\) TAGs, is dispersed in globules with a mean diameter of 4 \(\mu\text{m}\) stabilised by a complex membrane. The concentration of fat globules in milk leads to cream. The increased knowledge of crystallisation of natural milk fat globules might be of a certain value for technical applications on milk fat crystallisation. Thus, it is interesting to compare crystallisation of fat dispersed in emulsion, such as milk or cream, with crystallisation of bulk milk fat, devoid of the physical barrier of membranes. Lopez et al. (2001) studied the thermal and structural properties of TAGs in fat globules of cream and in AMF using X-ray diffractometry and differential scanning calorimetry (DSC). They showed that similar structures were formed when crystallising cream and TAGs at fast cooling rate, but they also showed that TAG crystallisation is more disordered in emulsions, showing broader peaks attributed to constraints due to interface curvature in emulsion droplets.

4.7.2 Emulsion stability

The process of controlled fat destabilisation of an emulsion during whipping and air incorporation is responsible for the establishments of structure in two notable dairy products – whipped cream and ice cream – leading to complex products described both as protein-stabilised emulsions and fat-stabilised foams. This process has been studied by several researchers in whipped cream (Schmidt & van Hooydonk, 1980; Brooker et al., 1986; Noda & Shiinoki, 1986; Bruhn & Bruhn, 1988; Brooker, 1990; Needs & Huitson, 1991; Stanley et al., 1996; Smith et al., 1999, 2000a,b) and ice cream (Goff & Jordan, 1989; Gelin et al., 1994; Goff et al., 1997). Fat destabilisation is also responsible for structure formation in a variety of whipped non-dairy dessert toppings (Büchheim et al., 1985; Barfod & Krog, 1987). ‘Fat destabilisation’ (sometimes known as ‘fat agglomeration’) is a general term that describes the summation of several different phenomena (Goff, 1997), and the factors involved are as follows.

**Creaming**

During storage, due to the density differences between most edible oils and water, there is a tendency for the oil phase to concentrate at the top of the food emulsion. The rate of creaming can be lowered by reducing the droplet size, lowering the density difference between oil and the aqueous phase, and increasing the viscosity of the medium. In addition, the creaming rate is dependent on the volume fraction of the dispersed phase, and is usually slow in concentrated emulsions (volume fractions, \(\Phi > 0.74\)). Creaming is dominating at \(\Phi\) between 0.1 and 0.5 and droplet size range from 2 to 5 \(\mu\text{m}\) (Fig. 4.14a).

**Flocculation**

Flocculation is defined as a process by which two or more droplets aggregate without losing their individual identity. In practical food emulsions, larger droplets (> 2 \(\mu\text{m}\)) flocculate
faster and flocculation is promoted by creaming. Flocculation is dominant at $\Phi < 0.05$ and droplet size less than 1 μm. Bridging flocculation occurs in the presence of macromolecular emulsifying agents in an emulsion. Emulsion droplets flocculate through interaction of the adsorbed macromolecules between droplets. Bridging flocculation is a very complex phenomenon and depends greatly on the size, type and amount of the macromolecules used in the system. In addition, the rate of flocculation can be affected by the pH and ionic strength of the aqueous environment. Interactions among protein, polysaccharide and water-soluble surfactants can also affect the stability of the emulsion (Fig. 4.14b).

**Coalescence**

Coalescence is the process when two or more droplets collide with each other, resulting in the formation of one larger droplet, and is dominant when $\Phi$ is high. Coalescence involves breaking the interfacial film, and is irreversible. At the extreme, a planar interface exists between the homogeneous lipid phase and the homogeneous aqueous phase. Various factors, such as solubility of the emulsifier, pH, salts, emulsifier concentration, phase-volume ratio, temperature and properties of the film, affect the coalescence stability of an emulsion (Fig. 4.14c).

---

**Fig. 4.14** Emulsion destabilisation mechanisms.
Partial coalescence

Partial coalescence is an irreversible agglomeration or clustering of fat globules, held together by a combination of fat crystals and liquid fat, and a retention of identity of individual globules as long as the crystal structure is maintained (i.e. temperature-dependent; once the crystal melts, the cluster coalesces). Partial coalescence dominates structure formation in whipped, aerated dairy emulsions (Boode et al., 1991, 1993; Boode & Walstra, 1993; Goff et al., 1999), and occurs because of the presence of fat crystals within the emulsion droplets (Rousseau, 2000).

Ostwald ripening

Ostwald ripening occurs in emulsions with polydispersed droplets. Collisions between two droplets may lead to one bigger droplet and one smaller droplet. As a result, small droplets become smaller. Eventually, the small droplets become very small and become solubilised in the continuous medium. Since a high solubility of the oil in the aqueous phase is required, Ostwald ripening is uncommon in food systems where triglycerides are not normally soluble in water. Nevertheless, Ostwald ripening occurs rapidly in frozen foods and in water–oil emulsions where water is partially soluble in polar triglyceride oils (Fig. 4.14d).

In products such as whipped cream and ice cream, the emulsion must be stable in the liquid form, but must partially coalesce readily upon foaming and the application of shear. The structural and physical properties of whipped cream and ice cream depend on the establishment of a fat globule network. In cream whipped to maximum stability, partially coalesced fat covers the air interface. In ice cream, partially coalesced fat exists both in the serum phase and at the air interface; also, there is more globular fat at the air interface with increasing fat destabilisation. Partial coalescence occurs due to the collisions in a shear field of partially crystalline fat emulsion droplets with sufficiently weak esteric stabilisation (low level of surface adsorption of amphiphilic material to the interface per unit area). To achieve optimal fat crystallinity, the process is very dependent on the composition of the TAGs and the temperature. It is also possible to manipulate the adsorbed layer to reduce steric stabilisation to an optimal level for emulsion stability and rapid partial coalescence upon the application of shear. This can be done either by the addition of a small molecule surfactant to a protein-stabilised emulsion or by a reduction of protein adsorption to a minimal level through selective homogenisation (Goff, 2001). Figure 4.15 shows the colloidal structure of ice cream mix (Figs 4.15A,B), ice cream (Figs 4.15C–F) and melted ice cream (Figs 4.15G,H), as viewed by electron microscopy, focusing specifically on the effect of added emulsifiers on fat structure formation (Marshall et al., 2003). Figures 4.15(A,C,E,G) show micrographs of the samples without surfactants, and Figs 4.15(B,D,F,H) with the addition of surfactants.

From Fig. 4.15 it can be seen that the colloidal structure of ice cream begins with the mix as a simple emulsion, with a discrete phase of partially crystalline fat globules surrounded by an interfacial layer comprised of proteins and surfactants. The continuous, serum phase consists of the unadsorbed casein micelles in suspension in solution of sugars, unadsorbed whey proteins, salts and high molecular-weight polysaccharides. During the ‘freezing’ stage of manufacture, the mix emulsion is foamed, creating a dispersed phase of air bubbles, and is frozen, forming another dispersed phase of ice crystals. Air bubbles and ice crystals
Fig. 4.15  Electron micrographs showing the microstructure of ice cream: (A, B) ice cream mix; (C–F) ice cream; (G, H) melted ice cream; (A, C, E, G) without added surfactant; (B, D, F, H) with added surfactant. f = fat globule, c = casein micelle, arrow = crystalline fat, a = air bubble, fc = fat cluster, fn = fat network. Source: Marshall et al. (2003) Ice Cream, 6th edn, Kluwer Academic/Plenum Publishers. Reproduced by kind permission of Dr H.D. Goff and Springer Science and Business Media, Heidelberg.
usually range in size from 20 to 50 μm and are surrounded by a temperature-dependent unfrozen phase. In addition, the partially crystalline fat phase in the mix at refrigerated temperatures undergoes partial coalescence during the concomitant whipping and freezing process, resulting in a network of agglomerated fat, which partially surrounds the air bubbles and gives rise to a solid-like structure (Goff & Jordan, 1989; Boode & Walstra, 1993; Boode et al., 1993).

### 4.8 Conclusions

The microstructure of food products determines to a large extent the physical, textural and sensory properties of these products. Developing a proper understanding of the microstructure, particularly the spatial distribution and interaction with food components, is a key tool in developing products with desired mechanical and organoleptic properties. The microstructure of a food product, specifically milk fat and their derivatives, can be affected by several factors. The first factor that should be taken into account is the chemical composition of the starting material. In the case of milk fat, the TAG composition, together with the presence of minor components or impurities, strongly affects the crystallisation behaviour and, therefore, the microstructure of the final product. Processing conditions are also significant parameters that should be taken into account in the elaboration of a product. Cooling rate, crystallisation temperature and agitation are some of the variables that affect the microstructure of fats. The microstructure found in each of these cases can be quantified by means of fractal dimension analysis, and usually a correlation between microstructure and textural properties of the material can be found. Understanding which factors control the microstructure of a product and being able to control them during processing are of major importance in obtaining a high-quality product with the desired physicochemical and sensory attributes.

### Acknowledgements

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### References


5 Microstructure of Concentrated and Dried Milk Products

A.Y. Tamime, R.K. Robinson and M. Michel

5.1 Introduction

Concentrated milks, such as evaporated milk or sweetened condensed milk, are liquid milk products with considerably reduced water content and, as a consequence, have a long-shelf. The water content in milk is primarily removed by evaporation under vacuum but, in theory, other methods could be used to remove water, such as sifting, centrifugation, extraction, lyophilisation and membrane filtration (Carić, 1994). Under commercial application, vacuum evaporation and membrane filtration methods are widely used to concentrate the milk. In principle, the preservation of evaporated milk is achieved by in-container sterilisation or ultra high temperature (UHT) processing followed by aseptic packaging. However, by adding a large amount of sugar to evaporated milk, a condition is created in the product such that micro-organisms are unlikely to grow, and the sweetened condensed milk is thus preserved for long periods. These products are widely used in tea and coffee; however, evaporated milk can be diluted and consumed as liquid milk, while sweetened condensed milk can be also used in sandwiches to replace jam.

In contrast, dried milk powders (e.g. whole, skimmed or retentate), dried buttermilk, and other dairy powders, such as cheese whey powder (WP), whey protein concentrates (WPC), whey protein isolates (WPI), caseinates and lactose, are products made from milk and whey where practically all the water is removed, i.e. to < 4 g 100 g\(^{-1}\) of water. These dried products have a very long shelf-life, they can be stored at ambient temperature, and can be exported to countries that have a shortfall in milk production. After rehydrating milk powders, the reconstituted products may be similar to fresh milk (whole or skimmed), and the rest of the dairy powders have different applications in the dairy, food and pharmaceutical industries. The following are recommended for further reading regarding aspects of milk concentration, drying, heat stability of proteins and membrane processing (Masters, 1985; Mujumdar, 1987; Sienkiewicz & Riedel, 1990; Carić, 1994; Grandison & Glover, 1994; Knipschildt & Andersen, 1994; Southward, 1994; Zadow, 1994; Pisecky, 1997; Cheryan, 1998; Tamime & Robinson, 1999; Nielsen, 2000; Verdurmen & de Jong, 2000; Bimbenet et al., 2002; Ilari, 2002; Kelly et al., 2002; Anon., 2003; Fox & McSweeney, 2003; Birchal et al., 2005; Walstra et al., 2006).

This chapter will briefly review the processing methods that are used commercially to manufacture concentrated and powder products, and the changes that may occur in the structures (i.e. using differential interference contrast light microscopy – DICLM, polarised light microscopy, scanning electron microscopy – SEM and transmission microscopy – TEM) of the milk components.
5.2 Patterns of production

The world production figures for condensed and evaporated milk and milk powders have fluctuated over the past couple of decades. According to IDF (2004), condensed and evaporated milk production declined in some industrialised countries (e.g. the European Union, Eastern European countries, Canada and South Africa) mainly due to reduced local demands and export opportunities, while production of these products is growing slowly in the USA and some other countries (Table 5.1).

Recently, the production of powders in countries where data are available has shown resumed growth, in particular in Europe, New Zealand, Argentina and Brazil, in response to demand for these products in many countries worldwide (Tables 5.2 and 5.3). It is of interest to note that the improvements in the functionality of certain powders are the major driving force in demand. It is evident that the strongest increase in production of powders in recent years is in China, the world’s largest producer. In some countries, the possible increase in whole milk powder production is mainly attributed to local demand, especially in remote regions within a country and where milk production is not available. However, the fluctuation in production of skimmed milk powder in many countries is mainly attributed to fluctuations in milk supplies (IDF, 2004). The EU is by far the biggest producer and utiliser of skimmed milk powder.

No data are available on world production figures for whey and caseinate powders, but sweet whey powder production in the EU and North America accounted for 1.3 million tonnes and 550 000 tonnes, respectively (IDF, 2004). Other derivatives of whey are whey protein concentrates, lactose, demineralised whey, delactosed whey and/or whey protein isolates, but the production data for these products are not available.

Table 5.1 Total production (000 tonnes) of condensed milk products in 1995, 2000 and 2003.

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<td>USA</td>
<td>220.3</td>
<td>211.1</td>
<td>270.0</td>
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*a Fifteen countries within the European Union.
NR = not reported.
5.3 Milk as a raw material

Most concentrated and dried products are made from cow’s milk, and initial on-farm handling of the milk is important. Figures 5.1(a) and 5.1(b) illustrate the main differences between the fat globules in raw and homogenised milk. The size of a fat globule is up to 10 μm, and they are easily recognisable in raw milk. If raw milk is stored for several hours after milking, the presence of large fat globules, in combination with cold agglutination (aggregate formation), causes ‘creaming’ to occur; this effect may be reduced by stirring the raw milk in the farm bulk tank. However, homogenisation reduces the size of fat globules to ~1 μm, giving the milk its long-term physical stability (Fig. 5.1b).

Normally the milk is cooled to < 10°C before it is transported to the factory, and the TEM structure of the milk components, which are affected by cooling, are the fat globules and casein micelles (Fig. 5.2). However, if raw milk is stored at room temperature, the fat globules are covered by a membrane known as the milk fat globule membrane (MFGM),

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Table 5.2 Total production (000 tonnes) of whole and semi-skimmed milk powder in 1995, 2000 and 2003.

a Fifteen countries within the European Union.
b All milk powders.
NR = not reported.
which consists mainly of phospholipids and proteins. The MFGM is rather thick and shows a high electron density (i.e. black rim around the globules) (Fig. 5.2a). Lowering the temperature to 4°C has no influence on the MFGM, but leads to a partial crystallisation of the fat present in the fat globules (Fig. 5.2c). In contrast, the structure of casein micelles in

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<td>USA</td>
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<td>661.0</td>
<td>723.3</td>
</tr>
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</table>

\(^a\)Fifteen countries within the European Union.
NR = not reported.

![Fig. 5.1](image)

**Fig. 5.1** Structure of (a) unhomogenised and (b) homogenised raw milk using differential interference contrast light microscopy (DICLM). Reproduced by permission of Nestlé Research Centre, Switzerland.
milk stored at 20°C are round, have a smooth surface, and their interior structure is homogeneously stained (Fig. 5.2b), while in cold milk (e.g. at 4°C) the micelles are irregular in shape, exhibit a hairy surface and a rather ‘loose’ internal structure (Fig. 5.2d). In addition, fine needles exhibiting a high electron density are observed around and in between the casein particles.

When milk is subjected to homogenisation and heat treatment, for example pasteurisation at 72°C or UHT, the structure of the MFGB cannot be observed and most of the globules at the fat interface are stabilised by the proteins (Figs 5.3a,c). Pasteurisation (e.g. 72°C for 15s) causes changes in the micelles, leading to a more irregular shape and ‘looser’ internal structure (Fig. 5.3b) compared to the more compact structure in UHT milk (Fig. 5.3d). Although pasteurisation and UHT treatments are not used during the manufacture of concentrated and dried products, the high heating temperature of milk > 85°C may induce changes in the MFGM and the casein micelles, similar to those shown in Fig. 5.3.

Fig. 5.2 TEM structure of raw milk stored (a, b) at room temperature and (c, d) at 4°C. Reproduced by permission of Nestlé Research Centre, Switzerland.
5.4 Concentrated milk products

As mentioned elsewhere, concentrated milk products are known as evaporated milk and sweetened condensed milk. The former product is heat sterilised, while sweetened condensed milk contains high concentrations of sugar to restrict the growth of micro-organisms.

5.4.1 Evaporated milk

This concentrated product may have different chemical compositions in different countries based on existing statutory instruments. For example, in the USA, the American standard states that the whole-fat product must contain (g 100 g⁻¹) 7.8 fat and 18.1 solids-not-fat.

Fig. 5.3 TEM micrographs showing effect of heating by (a, b) pasteurisation and (c, d) ultra high temperature, on the structure of the fat globule membrane and casein micelles. Reproduced by permission of Nestlé Research Centre, Switzerland.
(SNF), with concentration factor ($Q$) 2.1, while the British Standards stipulate that the product should contain 9 fat and 22 SNF, with $Q$ 2.6 (Walstra et al., 2006). However, reduced-fat evaporated milk is also produced; for example (1) low-fat product should contain 4 fat and 20 SNF with $Q$ 2.25, and (2) skimmed milk evaporated milk should contain 0.1 fat and 22 SNF with $Q$ 2.35 (see Table 5.4).

**Method of manufacture**

The fat content in the milk base is standardised, preheated to < 100°C for 20 min (old method) or 130°C for 30 s (current method of processing), followed by concentration using vacuum evaporation or membrane filtration, for example, reverse osmosis. The application of heat inactivates the micro-organisms and enzymes present in the milk, and it improves the heat stability of milk when subjected to high-temperature processing of the concentrate (Carić, 1994; Walstra et al., 2006). Two methods of processing are available. In the first method (in-container sterilisation), the concentrated milk is homogenised (e.g. two-stage homogenisation at 22 and 5 MPa pressure, respectively) to prevent creaming and coalescence of the fat globules, cooled to 10°C, stabilised with sodium phosphate ($\text{Na}_2\text{HPO}_4$), packaged and heat sterilised at 120°C for 15 min. In the second method (continuous sterilisation of evaporated milk, i.e. UHT method), the concentrated milk is stabilised with $\text{Na}_2\text{HPO}_4$, heated to 140°C for 15 s, cooled to 60°C, homogenised at a pressure of 45 MPa using a single-stage homogeniser, cooled to 10°C and aseptically packaged.

In some developing countries where there is a shortage of fresh milk, evaporated milk is produced by the recombination of skimmed milk powder (SMP), anhydrous milk fat (AMF) and water; however, 10% of the skimmed milk powder may be replaced by butter-milk powder to improve the flavour of the product (Carić, 1994; Walstra et al., 2006). The dairy ingredients are recombined at 40°C to achieve the desired chemical composition of the product, filtered to remove any undissolved particles, heated to 62°C, homogenised at pressures of 20 and 4 MPa, and cooled to 10°C. Stabilising salt(s) (e.g. $\text{Na}_2\text{HPO}_4$ and/or carrageenan) is added to the cooled concentrate, which is packaged, sterilised at 117°C for 13 min and finally cooled (Carić, 1994; Schuck, 2002; Walstra et al., 2006). Incidentally, in some instances the dairy fat is replaced by vegetable fat and the product is known as ‘filled’ evaporated milk.

**Structure**

The structure (using DICLM) of evaporated milk consists of visible aggregated particles (Fig. 5.4a); by comparison sweetened condensed milk contains sucrose crystals up to 10–20 μm (Fig. 5.4b). At higher magnification, the TEM structure of evaporated milk consists of very small fat globules embedded in the protein matrix (Figs 5.5a,b).

**5.4.2 Sweetened condensed milk**

This product is concentrated by evaporation and sugar (i.e. sucrose) is added to the concentrate. The composition of standards (g 100 g⁻¹) of full-fat sweetened condensed milk in the
Table 5.4  Average gross chemical composition (g 100 g⁻¹) of commercial concentrated and dried dairy products.

<table>
<thead>
<tr>
<th>Product</th>
<th>Component</th>
<th>Moisture</th>
<th>Protein</th>
<th>Fat</th>
<th>Lactose + sucrose¹</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaporated milk (full fat)</td>
<td></td>
<td>69.1</td>
<td>8.7</td>
<td>10.1</td>
<td>8.5</td>
<td>1.9</td>
</tr>
<tr>
<td>(reduced fat)</td>
<td></td>
<td>74.7</td>
<td>6.5</td>
<td>7.6</td>
<td>9.2</td>
<td>1.5</td>
</tr>
<tr>
<td>(skimmed)</td>
<td></td>
<td>70.0</td>
<td>11.1</td>
<td>0.2</td>
<td>16.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Evaporated buttermilk</td>
<td></td>
<td>70.0</td>
<td>11.2</td>
<td>1.4</td>
<td>15.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Evaporated whey</td>
<td></td>
<td>32.0</td>
<td>10.1</td>
<td>0.6</td>
<td>51.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Sweetened condensed milk (full fat)</td>
<td></td>
<td>26.1</td>
<td>8.2</td>
<td>8.8</td>
<td>55.1</td>
<td>1.8</td>
</tr>
<tr>
<td>(skimmed)</td>
<td></td>
<td>29.0</td>
<td>10.0</td>
<td>0.2</td>
<td>56.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Sweetened condensed buttermilk</td>
<td></td>
<td>28.0</td>
<td>11.2</td>
<td>1.4</td>
<td>57.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Sweetened condensed whey</td>
<td></td>
<td>24.0</td>
<td>5.6</td>
<td>0.3</td>
<td>66.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Milk powder (full fat)</td>
<td></td>
<td>3.5</td>
<td>25.2</td>
<td>26.2</td>
<td>38.1</td>
<td>7.0</td>
</tr>
<tr>
<td>(retentate)</td>
<td></td>
<td>NR²</td>
<td>41.7</td>
<td>41.7</td>
<td>9.3</td>
<td>NR</td>
</tr>
<tr>
<td>Milk powder (skimmed)</td>
<td></td>
<td>4.3</td>
<td>35.0</td>
<td>0.9</td>
<td>51.9</td>
<td>7.8</td>
</tr>
<tr>
<td>(retentate – UF)</td>
<td></td>
<td>NR</td>
<td>62.8</td>
<td>0.9</td>
<td>23.9</td>
<td>NR</td>
</tr>
<tr>
<td>(retentate – UF and DF1)</td>
<td></td>
<td>6.3</td>
<td>75.8</td>
<td>2.1</td>
<td>9.0</td>
<td>6.8</td>
</tr>
<tr>
<td>(retentate – UF and DF3)</td>
<td></td>
<td>6.4</td>
<td>81.5</td>
<td>2.3</td>
<td>3.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Buttermilk powder</td>
<td></td>
<td>3.5</td>
<td>36.0</td>
<td>4.5</td>
<td>47.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Whey powder</td>
<td></td>
<td>4.0</td>
<td>14.2</td>
<td>0.6</td>
<td>72.7</td>
<td>8.5</td>
</tr>
<tr>
<td>Whey protein concentrate powder</td>
<td></td>
<td>8.3</td>
<td>46.7</td>
<td>4.5</td>
<td>37.1</td>
<td>3.4</td>
</tr>
<tr>
<td>(commercial sample)</td>
<td></td>
<td>NR</td>
<td>73.2</td>
<td>0.2</td>
<td>12.0</td>
<td>NR</td>
</tr>
<tr>
<td>Caseinate powder (Na)</td>
<td></td>
<td>4.0</td>
<td>94.0</td>
<td>1.5</td>
<td>0.2</td>
<td>4.0</td>
</tr>
<tr>
<td>(Ca)</td>
<td></td>
<td>4.0</td>
<td>93.5</td>
<td>1.5</td>
<td>0.2</td>
<td>4.5</td>
</tr>
<tr>
<td>(acid)</td>
<td></td>
<td>10.0</td>
<td>95.0</td>
<td>1.5</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td>(rennet)</td>
<td></td>
<td>12.0</td>
<td>89.0</td>
<td>1.5</td>
<td>NR</td>
<td>7.5</td>
</tr>
<tr>
<td>(co-precipitate)</td>
<td></td>
<td>5.0</td>
<td>92.0</td>
<td>1.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Lactose powder (crude grade)</td>
<td></td>
<td>0.3</td>
<td>0.8</td>
<td>0.1</td>
<td>98.4</td>
<td>0.4</td>
</tr>
<tr>
<td>(pharmaceutical grade)</td>
<td></td>
<td>0.1</td>
<td>0.01</td>
<td>0.001</td>
<td>99.85</td>
<td>0.03</td>
</tr>
</tbody>
</table>

¹ Sucrose is only present in sweetened condensed milk.
² NR = not reported.
UF = ultrafiltration of the milk was used to concentrate the milk and reduce the lactose before evaporation and drying.
DF1 = diafiltration of the retentate once to reduce the lactose level.
DF3 = diafiltration of the retentate three times to reduce the lactose level.
USA and UK are: 8 or 9 fat, 20 or 22 SNF, 10.3 or 11.4 lactose, 45 or 43.5 sucrose, and 27 or 25.5 moisture, respectively (Walstra et al., 2006). Skimmed milk sweetened condensed milk contains 23.5 g SNF 100 g⁻¹ and ~ 0.5 g fat 100 g⁻¹ (see also Table 5.4).

**Method of manufacture**

The milk is heated to 135°C for 5 s, cooled to 70°C, homogenised at 4 MPa of pressure, and concentrated by vacuum evaporation. A sugar solution (refined and devoid of any invert sugar to restrict or minimise Maillard reactions) should be heat-treated to inactivate any yeasts before adding to the concentrate. The latter is then cooled to 50°C, and seeded with very fine lactose crystals (i.e. ~ 30°C) to induce the formation of small lactose crystals; otherwise, if large crystals are formed, the product will have a ‘sandy’ texture. Afterwards, the product is cooled to 18°C, and packaged in metal containers. It is recommended that the metal cans and lids should be sterilised before packaging with the sweetened condensed milk, and the air in the packaging area should be filtered using high-efficiency particulate air (HEPA) filters to remove any bacteria from the air and minimise contamination of the product (Carić, 1994; Tamime, 2002; Walstra et al., 2006).

**Structure**

Sucrose crystals are evident in the structure of sweetened condensed milk, and also crystalline ‘needle’-shaped structures are present, which are made of calcium phosphate (Fig. 5.4b). The TEM structure of sweetened condensed milk shows that the fat globules appear as single entities and their interface is stabilised with a thin protein layer (Figs 5.5c,d).

### 5.4.3 *Bulk concentrated milk*

This product is used as a source of milk solids for candy, bakery products, ice cream, concentrated yoghurt and a number of other manufactured foods. Prior to condensing, the milk is heated in a continuous pre-heater to 65.6–76.7°C or 82.2–93.3°C for up to 15 min to
impart desirable characteristics to the product. The pre-heated milk is then concentrated in a vacuum pan or multiple-effect evaporator at 54.4–57.2°C. To our knowledge, the structure of this type of concentrated milk has never been reported, and it will not be reviewed in this chapter.

5.5 Dairy powders

Some examples of dairy powders are whole milk, skimmed, buttermilk and whey powders including high-protein products. Detailed chemical compositions of these powders have been reported by Tamime & Robinson (1999) and Walstra et al. (2006), and on average the moisture content of these powders is $\sim 3 \text{ g } 100 \text{ g}^{-1}$ (Table 5.4).
5.5.1 Whole and skimmed milk powders

Method of manufacture

The manufacturing stages of milk powders (e.g. full-fat where the fat content is standardised or skimmed milk) are: the milk is pre-heated (at 95°C for 1 min), evaporated (i.e. ~ 45 g 100 g⁻¹ total solids), homogenised at 15 MPa of pressure, and cooled. Afterwards, the concentrated milk is heated to 78°C, spray-dried (~ 8 g 100 g⁻¹ moisture) followed by fluid-bed drying and packaged in bulk or retail containers (Carić, 1994; Pisecky, 1997; Walstra et al., 2006; see also Ozkan et al., 2003; Ozmen & Langrish, 2003a,b; Birchal et al., 2005). An additional process after spray-drying is agglomeration and final drying (Table 5.4).

Structure

Although roller-dried milk powder is not currently produced commercially in large quantities, the structure of this powder consists of casein particles that are preserved as individual globular entities and fused casein micelles, which occur as clusters. Incidentally, this method of drying produces flakes of powder that are indicative of casein aggregates, and the structure is folded (Kaláb & Emmons, 1974).

In the early 1970s, the detailed structure of spray-dried whole milk powder was studied by researchers in the Netherlands and, according to Kaláb (1979a,b), the following observations were reported: (1) the processing conditions can affect the size and shape of the powder particles; (2) variations in the diameters of the particles occur; (3) the particles have wrinkled exterior surfaces and many were hollow and porous (see also Hogekamp & Pohl, 2003); (4) smaller particles were sometimes located within the large vacuole in the large particles; (5) homogenisation of the concentrated milk before drying affected the pore sizes of the particles; and (6) the wrinkles, which appear on the surfaces of the dried particles, were caused by the contraction of the casein micelles during drying (Fig. 5.6) (see also Hardy et al., 2002). Kaláb & Emmons (1974) also reported that freeze-drying deformed the shape of the casein micelles, and concluded that the casein micelles were best preserved by spray-drying. Furthermore, to improve hydration of the powder during the reconstitution stage with water, instant powders were developed (i.e. wetting the dried powder followed by drying); this resulted in agglomeration of the sticky particles. However, the agglomerated dried milk powder particles may have a smooth surface with small lactose crystals (see Fig. 5.9 and Verdurmen et al., 2002) or rough surfaces covered with relatively large lactose crystals. The differences in the structure of the instant-type powder particles could be attributed to the processing method used. In addition, the particle shape of milk powder is also influenced by the type of atomiser used in the drying chamber (Carić & Kaláb, 1987; Kaláb, 1993; Pisecky, 1997; Verdurmen et al., 2002).

The structure of skimmed milk powder particles is slightly different from whole milk powder particles due to the higher lactose content in the former product (Kaláb, 1979b, 1992; Roos, 2002; Schuck & Dolivet, 2002). During the drying stage, the lactose solution will be dried very quickly and, as a consequence, the viscosity of the solution increases rapidly for crystallisation to take place and an amorphous mixture of α- and β-lactose is produced. The formation of these lactose crystals is influenced by the drying temperature, for example,
α-hydrate crystals are formed at low temperature while the β-anhydride crystals are formed above 93.5°C. In addition, prism-shaped crystals are normally formed by rapid crystallisation and high precipitation pressure and, by decreasing the pressure, the shape of the crystals changes to diamonds, pyramids, tomahawks and 13-sided crystals (Kaláb, 1979b; see also Saito, 1985; Sargent, 1991; Aguilar & Ziegler, 1993, 1994a,b).

In freshly made skimmed milk powder, small prism-shaped (5–30 μm in length) lactose crystals are very common, α-hydrate type, the surfaces of the particles are smooth and they are located inside the milk powder particle. In 1- to 3-year-old skimmed milk powder, tomahawk-shaped crystals are more evident, the sizes ranging between 60 and 170 μm, and the numbers of prism-shaped crystals are substantially reduced; hence, while the presence of these large crystals in powder may reflect its age, they can be also present in high-moisture products (Kaláb, 1979b, 1992). The interior structure of whole milk powder is shown in Fig. 5.7, where the casein micelles are darkly stained and embedded in an amorphous lactose matrix. Some lactose crystals can be recognised at the particle surface, and the fat globules are recognised as ‘ghost areas’ showing no internal structure as the fat is lost during sample preparation.

**Fig. 5.6** Scanning electron microscopy (SEM) of spray-dried skimmed milk powder. Note the wrinkles (large black arrow), cracked shell-like structure (white arrow), and small round and broken aggregates showing minute pores (small black arrow). Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
Fig. 5.7 TEM micrographs showing (a) interior structure of the whole milk powder particle and (b) agglomeration of smaller particles into larger complex aggregates (i.e. shown at higher magnification). Reproduced by permission of Nestlé Research Centre, Switzerland.
In some instances, the nutritional properties of skimmed milk powder are improved by fortifying the product with vitamins A, vitamin D and coconut oil, as these fat-soluble vitamins are present in the milk fat component. Figure 5.8 shows the structure of a fortified powder (i.e. commercial product) with these vitamins, and it also contains coconut oil. In general, agglomeration of the powder particles is evident, and there is no sign of oil, which is either well incorporated, or removed when the powder sample was prepared for SEM analysis. In addition, the surface of the powder particle is slightly rough, and evidence of fractured particles is very low.

As mentioned earlier, different processing conditions can cause particles to agglomerate and, in whole milk powder, agglomeration of smaller particles into larger complex aggregates is clearly seen in Fig. 5.9. In addition, by using polarised light microscopy of these agglomerated powder particles, lactose crystals are evident at the particle surface (Fig. 5.10).
5.5.2 Retentate milk (whole and skimmed) powders

Method of manufacture

Retentate powders, which are manufactured from whole or skimmed milk, are the materials produced when selective membranes are used to concentrate desirable components. Reverse osmosis permits passage into the permeate of only low-molecular-weight materials, such as water and some salts; ultrafiltration retains fat and proteins in the retentate, but the membrane is permeable to lactose, ash and non-protein nitrogen (NPN). In principle, the latter membrane filtration method is used to produce high-protein powders. The filtrate (i.e. the liquid and milk components passing through the membrane) is known as the permeate, and the retained material is called the retentate. In order to increase the protein content in the final powders, the retentate is diafiltered where it is diluted with water (e.g. same volume of the permeate) followed by ultrafiltration; the number of diafiltration stages performed will ultimately affect the level of the protein in the powder (see Table 5.4). The heat-treated milk (whole or skimmed) is ultrafiltered, and the retained fraction is diafiltered (i.e. once to three times); the final retentate is then processed in a similar manner to that described earlier for the manufacture of milk powder.
Structure

The structures of retentate powders have been reported by Kaláb et al. (1989), who described the following aspects. (1) The overall visual appearance of the powder was moist-looking, and the small particle aggregates separated easily on contact. (2) The structure of freshly made powder viewed by SEM had a relatively smooth appearance, shallow dimples and occasional simple ‘venation’ irrespective of the extent of diafiltration. Figure 5.11 shows the structures of three different commercially available high-protein milk powders. In general, all of these powder products look the same, but the main differences are the particle shape. For example, most powder particles have a smooth surface appearance, but some are collapsed (Fig. 5.11a). The internal structure of well-aerated particles is shown in Fig 5.11b, and plenty of milk protein material (i.e. not aerated) is shown in Fig. 5.11c. However, Mistry et al. (1992) reported deep dents in high-protein milk powder particles, which could be attributed to the inlet and outlet air temperatures in the spray drier (e.g. 120–125 and 75–80°C, respectively – compared to industrial operations using an inlet temperature of 220°C and outlet temperature of 90°C). (3) When the powders were exposed to humidity, the products exhibited less lactose crystallisation than ordinary milk powders. (4) The retentate powders differed from ordinary skimmed or whole milk powders, which contain high levels of lactose (see also Kim et al., 2003).

5.5.3 Buttermilk powder

Method of manufacture

Buttermilk (i.e. sweet or acid), which is a byproduct of buttermaking, can be easily spray-dried to produce a powder that is utilised in the food industry or for animal feed. According to Masters (1985), sweet buttermilk is concentrated to 45–50 g total solids (TS) 100 g–1, and dried in a co-current flow drier either with a pneumatic powder-conveying or a fluid-bed drier. The residual moisture content is ~ 4 g 100 g–1 (Table 5.4). When drying acid buttermilk (i.e. pH 4–5), the concentration temperature is kept low (57–58°C) in order to avoid coagulation of the proteins and, since the viscosity of the product is high, there is a danger of scorching on the hot surfaces of the evaporator (Masters, 1985). It is also recommended by the same author that there must be no high temperatures in the first and second effects of the evaporator or pre-heating of the buttermilk. The circulation of the product in the first two effects of the evaporator should be at low capacity. In ordinary evaporators, the product is concentrated to 25 g TS 100 g–1 but, by using a special type of evaporator fitted with a de-gassing stage, the buttermilk is concentrated to 36 g TS 100 g–1 before drying. The concentrated acid buttermilk is highly viscous, and pumped to the spray drier at 43°C. Incidentally, skimmed milk drier designs can be used. The recommended inlet drying temperatures range between 175 and 190°C, and light deposits of dried buttermilk in the chamber may occur; however, by using low outlet temperatures, discoloration of the powder is minimised. It is also recommended to use a drying chamber with an integrated fluid bed drier, which acts as a cooler. The moisture content is ~ 4 g 100 g–1 and the tapped bulk density is 0.77–0.83 g cm–3 (Masters, 1985; see also Carić, 1994, regarding the chemical composition of buttermilk powder including evaporated buttermilk and sweetened condensed buttermilk).
Fig. 5.11  SEM micrographs showing structure of three different commercial high-milk-protein spray-dried powders. (a) Arrow shows small and fused powder particles; (b) arrow shows well-aerated powder particle; (c) white arrows show egg shell-like structures and black arrow illustrates very compact and solid milk protein material. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
According to Kaláb (1981, 1983, 1993), the structure of spray-dried buttermilk powder viewed by SEM showed intact and crushed particles (Fig. 5.12a). Shallow wrinkles are evident on the surface of the large particles, while rims around small globules emerging from the large particles and crater-like scars on the surface of the particle are also evident. However, recent analysis (M. Kaláb, personal communication) of freshly made buttermilk powder produced in 2005/06 suggests a different surface appearance of the particle (i.e. the wrinkles and crater-like scars are not evident (Fig. 5.12b), which could be attributed to processing and drying conditions.

*Fig. 5.12*  SEM micrographs showing the structure of buttermilk powder. (a) Bar = 20 μm. (b) Bar = 50 μm. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
**5.5.4 Whey powder**

*Method of manufacture*

Whey powder is manufactured in a similar manner to skimmed milk powder but, at the beginning of the production stages, the whey is filtered to remove any residual curd fines and the fat is separated. Acid whey should be neutralised with alkali; otherwise it causes rapid fouling of the equipment. In brief, the preliminary treated whey is evaporated to 60 g TS 100 g⁻¹ and the lactose is crystallised. The concentrated whey is atomised in the drying chamber using a disk rather than a nozzle to minimise blockage of the atomiser (Carić, 1994; Pisecky, 1997; Schuck *et al*., 2004; Walstra *et al*., 2006). Typical chemical compositions of different types of whey powders are shown in Table 5.4. However, the whey may be demineralised before drying to de-acidify the product, or acidified to precipitate the protein, and then separated (30–35 g protein 100 g⁻¹) and dried for the production of whey protein concentrate. An alternative method of whey protein concentrate production is using ultrafiltration to retain the protein content in the retentate, and to reduce the lactose, mineral and non-protein nitrogen contents in the whey, which are lost in the permeate. According to Carić (1994), the ultrafiltered retentate may be evaporated under vacuum followed by drying; alternatively the retentate is first concentrated using reverse osmosis, followed by evaporation and drying. Depending on the method used to produce whey protein concentrate, the protein level in the dried product ranges between 30 and 88 g 100 g⁻¹ (Carić, 1994; Walstra *et al*., 2006).

*Structure*

The structure of whey powder (Fig. 5.13) is quite different from that of whole or skimmed milk powders, which could be attributed to its chemical composition, i.e. mainly composed of lactose, whey protein and minerals (see Table 5.4). The surfaces of the particles are not smooth, and each is filled with holes and has a honeycomb structure; the plateau or angular-shaped particles may indicate lactose crystals.

![Fig. 5.13  SEM micrograph showing the structure of whey powder. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.](image)
The water-holding capacity (WHC) and structure of a curd made from heat-coagulated whey protein powders were influenced by the method of drying (freeze-drying versus spray-drying versus roller-drying versus air-drying) (Jelen et al., 1979). The same authors concluded: (1) the loss of WHC in the dried whey products appeared to be caused by physical shrinkage of the protein matrix during drying; (2) only the freeze-dried method minimised the loss of WHC (i.e. by 24%) compared with the other drying methods that caused a loss of 70%; and (3) the presence of a surface crust in whey protein powders produced by all of the drying methods except freeze-drying confirmed the ‘case-hardening’ phenomenon, which is the major cause of poor rehydration of dried whey proteins.

In addition, the structure of demineralised spray-dried milk permeate powder consisted of globular and partly sharp-edged particles (Fig. 5.14) (Kaláb et al., 1991). The proportion of globular to sharp-edged particles was influenced by the degree of demineralisation, being highest at 51% demineralisation and lowest at 93% demineralisation. The globular-shaped particles were formed by minute lactose crystals cemented by amorphous material, while the sharp-edged particles were lactose crystals covered with dried collapsed foam consisting of mother liquor. However, X-ray microanalysis of the powders showed that the minerals, such as potassium, calcium and phosphorus, were concentrated in the globular particles. In addition, the lactose was mostly in the form of $\beta$-monohydrate crystals (Kaláb et al., 1991; see also Saito, 1988; Moreau & Rosenberg, 1993; Rosenberg & Young, 1993).

### 5.5.5 Whey protein concentrates and whey protein isolates

**Method of manufacture**

These products are made from de-lactosed and de-salted whey where the protein content may range between 35 and 80 g 100 g$^{-1}$. According to Carić (1994), the whey (i.e. de-fatted and free from cheese curd fines) is pasteurised, ultrafiltered, evaporated and finally spray-dried; some examples of the chemical composition of whey protein concentrate are shown.
in Table 5.4. In contrast, whey protein isolate contains around 90 g 100 g\(^{-1}\) whey protein; it is made in a similar manner to whey protein concentrate, but diafiltration (i.e. at least once) should be used to obtain a high level of protein in the dried product. Walstra et al. (2006) also reported that whey protein isolate can be produced from the permeate of microfiltered skimmed milk.

Structure

The overall structures of whey protein concentrate powders are different from those of whey powder as they contain more protein and less lactose. The structures of whey protein concentrate powder samples obtained from different manufacturers are shown in Fig. 5.15; they are somewhat similar, and the differences in the structures could be influenced by the chemical composition of the product and processing conditions. For example, the structures of three whey protein concentrate products, which do not contain any fat (Fig. 5.15a–c), consist of small particles fused in dimples, and, in some instances, the surface of the particle is smooth or wrinkled, similar to skimmed milk powder. Some cracked particles (i.e. shell-shaped) are dense or thin and reflect the degree of aeration during the drying stage. However, the structure of a commercial whey protein concentrate sample containing 2.5 g fat 100 g\(^{-1}\) and 86 g protein 100 g\(^{-1}\) (Fig. 5.15d) consists of some particles that are similar to caseinates (see Fig. 5.18) and some that are fused; in general, the particles are reasonably aerated.

In contrast, the structures of different whey protein isolate powders suggest the following: (1) powder particles are well aerated and plenty are fractured (Fig. 5.16a), and the differences in the structure compared with other samples could be the effect of demineralisation during the preparation of the product; (2) the degree of particle fragmentation is less (Figs 5.16b,c) and the product contains plenty of small to intermediate-sized particles.

![SEM micrographs showing structure of four different commercial whey protein concentrate (WPC) powders. (a, b) White arrows show shell-shaped cracked particles that are thin and dense, and black arrow shows small particles fused in dimple of a large particle; (c) arrow shows wrinkles similar to SMP; (d) arrow shows a particle similar to caseinates. Data by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada. (Continued.)](image)
Fig. 5.15  (Continued.)
Fig. 5.16  SEM micrographs showing structure of three different commercial whey protein isolate (WPI) powders. The protein content of these products (g $100 \, g^{-1}$) is: (a, c) not reported; (b) 90; (d) 86. Data by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada. (Continued.)
Overall, the surfaces of particles of whey protein concentrate powders are generally smoother and the number of collapsed particles are greater compared to whey protein isolate particles.

### 5.5.6 Lactose

#### Method of manufacture

Lactose powder (crude or refined) is mainly produced from whey (i.e. normal whey or deproteinised whey, for example using ultrafiltration or heat coagulation) by crystallisation; an improved method to increase the yield of lactose is to use chromatographic separation (i.e. based on ion exclusion and a molecular sieve effect) (Carić, 1994). Protein-free whey is concentrated by evaporation up to 70 g TS 100 g$^{-1}$ followed by crystallisation of lactose using a nuclei and separation of the crystals in two stages. The lactose crystals are dissolved in water to 30 g TS 100 g$^{-1}$ at 105°C, filtered to remove any sediments, and evaporated to 65–70 g TS 100 g$^{-1}$. The concentrate is crystallised, separated, dried at 70°C, ground, sifted and packaged (Pisecky, 1997) (see also Table 5.4).

#### Structure

The structure of lactose powder (Fig. 5.17) consists of arrowhead and irregular-shaped particles; the former particles could be α-lactose monohydrate (see also Chapter 9; Saito, 1985, 1988; Bronlund & Paterson, 2004; Foster et al., 2005).

### 5.5.7 Caseinates

#### Method of manufacture

Several casein preparations are available on the market (Table 5.4), and these products are made from heat-treated skimmed milk. The casein fraction in milk is precipitated using different methods, and the powder obtained is always known by the method of manufacture (Carić, 1994; Pisecky, 1997; Walstra et al., 2006).
Rennet casein is manufactured from enzymatically (e.g. chymosin, pepsin, microbial coagulants, genetically engineered chymosin) coagulated milk, followed by cutting or stirring and increasing the temperature to ~ 55°C. Syneresis takes place and the curd particles are separated by centrifugation or vibrating sieve, washed with potable water, pressed to remove the moisture and dried using a drum or belt drier. The product is ‘sweet’ and insoluble, with a high ash content, and is devoid of caseinomacropeptide, which is lost in the whey and this means that the yield is reduced by ~ 4% (Walstra et al., 2006). This product can be later treated with acid to produce acid casein (Carić, 1994).

Acid casein is made by chemically acidifying skimmed milk using organic acids, such as hydrochloric, lactic (sometimes using starter cultures) or sulphuric acid. The acid is added while stirring the skimmed milk at 30 or 45°C until the isoelectric point is reached (pH 4.6). The temperature used is critical as it can affect the speed of coagulation of the milk and the production of large lumps. The precipitated casein is processed in a similar manner to rennet casein; however, after curd drying, the dried casein is tempered, ground, sifted and packaged. The casein yield is higher than for rennet casein (see Table 5.4), and the product can be further purified by dissolving in alkali (see below).

Sodium, potassium, ammonium, calcium and magnesium caseinates are made from acid casein which has been dissolved in alkali – e.g. NaOH, KOH, NH₄OH, Ca(OH)₂ or Mg(OH)₂ (see Table 5.4). These products are soluble in water, flavourless if the pH during manufacture was maintained at ~ 7, and each type has different characteristics. For example, sodium caseinate is the most widely produced, potassium caseinate is preferred for its nutritional quality, and calcium caseinate has different physicochemical properties as compared to sodium and potassium caseinates (Walstra et al., 2006).

Phosphocaseinate is manufactured from microfiltered skimmed milk using a pore size of 0.1 μm to retain all the casein and wash out the serum proteins; diafiltration with water is used to remove any soluble material present in the milk. The casein micelles of phosphocaseinate have similar properties to the natural micelles in milk; this product is only used in research and is not yet commercialised (Walstra et al., 2006).
Structure

Kniefel & Seiler (1993) reported that the structure of spray-dried sodium caseinate powder particles from different manufacturers varied in terms of particle dimensions and surface structure, the latter being either smooth with indentations, or rough. Recently, M. Kaláb (personal communication) analysed different calcium and sodium caseinate powders, and described the structures as follows. (1) The particles of two samples of calcium caseinate powders (Figs 5.18a,b) are dented or collapsed and have smooth surfaces; the broken pore hole (Fig. 5.18a) has a more dense structure compared to skimmed milk powder. (2) The particles of two of the sodium caseinate samples (Figs 5.18c,d) are similar and they closely resemble calcium caseinate, but Fig. 5.18(d) shows sodium caseinate particles that are irregular in shape (i.e. some elongated while others are folded), and a few are cracked, collapsed or stringy/elongated in shape (Fig. 5.18e). It is most likely that the drying conditions may have caused these differences in the structure of the calcium and sodium caseinates.

Fig. 5.18  SEM micrograph showing structure of different commercial Ca-caseinate (a, b) and Na-caseinate (c–e) powders. Data by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada. (Continued.)
Fig. 5.18 (Continued.)
5.6 Conclusion

The detailed examination of biological materials using different microscopic techniques is always susceptible to the production of artefacts and, when the materials have been subjected to heating and drying and perhaps, further drying before examination, the interpretation of the images is never easy. Nevertheless, the use of TEM, SEM, DICLM or polarised light microscopy to study milk and milk products has provided valuable confirmation of the reasons behind some widely observed phenomena. The physical stability of homogenised cow’s milk, for example, is easily explained once the size of the fat globules is compared to those in fresh milk, especially as the protein can be observed replacing some of the fat globule membrane.

The reason for the rapid solubility of ‘instant’ spray-dried milk powders in comparison with standard powders is evident from SEM examination of the two types of powder, while the poor rehydration properties of whey protein powders can also be supported by their physical appearance. The revelation of more subtle differences between dried products may also be possible. Kaláb (1992) suggests that it may be possible to tell the age of a sample of skimmed milk powder by the shape of the lactose crystals, and standardisation of such technique could provide a useful means of checking the history of a given batch of product. Similarly, it may be possible to employ the structural details of powder particles to determine the drying temperatures to which the material has been exposed. Moreover, as new techniques of microscopy reveal more about the fine structure of milk products, it may be possible for dairy scientists to exploit their properties with more precision.

References


6 Structure of Fermented Milks

A.Y. Tamime, A. Hassan, E. Farnworth and T. Toba

6.1 Introduction

Many different types of fermented milks have been produced in many countries worldwide for thousands of years (Kurmann et al., 1992), and consumers in these communities incorporated fermented milks into their diets almost as soon as man began to domesticate animals. However, it was not until the beginning of the 20th century when Metchnikoff (1910) published his work about the health-promoting properties of yoghurt that small sections of the general public began to take serious interest in fermented milks. Natural (plain) yoghurt and/or cultured buttermilk have been very popular products in the Balkans and many Middle Eastern countries, while buttermilk became popular in Scandinavia and, through immigration, in North America as well. Acidophilus milk was marketed in North America as a beneficial health food, while in Russia the traditional drinks of kefir and koumiss received more serious attention from the medical authorities (Koroleva, 1991).

According to Tamime & Robinson (1999), fermented milks can be divided into three broad categories based on the microbial metabolic products, namely (1) lactic acid fermentations, which can in turn be subdivided into three groups (mesophilic, thermophilic and health-promoting products known as probiotic products), (2) yeast-lactic acid fermentations, and (3) mould-lactic acid fermentations. Although these fermented dairy products are well known in some countries, and are often an important part of the diet, many factors can affect their physical structure, chemical composition and nutritive value. Hence, it will be most appropriate that the classification of fermented milks proposed by Tamime & Robinson (1999) is used in this chapter to review the factors that can influence the microstructure of these products.

6.2 Background to manufacturing practice

In general, two types of fermented milk product are available on the retail market. One variant has a firm gel-like structure where the milk base is fermented in the retail container (set product), while the other has a thick consistency and the milk base is fermented in large tanks, followed by breaking the coagulum, partial cooling, blending with fruit and packaging (stirred type). Incidentally, drinking-type fermented milks are made from milk without fortification of the milk solids-not-fat (SNF) or may be diluted, and it is safe to consider these products as stirred type, but the viscosity is rather low. Despite the apparent contrasted nature of the end-products, the manufacturing stages are broadly similar. A multitude of factors can affect the stability, viscosity, texture and microstructure of the acidified gel and, according
to Walstra (1998), Tamime & Robinson (1999), Jaros & Rohm (2003a,b) and Sodini et al. (2004), these can be summarised as: (1) fortification level of the SNF (12–14 g 100 g⁻¹), fat content (0–3.5 g 100 g⁻¹) and other added material(s) used in the milk base; (2) the addition of stabilisers (optional); (3) processing conditions of the milk base, for example, the homogenisation pressure and heat treatment level used; (4) starter culture used (type, rate of acid development and production of exopolysaccharides – EPS), incubation temperature employed (influences growth of starter cultures, gel aggregation, bond strength), and pH at breaking of the gel (stirred) and/or start of cooling (set); (5) mechanical handling of the gel, cooling rate and conditions of the cold store; and (6) post-manufacture handling of the product (e.g. physical abuse such as vibration), post-fermentation heat treatment of the product, and/or temperature fluctuations during storage, transport and retailing (i.e. if the product is not maintained at \( \leq 5^\circ C \)).

The recipe for most commercial fermented dairy products in times past was similar – prepare milk (pasteurise, homogenise), add starter culture, terminate fermentation when desired characteristics (viscosity, pH, taste) were attained. The desire to produce more homogeneous products with a wider variety of textures, flavours and nutritional values has meant that manufacturers have developed unique starter cultures, have altered fermentation conditions, and have also added additional milk proteins to their recipes.

The action of bacteria, yeasts and moulds in the fermentation process(es) results in a large variety of fermented dairy products worldwide. The possibility of using different milks (cow, sheep, goat, buffalo, mare or camel) adds to the number of possible products. However, the enzymes of the starter microflora added to milk act on the native proteins to produce substrates that can be used for metabolism and growth by other starter micro-organisms. In complex starter culture mixtures, an associative relationship often develops whereby the rate of growth of one organism is dependent on the metabolic products of a second organism. Secondary metabolites produced during the fermentation cause changes in the pH, texture and taste of the fermented product, and may affect the structure of the product. Depending on the fermentation conditions (temperature, time and starter culture) either a thick drink or a soft pudding-like solid results.

### 6.3 Patterns of production and consumption

Outside Eastern Europe and the Middle East, serious interest in fermented milks has been limited, mainly due to the extremely sharp and acidic taste of the product(s), for example yoghurt, to Western palates. A breakthrough in consumer acceptability was realised when yoghurt was sweetened and fruit flavour added to mask the sharp acidic taste; these yoghurts went on sale in urban areas in Switzerland in the mid-1950s. As a consequence, the pattern of per capita consumption of fermented milks is still rising in many countries, a trend confirmed by the data shown in Table 6.1. It is of interest to note that in some Asian countries where fermented milks are not traditional products and were not consumed in the past, annual production figures have risen from 12 000 to 710 000 tonnes between 1998 and 2003 in China, while in Thailand total production increased from 300 000 to 650 000 tonnes between 1998 and 2000 (IDF, 2002, 2004).
Table 6.1  Per capita annual consumption of milk drinks and fermented products including yoghurt in some selected countries in 2000 and 2003.

<table>
<thead>
<tr>
<th>Country/consumption level</th>
<th>2000</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>&lt; 5 kg per person</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>4.9</td>
<td>6.2</td>
</tr>
<tr>
<td>China</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Mexico</td>
<td>3.0</td>
<td>NR</td>
</tr>
<tr>
<td>South Africa</td>
<td>3.2</td>
<td>NR</td>
</tr>
<tr>
<td>Ukraine</td>
<td>3.2</td>
<td>5.7</td>
</tr>
<tr>
<td><strong>5–10 kg per person</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Argentina</td>
<td>6.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Australia</td>
<td>5.6</td>
<td>NR</td>
</tr>
<tr>
<td>Cyprus</td>
<td>NR</td>
<td>10.8</td>
</tr>
<tr>
<td>Estonia</td>
<td>5.7</td>
<td>NR</td>
</tr>
<tr>
<td>Portugal</td>
<td>9.7</td>
<td>NR</td>
</tr>
<tr>
<td>Thailand</td>
<td>5.8</td>
<td>NR</td>
</tr>
<tr>
<td><strong>10–20 kg per person</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>17.3</td>
<td>21.2</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>13.8</td>
<td>14.8</td>
</tr>
<tr>
<td>Hungary</td>
<td>12.2</td>
<td>11.9</td>
</tr>
<tr>
<td>Norway</td>
<td>16.6</td>
<td>20.7</td>
</tr>
<tr>
<td>Poland</td>
<td>NR</td>
<td>11.9</td>
</tr>
<tr>
<td>Slovakia</td>
<td>11.7</td>
<td>16.0</td>
</tr>
<tr>
<td>Spain</td>
<td>15.7</td>
<td>NR</td>
</tr>
<tr>
<td><strong>20–30 kg per person</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>21.1</td>
<td>NR</td>
</tr>
<tr>
<td>Germany</td>
<td>26.5</td>
<td>28.8</td>
</tr>
<tr>
<td>France</td>
<td>20.2</td>
<td>21.9</td>
</tr>
<tr>
<td>Switzerland</td>
<td>22.8</td>
<td>26.0</td>
</tr>
<tr>
<td><strong>&gt; 30 kg per person</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>35.8</td>
<td>43.0</td>
</tr>
<tr>
<td>Finland</td>
<td>39.0</td>
<td>38.8</td>
</tr>
<tr>
<td>Israel</td>
<td>28.0</td>
<td>NR</td>
</tr>
<tr>
<td>Netherlands</td>
<td>46.4</td>
<td>42.5</td>
</tr>
<tr>
<td>Sweden</td>
<td>32.1</td>
<td>34.9</td>
</tr>
</tbody>
</table>

NR = not reported.
Although sweetened and fruit-flavoured fermented milks became accepted by consumers and are now commonplace in many countries, the popularity of these products has been enhanced by the health-promoting properties of some non-traditional and specific strains of starter cultures, such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* spp. (known as probiotic or health-promoting cultures), which are blended with traditional starter cultures belonging to the genera *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* (Tamime, 2005). In some products, such as kefir and koumiss, yeasts are blended with the starter culture and mould in viili (fermented milk from Finland) (Tamime, 2006).

### 6.4 Lactic acid fermentations

#### 6.4.1 Background

The technological developments of fermented milks, including the health aspects attributed to special probiotic micro-organisms used in fermented milks and other dairy products, have been detailed in separate books (*Fermented Milks* and *Probiotic Dairy Products*) published within this Technical Series prepared on behalf of the Society of Dairy Technology in the UK (Tamime, 2005, 2006). The manufacturing stages of all the products falling into the category of fermented milks are very similar and well established. In general, the milk base is homogenised (17–20 MPa pressure) at 60°C, heated to 90–95°C for up to 5 min, cooled to the incubation temperature of the starter culture, fermented, partially cooled, blended with fruits (optional) and packaged. For buttermilk production, the heated milk is cooled to 60°C, homogenised at 18–20 MPa pressure, cooled and fermented at 20°C and, after acidification, the fermentate is vigorously agitated, homogenised at 5–10 MPa pressure, cooled to 4°C and packaged (Tamime *et al*., 2001). Various microscopic means have been used to study the factors affecting the stability of fermented milks. Direct microscopic observations provide more complete information on various aspects of the quality and characteristics of the final product. Conventional light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM) and fluorescence microscopy (FM) have been used to study the microstructure of fermented milks, and the factors affecting the structure of these products.

#### 6.4.2 Liquid-type fermented milks

*Ayran and dough*

Traditional fermented milk drinks, such as drinking yoghurt, ayran (in Turkey and Lebanon) or dough (in Iran) are made from whole milk where the SNF of the milk base is not fortified and is later diluted with water or whey from labneh-making after the fermentation stage. In the industrialised countries, drinking yoghurt can be classified as stirred yoghurt of low viscosity. To reduce the fault of whey separation, stabiliser(s) is normally added to the milk base. To our knowledge the structure of drinking yoghurt has never been reported by researchers; however, a closely related product is buttermilk or Yakult® (see subsequent sections), and the overall structure of drinking yoghurt may be similar with the exception that the protein matrix is less dense when compared with traditional yoghurt. The use of pectin in drinking yoghurt will protect the protein after the homogenisation stage and will help to prevent the re-aggregation of the protein which leads to whey separation (Olsen, 2003).
**Buttermilk**

The manufacturing stages of buttermilk have been reviewed by Tamime and Marshall (1997), Tamime et al. (2001) and Nilsson et al. (2006). Traditional or natural buttermilk is the byproduct of ripened cream buttermaking, and normally *Lactococcus* spp. and *Leuconostoc* spp. are used as mixed blend starter cultures. Currently, the product is made from skimmed milk and, after the fermentation period, the coagulum is stirred and homogenised before cooling and packaging. The overall structure is shown in Fig. 6.1, and the SEM micrograph illustrates protein matrices composed of casein micelle chains and clusters, void spaces or pores and bacterial cells.

**Yakult®**

Yakult® is a fermented milk drink that was developed in Japan and launched in 1935. It is fermented by *Lactobacillus casei* Shirota strain which had been isolated from human faeces. Today it is sold worldwide in 23 countries. Pasteurised skimmed milk inoculated with *L. casei* is fermented until the titratable acidity reaches 2.0 mL 100 mL⁻¹. The resulting curd is broken and mixed with sweeteners (glucose-fructose liquid sugar and sucrose) and flavours. The mixture is homogenised at 15 MPa and is diluted with water. The resulting product is packed into 65-mL plastic bottles; the chemical composition (g 100 g⁻¹) of the product is 82.6 moisture, 1.2 protein, 0.1 fat and 15.8 carbohydrate, with pH 3.8–3.9 (see Fig. 6.2) (see also Tao et al., 2005).

![SEM micrograph showing structure of buttermilk](image)

Fig. 6.1  SEM micrograph showing structure of buttermilk. Chemical composition (g 100 g⁻¹) of the product as stated on the carton: protein, fat and carbohydrates averaged 3.6, 1.0 and 4.8 respectively; buttermilk was made from milk ingredients, salt and bacterial cultures. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
6.4.3 Gel-type fermented milks

The structure of the fermented milks is unique. Although they contain high moisture levels (almost the same as that of milk), they behave as solid-like materials. This behaviour is due to the aggregation of casein micelles in the milk and formation of a three-dimensional network (see subsequent sections).

Yoghurt is made with thermophilic cultures consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Two types of yoghurts are produced, set-style and stirred products. In set yoghurt, milk inoculated with the appropriate culture is packaged and incubated until the desired pH and texture are obtained. This type of yoghurt has a firm gel-like structure. In the stirred yoghurt, the protein network is broken into small aggregates after it has been formed. The difference in the microstructure of these yoghurts is shown in Fig. 6.3; the SEM micrographs show that the casein matrix of set yoghurt is continuous and uninterrupted except for the void spaces occupied by milk serum starter culture cells (Fig. 6.3a), whilst in stirred yoghurt the casein matrix is interrupted (Fig. 6.3b).

Structural components in fermented milks include fat globules, water, colloidal protein aggregates and additives such as stabilisers. Textural properties of the product depend on its microstructure and physicochemical interactions between different structural elements. The two main microstructural elements in milk that make it opaque are casein micelles and fat. Casein micelles are protein aggregates with dimensions ranging from 50 to 500 nm, which are too small to be seen under light microscopy. Fat is present in the form of large globules with an average diameter of 3 to 5 μm. Other milk constituents, such as lactose, minerals, whey proteins and vitamins, cannot be seen under electron microscopy.
Fig. 6.3  SEM micrographs showing microstructure of (a) set and (b) stirred yoghurts. Chemical composition (g 100 g⁻¹) of the products as stated on the carton: (a) set yoghurt where the protein, fat and carbohydrates averaged 4.3, 1.4 and 1.6 respectively, and the product was made from milk ingredients, sugar, modified milk ingredient, pectin, agar, vanilla extract and bacterial cultures; (b) stirred yoghurt where the protein, fat and carbohydrates averaged 3.3, 0.0 and 8.6 respectively, and the product was made from skimmed milk, concentrated skimmed milk, fructose, modified corn starch, whey protein concentrates, gelatin, pectin, locust bean gum, vanilla extract, aspartame, potassium sorbate, calcium lactate, natural colour, vitamins A and D and bacterial cultures; the dense structures embedded in the gel matrix could be aggregates of casein particles and stabilisers. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
Factors affecting the structure formation in yoghurt

The protein fraction in milk is the building block of the structure of the gel (Brooker, 1987), and milks from different species of mammals have been used during the manufacture of yoghurt. The porosity of the gel is more compact or dense for sheep’s milk yoghurt compared with a similar product made from cow’s milk without fortifying the SNF level.

The fortification method used to increase the SNF content of the milk base can affect the structure of the yoghurt. Tamime et al. (1984) reported that the SEM structure of yoghurts made from low-fat cow’s milk fortified by the addition of skimmed milk powder or concentrated by evaporation or reverse osmosis were similar. The densities of the protein matrices were as follows: skimmed milk powder yoghurt was the densest, followed by the ultrafiltered and sodium caseinate yoghurts with medium dense matrices. However, the yoghurts made with the addition of sodium caseinate or concentrated by ultrafiltration had the most open matrices. TEM structures of the same yoghurts revealed differences in the superficial features of the casein micelles forming the protein matrix; whereas all the yoghurts had ‘appendages’ or ‘spikes’ (i.e. heat-denatured β-lactoglobulin and complexing with κ-casein) appearing on the surface of the casein micelles structures, the product made with sodium caseinate had a smooth surface and was coarse. The reason for this difference was not known, and it was hypothesised by the same authors that their absence could be associated with high casein to non-casein ratio (4.6 for the sodium caseinate product versus 3.3 for the other yoghurts).

It is of interest to point out that, currently, different powders (e.g. skimmed milk retentate, whole milk retentate and whey protein concentrates) including instant-type caseinates are available on the market, and can be blended with each other depending on the recipe used for the manufacture of yoghurt. However, to our knowledge there are limited studies available to demonstrate the effect of these powders on the structure of yoghurt (see Guinee et al., 1995; Sodini et al., 2004, 2005). Recently, Bhullar et al. (2002) reported that fortification of the milk base with whey protein concentrates (2 g 100 g⁻¹) produced the firmest yoghurt, and the microstructure showed that the product had a more regular and dense protein network compared to yoghurts fortified with whey powder or skimmed milk powder; also the same yoghurt contained fewer void and interstitial spaces compared with other products.

Addition of stabiliser(s) can affect the rheological properties of the yoghurt gel, and both SEM and TEM studies have shown differences in the structures of yoghurt. These compounds stabilise the protein molecules in the form of a network that retards the free movement of other components of yoghurt. The structures of yoghurts in the presence of different stabilising agents are different. According to Kaláb et al. (1975), the addition of carrageenan resulted in the formation of a fibrillar structure, which was connected to large clusters of casein micelles, and the fibres had no free terminations but were thin and long. However, the addition of starch to the yoghurt milk gave rise to short fibres and sheets, and the fibres frequently had free terminations where some of them connected to small clusters of casein micelles. Recently, Everett & McLeod (2005) studied the effect of different stabilisers (low-methoxy pectin (LMP), λ-carrageenan, guar gum, locust bean gum and xanthan) in low-fat stirred yoghurt, and found that the stabilisation mechanism was influenced by the level and type of stabiliser used. As the level of LMP and λ-carrageenan was increased, the stabilisation mechanism was consistent with the model of casein aggregates passing through a region of bridging flocculation, followed by partial steric stabilisation. Although
these studies underpin and reflect the behaviour of each type of stabiliser during the manufacture of yoghurt, it may be difficult to visualise the identity of each type of stabiliser using microscopy studies during the industrial production of yoghurt because the commercial stabilisers are blends of different types.

**Homogenisation** of the milk base during the manufacture of yoghurt reduces the fat globule size, enhances the firmness of the product and minimises syneresis. However, to our knowledge there are no reported microstructure studies of the effect of homogenisation on the micellar matrix of the yoghurt gel.

**Heat treatment** of the yoghurt milk base revealed some distinctive characteristics of the casein micelle as studied by SEM. In heated milks, the gel is formed as the casein micelles gradually increase in size and form a chain matrix. This behaviour results in an even distribution of the protein throughout the yoghurt, and the aqueous phase is immobilised within the network; the resultant coagulum is firm and not susceptible to syneresis. By comparison, yoghurt made from unheated milk had aggregates or clusters of casein micelles unevenly distributed, and this heterogeneity impairs the immobilisation of water within the micellar matrix; the gel strength was much weaker (by 50%) compared with a similar coagulum made from heated milk (see also Lucey et al., 1998; Tamime & Robinson, 1999; Ipsen, 2003).

**Microbial acidification** of milk during the manufacture of yoghurt and other related products results in a gel, which is built of clusters of aggregated particles (particle gels) that are connected to form a continuous structure. Under the microscope, this structure is sponge-like with fat and serum entrapped. Ideally, all serum is immobilised within the protein network. However, whey separation (syneresis) is a common defect in fermented milk products and preventing it is still a major challenge for fermented milk manufacturers. The mechanisms involved in the formation of acid gels in milk have been extensively researched worldwide, and the following publications are recommended for further reading regarding the gelation aspects (Horne, 1999; Tamime & Robinson, 1999; Lucey, 2004; Lee & Lucey, 2004; Robinson et al., 2006).

**High pressure** application has been used during strategic studies of fermented milks. Since such technology is not used in the industry, the topic will not be reviewed. Detailed studies on the use of high pressure have been reported by Sandra & Dalgleish (2005), Dalgleish et al. (2005) and Robinson et al. (2006).

**Monitoring milk coagulation using confocal scanning laser microscopy**

Milk gelation is a critical step in making fermented milks. The overall sensory perception and functionality of fermented milks are affected by characteristics of the milk gel. Understanding the gelation process during fermentation would allow manipulation of the physical properties of fermented milks. Various techniques such as dynamic rheology, light scattering and permeability have been used to study milk gelation. However, microscopic observations provide a more accurate picture of changes in structure during formation. SEM is a powerful technique because of its high resolution and ability to characterise surfaces as well as internal structures. However, this technique is of limited importance in studying milk gelation. Part of the problem is that samples are dehydrated or frozen prior to electron microscopy, which can cause major changes in the initial structure, especially
when dealing with a very weak gel during its formation. In addition, it is impossible to obtain samples during formation of milk gels without causing damage to the structure and the induction of syneresis.

The possibility of examining fully hydrated samples made CSLM a potentially useful technique for studying the gelation process in milk. This technique was first used to monitor milk coagulation in real time and relate structural development to pH by Hassan et al. (1995b; see also Andersen et al., 2003), and Fig. 6.4 shows the process of monitoring milk gelation using such a technique. Milk inoculated with yoghurt culture was dispensed into a glass chamber in which a pH microelectrode was inserted. Temperature was maintained at 40°C on the stage and the focal plane was held constant during the fermentation process. The pH was monitored as the structure developed and images at various pH values were captured. This technique made it possible simultaneously to monitor changes in the pH and microstructure of yoghurt.

Casein micelles in milk can be observed with CSLM in two modes. Casein micelles and bacterial cells have the ability to reflect laser beams, and this property can be used to observe casein in milk and dairy products. Another option is to stain protein using protein-specific fluorescent dyes. Plate 1 shows microstructure development in milk acidified with glucono-δ-lactone (GDL) obtained using CSLM in the reflectance mode. In this mode, non-capscule-forming cells could not be seen as they become obscured by the casein micelles. Casein micelles in milk were seen as white dots whereas milk serum appeared

![Fig. 6.4](image_url) Monitoring microstructure development in yoghurt using confocal scanning laser microscopy. ~ 7 mL of milk mixed with starter cultures were dispensed into a glass chamber in which a pH microelectrode was inserted. The chamber was incubated at 40°C on the microscope stage. Source: N. Hassan, J.K. Frank, K.A. Schmidt & S.I. Shalabi (unpublished).
as a dark background. Three stages were observed: stage 1 (pH 6.6–5.5) – no changes in size of casein micelles could be seen; stage 2 (pH 5.5–5.3) – the casein micelles became coarser, indicating the initiation of the aggregation; and stage 3 (pH 5.3–5.0) – casein appeared as large micelle aggregates separated by non-reflecting (serum) spaces, which indicated the initiation of a three-dimensional network. At this stage, a contraction within the casein aggregates is initiated leading to the formation of wider non-reflecting spaces (serum cavities) and protein clusters that are connected via thinner strands, forming a well-defined network.

Effect of capsular exopolysaccharides (EPS) on structure formation and origin of void spaces around starter cultures

The microstructure of yoghurt is affected by several factors such as milk composition (fat, proteins and total solids), processing variables (heat treatment, incubation temperature and additives) and the type of starter culture used. The yoghurt microstructure affects the physical properties of the final product, and therefore microstructure studies may provide information on process conditions needed to improve body, texture and stability.

Exopolysaccharide (EPS)-producing lactic cultures cause profound modifications in the physical and sensory properties of yoghurt. EPS can either be attached to the producing cell as capsules or excreted as unattached materials into the growth medium. Capsular EPS does not cause ropiness, nor does production of unattached EPS ensure ropiness (Hassan et al., 2003a). Both types of EPS can be produced by the same bacterial cell. Whereas some ropy strains are not encapsulated, production of only the capsular form has not been confirmed. Some non-ropy strains of lactic acid bacteria produce amounts of EPS comparable to those produced by non-ropy strains (van Marle & Zoon, 1995). The effect of EPS on the physical and sensory properties of yoghurt depends on various factors, such as the type of EPS (capsular or unattached) and the molecular and chemical characteristics of the EPS with the type of linkages, degree of branching and molecular weight being the most important factors (Kleerebezem et al., 1993).

Capsule size and production by lactic acid bacteria is strain dependent. Some strains produce capsules up to 5 μm. CSLM can be used in the reflectance mode for the direct observation of encapsulated lactic cultures growing in milk (Hassan et al., 1995a). Capsules appear as non-reflective (black) areas surrounding bacterial cells (Plate 2).

CSLM has been used to study the effect of capsular EPS on structure development in yoghurt (Plate 3) (Hassan et al., 1995a). Casein micelles cannot penetrate capsules. Therefore, the microstructure of yoghurt made with capsule-forming cultures depends on the distribution of the producing micro-organisms. During the fermentation stage of the yoghurt milk, the number of encapsulated bacteria increases, leading to formation of more and larger areas unoccupied by casein micelles. Direct CSLM observations showed that, as the pH reached 5.5, the movement of micro-organisms decreased and casein micelles became coarser, indicating the initiation of gelation. Gelation was defined as the instant when the motion of the capsule-forming cells was no longer visible, and this took place at pH 5.35. Low-shear dynamic measurements showed that the gelation point of milk fermented with a capsule-forming strain occurred at a higher pH value than in milk fermented by a non-
Plate 1  Photomicrographs of microstructure development in directly acidified milk obtained using confocal scanning laser microscopy in reflectance mode. (a) pH = 6.4; (b) pH = 5.35; (c) pH = 5.0; (d) pH = 4.2. Bar = 10 μm. Reproduced from Hassan et al. (1995b) Journal of Dairy Science, 78, 2629–2636, by permission of the American Dairy Science Association and Journal of Dairy Science.

Plate 3 Photomicrographs of microstructure development in yoghurt made with encapsulated starter cultures obtained using confocal scanning laser microscopy in reflectance mode. (a) pH = 6.4; (b) pH = 5.40; (c) pH = 5.35; (d) pH = 5.0. Bar = 10 μm. Reproduced from Hassan et al. (1995b) *Journal of Dairy Science*, 78, 2629–2636, by permission of the American Dairy Science Association and *Journal of Dairy Science*. 
Plate 4  Volume rendering of yoghurt made using encapsulated non-ropy cultures to give different views compiled from 60 optical sections obtained using confocal scanning laser microscopy in reflectance mode. The rendered image is approximately 36 μm thick. Reproduced from Hassan et al. (1995b) *Journal of Dairy Science*, 78, 2629–2636, by permission of the American Dairy Science Association and *Journal of Dairy Science*.

Plate 5  Optical sections of yoghurt made using (a) non-encapsulated and (b) encapsulated starter cultures at pH 5.0. Bar = 10 μm. Reproduced from Hassan et al. (1995b) *Journal of Dairy Science*, 78, 2629–2636, by permission of the American Dairy Science Association and *Journal of Dairy Science*. 
Plate 6  Distribution of exopolysaccharides in (a, c) set and (b, d) stirred yoghurts made with (a, b) a moderate ropy strain and (c, d) a highly ropy strain. Bar = 10 μm. Reproduced from Hassan et al. (2002b) *Journal of Dairy Science*, 85, 1705–1708, by permission of the American Dairy Science Association and *Journal of Dairy Science*. 
capsule-forming culture (Hassan et al., 2002a). The presence of large capsules forces the casein micelles to aggregate and occupy less space. This volume exclusion effect of capsules allows a high number of protein contacts, resulting in earlier gelation.

Once gelation is initiated, a contraction within the aggregated micelles results in more compact aggregates and wider casein-free zones around bacterial cells (Plate 3d). Plate 3 shows that bacterial cells and their capsules acted as nuclei for the formation of large pores. After coagulation, a layer of whey is usually formed on the surface of yoghurt made with capsule-forming cultures. This whey separation seems to occur as a result of the presence of large pores in such yoghurt, and the layer of whey is reabsorbed into the curd during cooling. When non-capsule-forming cultures were used, aggregation produced a more continuous structure with pores of uniform size and distribution. SEM showed that some micro-organisms were surrounded by ‘void’ spaces in yoghurt, and different explanations have been given as to the origin of such spaces. For example, solubilisation of casein surrounding bacterial cells by the action of acids or proteolytic enzymes has been proposed; however, no correlation was found between rate of acid production and formation of such zones. In addition, some proteinase-negative mutants were surrounded by void spaces. Plate 3 shows that void spaces originated with bacterial capsules and increased in size as casein aggregated and contracted away from bacterial cells and their capsules. Void spaces can reach sizes that affect the integrity of the structure and physical properties of the product.

The three-dimensional microstructure of yoghurt made with capsule-forming cultures is shown in Plate 4. Large pores were associated with the presence of encapsulated bacterial cells, while Plate 5 shows differences in microstructure between milk fermented with capsule-forming and non-capsule-forming yoghurt cultures.

How is ropiness formed?

Lectin conjugates were used in combination with CSLM to observe EPS in fully hydrated yoghurt (Plate 6) (Hassan et al., 2002b). Lectins are carbohydrate-binding proteins and their fluorescent conjugates are commercially available. EPS and protein appear to be segregated, with the former compound being found in the network pores. In yoghurt made with a highly ropy strain of starter culture, more and larger masses of EPS were observed. Production of this type of EPS prevented formation of a well-defined protein network. Stirring of the coagulated gel breaks the protein network, which facilitates more EPS–EPS interactions, leading to concentration of the EPS in the continuous liquid phase between protein aggregates. If the amount and/or type of EPS allows more EPS–EPS interactions, long strands of EPS are formed that give yoghurt a ropy appearance, texture and consistency. This is why yoghurt is stirred before testing for ropiness. Yoghurts made with EPS-producing and non-producing cultures seem to respond differently to stirring. Upon stirring, the dense protein network in yoghurt made with no EPS produces a granular structure containing dense aggregates separated by whey (Fig. 6.5). However, stirring EPS-positive yoghurt will concentrate the EPS in the continuous liquid phase, which may physically prevent syneresis. Syneresis is also reduced in EPS-positive yoghurt by the ability of EPS to bind water.
Stabilising mechanism of EPS in yoghurt

Functions of EPS in yoghurt have long been debated. Part of the problem was the unavailability of a suitable microscopy technique to observe fully hydrated samples. SEM is not a suitable technique for studying the microstructure of yoghurt containing EPS because of artefacts that arise during sample preparation. With conventional SEM, the EPS appeared as thin filaments attached to the protein network and bacterial cells. In most of the published images, EPS unattached to bacterial cells was not observed. This might be because it was difficult to differentiate between the casein micelles and EPS. Exopolysaccharides labelled with lectins appeared as masses thicker than those obtained by conventional SEM (Plate 6). Exopolysaccharides and protein seemed to be segregated with EPS found in network pores (Plate 6). The segregation of EPS and protein in yoghurt suggested incompatibility between the two components, and the segregation between these two components produces thicker and larger aggregates. This produces a more densely aggregated protein network in EPS-positive yoghurt. The presence of EPS decreases interactions between protein aggregates, which produce lower viscoelastic moduli, yield stress, and firmness. A polymer-like behaviour of the serum phase in yoghurt containing EPS increases the consistency index and yoghurt viscosity.

Cryo-scanning electron microscopy (cryo-SEM) is a useful tool for studying the distribution of EPS in yoghurt because of its high resolution and ability to study the microstructure of fully hydrated samples. With this technique, a specimen frozen in liquid nitrogen slush (−207°C) is transferred in a frozen state into a chamber under vacuum where it is fractured, etched and coated with gold. The specimen is then transferred under vacuum onto the cold stage and imaged. In this technique, a short sublimation time is needed to remove surface water with minimal sample dehydration. A sublimation that is too long will produce an effect similar to freeze-drying and will induce artefacts. Cryo-SEM confirmed the confocal images of EPS-positive yoghurt. A compact, well-defined protein network was observed in milk fermented with non-EPS-producing cultures (Fig. 6.6); however, an open structure was associated with the use of EPS-producing cultures (Fig. 6.7).

Fig. 6.5 Optical sections of (a) set and (b) stirred yoghurt made using an non-EPS-producing starter culture. Bar = 10 μm. Reproduced from Hassan et al. (2003a) *Journal of Dairy Science*, 86, 1632–1638, by permission of the American Dairy Science Association and *Journal of Dairy Science*.
Fig. 6.6 Microstructure of fully hydrated yoghurt made with a non-EPS-producing culture as observed by cryo-SEM. Bar = 2 μm. Reproduced from Hassan et al. (2003b) *International Dairy Journal*, 13, 755–762, by permission of *International Dairy Journal*.

Fig. 6.7 Cryo-SEM micrographs of milk (pH 4.6) fermented with moderately ropy (*Streptococcus thermophilus* CHCC 3534 and *Lactobacillus delbrueckii* subsp. *bulgaricus* CHCC 769) culture. Specimens were etched for 10 min. P = protein, E = EPS, W = casein aggregates covered with frozen water. Bar = 5 μm. Reproduced from Hassan et al. (2003b) *International Dairy Journal*, 13, 755–762, by permission of *International Dairy Journal*.
Exopolysaccharides partially or completely filled the pores within the microstructure of yoghurt. Although EPS and the protein network were segregated, evidence of an interaction between the two components was observed (Fig. 6.8). Because of its greater resolution, cryo-SEM provided more details of microstructure of the masses of EPS (Fig. 6.9) than did CSLM, with which EPS appeared as solid masses. Whereas EPS produced with a moderate ropy strain of *S. thermophilus* formed a well-defined porous network, a highly ropy type of EPS had a more densely entangled appearance with random distribution of thick filaments (Hassan *et al.*, 2003b). Such differences in microstructure of EPS itself might reflect variations at the molecular level.

6.5 Lactic acid–yeast fermentations

6.5.1 Kefir

Kefir (kephir, kiaphur, kefyr) is believed to have originated in the Caucasus Mountains (Kanbe, 1992; Robinson *et al.*, 2002; Farnworth & Mainville, 2003; MingJu *et al.*, 2005; Wszolek *et al.*, 2006). Milk from sheep, goats and buffalo has been used in the past to make kefir. At present, commercial kefir is made from cow’s milk. The largest markets for kefir
are in Eastern Europe; today several companies sell kefir in North America. Kefir is a thick drink that has a ‘sparkling’ mouth-feel due to the release in the mouth of bubbles of carbon dioxide produced by yeasts.

The traditional kefir starter culture is known as kefir grains. The grains themselves, or the fermentate of the grains, are used to inoculate milk (at a ratio of 2–10 g 100 g$^{-1}$ of inoculated milk) to produce kefir (Farnworth & Mainville, 2003). Fermentation is carried out at about 20–25°C for approximately 24 h or until a desired pH is attained. A maturing step (15–20 h at 8–10°C) is sometimes part of the production process. Since the maintenance of kefir grains is labour intensive, and grains have been shown to vary in composition, several companies now sell lyophilised mixtures of bacteria and yeasts that are used as kefir starters. Kefir grains are a mass of protein, polysaccharide, mesophilic, homofermentative and heterofermentative lactic acid cocci, thermophilic and mesophilic lactobacilli, acetic acid bacteria and yeasts.

The yeasts in kefir grains provide essential growth nutrients such as amino acids and vitamins, and produce both ethanol and carbon dioxide. *Lactobacillus kefir, Lactobacillus kefirgranum, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris* have been identified recently in kefir using molecular biology techniques (Mainville et al., 2006).

Kefir grains have been found to contain a unique EPS named kefiran (Kooiman, 1968; Fujisawa et al., 1988). Kefiran is made up of approximately equal proportions of galactose

![Cryo-SEM images of EPS produced by (a) highly ropy *Lactococcus lactis* subsp. *cremoris* (JFR1) or (b) moderately ropy (*Streptococcus thermophilus* CHCC 3534 and *Lactobacillus delbrueckii* subsp. *bulgaricus* (CHCC 769) culture. Specimens were etched for 10 min. Bar = 1 μm. Reproduced from Hassan et al. (2003b) *International Dairy Journal, 13*, 755–762, by permission of *International Dairy Journal*.](image-url)
and glucose. The EPS is composed of a variety of linkages including glucose-galactose \((1\rightarrow2(6))\), galactose-galactose \((1\rightarrow4)\), galactose-galactose \((1\rightarrow3)\). The EPS plus the effect of pH on the milk proteins in kefir produce the viscosity of the drink.

It is of interest to note that recently a French farmer has produced ‘milk’ beer known as Lactiwel from a blend of milk (75%) and malt (25%), and using kefir grains as a starter culture (http://www.backyardpub.com/blog/2005/09/farmers-drunk-on-milk.html). The beer contains 2 g 100 mL\(^{-1}\) of alcohol, and the farmer, Marcel Besnard, claims that such a product could help dairy farmers, who have been protesting against a fall in milk prices. The ‘milk’ beer could help to inject new life into the industry, but the levy paid to Customs and Excise could hinder the development of such a product on a large commercial scale.

**Structure**

Many electron microscopy studies have been performed on kefir grains (Molska *et al.*, 1980; Marshall *et al.*, 1984; Duitschaever *et al.*, 1988; Neve, 1992; Kuo & Lin, 1999); electron micrographs of the kefir drink are less common (Mainville *et al.*, 2001). Transmission electron micrographs of the drink (see Fig. 6.10) show a loose protein matrix, fat globules of various sizes and different types of bacteria and yeast. Many bacteria are surrounded by fine structures believed to be EPSs.

![Fig. 6.10](image)

TEM micrograph of structural detail of kefir showing the loose protein matrix. Fat globules vary in size; the bacteria are often surrounded by a structure (exopolysaccharide) preventing close association with the protein; the kefir was produced using kefir grains. P = protein, F = fat globule, B = bacteria. Reproduced by permission of D. Montpetit (personal communication) and Agriculture and Agri-Food Canada.
6.5.2 Koumiss

Koumiss (kumys, kumiss, coomys) was traditionally produced by the fermentation of mare’s milk. However, koumiss made from blending cow’s skimmed milk and cheese whey has been reported (Mann, 1985, 1989). In Russian literature quoted by Mann, the production of koumiss from camel’s milk (known as shubat) is reported. The Kazakhstan region (Kyrgyz Republic), Shkortostan, Bashkortostan and Mongolia are major koumiss production areas. Average annual koumiss production in Russia has been reported: (a) in 1965–1970, 1400 tonnes, (b) in 1971–1980, 1600 tonnes, (c) in 1990, 3000 tonnes, and (d) in 1991–1998, 1800 tonnes (Maksimenko & Petry, 2004).

Aged koumiss drink has a slightly sparkling mouth-feel, and a clean, refreshing and slightly yeasty taste (Puhan & Vogt, 1984; Mandukhai Urguu, 2004). Poorly made koumiss may upset the stomach, while old koumiss is very sour and intoxicating. To make koumiss, sugar (5 g 100 g⁻¹) is added to filtered milk, which is pasteurised and then cooled to 26–28°C. The milk is agitated while the starter culture is added, and agitation continues for 15–20 min after inoculation. Fermentation progresses until the desired level of acidity is attained. The fermented milk is cooled to 16–18°C, aerated (3–5 min) and agitated until the desired fluidity and fizziness is achieved. The koumiss is then bottled, and conditioned at 20°C to allow the production of gas and ethanol. The length of storage at 4°C determines the acidity and alcohol content of the final product (Boudier, 1985; Kanbe, 1992). Koumiss may contain up to 2.5% ethanol (Puhan & Vogt, 1984; Robinson et al., 2002).

A koumiss starter contains several types of yeasts, including lactose-fermenting, lactose non-fermenting and carbohydrate non-fermenting strains. Torula spp. and Saccharomyces lactis are found in koumiss together with Lb. delbrueckii subsp. bulgaricus and Lb. acidophilus. Yeasts and bacteria can be added together or in a staggered fashion to carry out the fermentation (Puhan & Vogt, 1984; see also Robinson et al., 2002; Wszolek et al., 2006).

6.5.3 Calpis®

Calpis®, a beverage developed in Japan, was first sold in 1919. After pasteurisation, skimmed milk is inoculated with a mixed culture containing Lactobacillus helveticus, Lactobacillus fermentum and Saccharomyces cerevisiae as the major organisms. The inoculated milk is fermented at 37°C for 24 h until the titratable acidity reaches 2.2 mL 100 mL⁻¹ or the pH reaches 3.3. The product contains (g 100 g⁻¹) 8.6 milk SNF, 2.9 protein, 0.01 fat and 0.1 mL alcohol mL⁻¹. Afterwards, the product is mixed with sucrose and undergoes further fermentation. After fermentation, the product is homogenised at 14 MPa of pressure, mixed with an approximately equal amount of sucrose, then soybean EPSs are added, and the mixture is pasteurised. Finally, flavours are added before the drink is filled into 500-mL glass bottles or laminated paperboard cartons. The resulting product contains approximately (g 100 g⁻¹) 49.9 water, 1.4 protein, 0.1 fat and 48.3 carbohydrate, and has a pH of 3.3–3.4. As Calpis® is a pasteurised product, it is distributed in the market at ambient temperature with a shelf-life of 12 (for glass bottles) or 9 (for paperboard cartons) months. Consumers drink Calpis® after diluting it five-fold with cold or hot water. Figure 6.11 shows Calpis® viewed by SEM.
6.6 Lactic acid–mould fermentations

6.6.1 Viili (viiliä)

Viili is the most common fermented milk product produced in Finland, although it originated in Sweden. Commercial production of viili started in Finland and, by 2002, per capita consumption in that country had reached 4.5 kg per year (Valio Foods and Functionals, 2003). The viscous product has a firm even consistency, mild flavour, with a stronger creamier flavour note at the surface.

Commercial production of viili uses pasteurised cow’s milk that is cooled and to which the viili starter culture is added. A mesophilic fermentation is carried out at 20°C for approximately 20 h in 200-g plastic cups. The final viscosity of the product is achieved without the addition of stabilisers or added milk solids. If viili is stirred, it becomes ropy (see also Fondén et al., 2006).

Viili starter culture contains *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis* biovar. *diacetylactis*, *Leu. mesenteroides* subsp. *cremoris* and the mould *Geotrichum candidum*. Tests have shown that the probiotic bacteria *Lb. acidophilus* and *Bifidobacterium bifidum* can survive for up to two weeks in traditional viili (Mantere-Alhonen & Kohtanen, 1995).

Traditional viili was made in small batches with unhomogenised milk. The cream layer that formed at the surface supported the growth of *G. candidum*, which formed a velvet-like mat. Modern production techniques use both homogenised and unhomogenised milk. In some products the limited oxygen in the product headspace reduces the growth of the mould on the surface of the product.

Fig. 6.11 SEM micrograph of Calpis® structure showing the yeasts, lactobacilli and homogenised milk fermentate. Calpis® was collected on a membrane filter and used for electron microscopy.
Japanese researchers reported that the *Lac. lactis* subsp. *cremoris* SBT 0495 isolated from viili produced an exopolysaccharide (slime) that contributed to the ropy consistency and decreased the susceptibility to syneresis of viili (Nakajima *et al*., 1990, 1992; ZhenNai *et al*., 1999). They isolated and characterised a polysaccharide that was composed of branched pentasaccharide repeating units of L-rhamnose, D-galactose, D-glucose and phosphate with a molecular weight of $1.7 \times 10^6$ Da and a molar ratio of 1.3 : 2.1 : 1.8 : 1.0 (rhamnose : glucose : galactose : phosphate). This unique polysaccharide has been named viilian. The ropiness of the fermented product depends upon the ratio of the ropy strains to non-ropy strains (Nakajima & Toyoda, 1994). Using electron microscopy they showed that the ropy character of viili could be attributed to strong polysaccharide–acid-coagulated milk interactions facilitated by the negatively charged phosphate group associated with the polysaccharide. In aqueous solutions, it was found that viilian displays characteristics of an entangled polymer as opposed to a weak gel, and most likely assumes a coiled conformation (Oba *et al*., 1999).

Transmission electron micrographs of viili (Fig. 6.12) show large fat globules – regular in shape, but with no surrounding membrane. The protein portion is open and not well structured. Bacteria are not as evident in the product as for other products.

![TEM micrograph showing structural details of viili. The bacteria are less evident; the structure consists of large fat globules with no external membrane evident because the milk base was not homogenised; clusters of casein particles are separated by large void spaces. P = protein, F = fat globule. Reproduced by permission of D. Montpetit (personal communication) and Agriculture and Agri-Food Canada.](image)
6.7 Concentrated fermented milk products

6.7.1 Labneh

Labneh (labaneh) is well known in the Middle East countries of Lebanon, Syria, Morocco and Iraq. It is traditionally made from cow’s milk, but goat’s milk labneh has also been produced (Tamime & Robinson, 1999; Abou-Donia, 2004; Nsabimana et al., 2005). Part of its popularity may be related to the fact that labneh has a longer shelf-life than yoghurt. Labneh is eaten in the stirred form with bread and garnished with olive oil or it can be shaped into a ball and stored under olive oil (Abou-Donia et al., 1992; Tamime & Robinson, 1999). Labneh is closely related to traditional yoghurt found in many Middle Eastern countries, and is referred to as yoghurt cheese in some places.

Traditional labneh is made by concentrating plain yoghurt by letting the whey separate from the fermented yoghurt held in a suspended cloth bag; a 1 kg pressing load per kg of curd for 24 h is used in many dairies. The remaining curd may be blended (smoothened) and then put into cups (Abou-Donia et al., 1992). Alternatives to the cloth bag method have been proposed. The fermented warm yoghurt can be processed with ultrafiltration systems, nozzle separators or reverse osmosis (Tamime et al., 1991a; Özer et al., 1999). The final total solids content of labneh is 23–29 g 100 g⁻¹. Normal stirred yoghurt has a final total solids content of at least 8.5 g 100 g⁻¹ (USFDA, 2003).

Electron microscopic studies of traditional labneh made from cow’s milk have shown the product to have a smooth uniform protein matrix with casein particles and chains, and occasional fluffy clusters before homogenisation (Tamime et al., 1989, 1991a). The firmness of labneh prepared by ultrafiltration from cow’s, goat’s or sheep’s milk was related to the relative uniformity of the protein matrix (cow > sheep > goat) (Tamime et al., 1991b). Void spaces or pores were reduced because the milk base was homogenised and the cool labneh was passed through an ALM structuriser to smooth the product; this resulted in minute fat particles embedded in the casein matrix; fat globules with intact and ruptured globule membranes can be found (Tamime et al., 1989, 1991a).

Fig. 6.13 SEM micrograph showing structure of protein matrices of labneh. The lactobacilli and streptococci are dispersed in the protein matrix consisting of coagulated casein micelles and whey proteins. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
Scanning and transmission electron micrographs of labneh (Figs 6.13, 6.14, 6.15) show uniform casein micelles with a variety of bacteria embedded in pockets throughout the protein mass. Higher-magnification transmission electron microscopy shows small, uniform-sized fat globules.

**Fig. 6.14** SEM micrograph of structure of labneh. Like yoghurt, the labneh is made from milk fermented with a mixed culture of lactic acid bacteria [*Lactobacillus delbrueckii* subsp. *bulgaricus* (top bacterium) and *Streptococcus thermophilus* (lower bacterium)]; hollow protein particles in the left half are the result of defatting the protein matrix for SEM; minute fat globules surrounded by protein have been removed but their protein envelopes remained. Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.

**Fig. 6.15** TEM micrograph of structural detail of labneh. Minute fat globule fragments, resulting from the homogenisation of milk (arrow) are surrounded by milk proteins (submicellar casein and whey proteins – dark structures). Reproduced by permission of M. Kaláb (personal communication) and Agriculture and Agri-Food Canada.
6.7.2  

Skyr production began in Iceland 900 years ago. Originally it was made from ewe’s milk, but now commercial skyr is made from cow’s milk. It has a soft texture, creamy taste and is often served with a mixture of milk and cream (Iceland Tourist Board, 2005) In 2003, per capita consumption of skyr in Iceland was 8.9 kg per year.

Skyr is produced commercially from skimmed milk or spray-dried skim milk powder (SMP). The milk is heated to 90–95°C for 30–40 min and then cooled to 40–43°C. Skyr starter is added together with cool skimmed milk or water. This mixture is stirred until smooth, and then cheese rennet is added. The rennet/starter/skimmed milk is then added to hot skimmed milk (40°C), and maintained at a temperature of 40–43°C for 4–5 h. At this stage, a curd starts forming. The mixture is cooled and stored overnight. The whey is then separated either by draining through a filter bag or using separators as for quark production. The skyr whey may be concentrated using ultrafiltration and then added back to the concentrated fermentate just before packaging (Gudmundsson, 1987; Wolpert, 1988). The final skyr contains 18–20 g 100 g⁻¹ solids and has a pH of 3.8–4.0 (Tamime & Robinson, 1988; see also Fondén et al., 2006).

Skyr starter is a mixture of micro-organisms that is maintained in-house. The most important bacteria include \( S. \) thermophilus and \( Lb. \) delbrueckii subsp. bulgaricus. The starter also contains yeasts.

Electron micrographs of skyr (Fig. 6.16) show dense, thick protein structures that can be single structures, chains or clusters. Skimmed milk is used to make skyr and, therefore, fat globules are not evident, but bacteria can be seen.

Fig. 6.16  TEM micrograph showing structural detail of skyr. Numerous bacteria are present; no fat globules are evident; the protein matrices are more compact and less delicate in the structure of the product. P = protein, B = bacteria. Reproduced by permission of D. Montpetit (personal communication) and Agriculture and Agri-Food Canada.
6.7.3  **Ymer**

Ymer is a Danish fermented milk product that resembles a soft milk pudding. Ymer is most often sold unflavoured and contains at least 6 g 100 g⁻¹ milk protein and at least 11 g 100 g⁻¹ SNF (Mogensen, 1980). The skimmed milk curd has a delicate aroma, and a smooth and silky texture. Ymer is related to the Swedish product lactofil (Anon., 1969).

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**Fig. 6.17** SEM micrograph showing structure of ymer. Uneven protein surface contains embedded bacteria; evidence of exopolysaccharides in void spaces. P= protein, B = bacteria, circles = possible exopolysaccharide networks. Reproduced by permission of D. Montpetit (personal communication) and Agriculture and Agri-Food Canada.

**Fig. 6.18** TEM micrograph showing structural detail of ymer. The protein structure is loose and dispersed; large irregular fat globules; some bacteria are evident. P= protein, B = bacteria, F = fat globule. Reproduced by permission of D. Montpetit (personal communication) and Agriculture and Agri-Food Canada.
Ymer is produced traditionally using an aromatic, gassy mesophilic starter culture (Anonymous, 1969; APV, 2005) that is added to skim milk which is kept at 18°C until the pH drops to 4.6 (within 18 h). The whey is broken up and drained after fermentation. Cream is added to produce either regular (minimum 3% fat) or high-fat ymer. Ultrafiltration methods have been used to increase the protein content and reduce the lactose content of the skimmed milk used for ymer production (Delaney, 1977). Ymer produced by ultrafiltration was found to have superior stability, and a more acceptable taste (more acidic, fresher) than the traditional product. The ymer starter contains 4–8% \( \text{Lac. lactis subsp. lactis} \), \( \text{Lac. lactis subsp. cremoris} \), \( \text{Lac. lactis subsp. lactis biovar diacetylactis} \) and \( \text{Leu. mesenteroides subsp. cremoris} \) (Danish Dairy Board, 2005). During fermentation the production of gas (carbon dioxide) causes the curd to float.

Scanning electron micrographs of ymer show bacteria embedded in the protein matrix that appears to have a surface containing numerous pockets. Structures can also be seen that resemble webs of polysaccharide material. TEM shows a widely dispersed open protein structure and irregular fat globules (see Fig. 6.18).

### 6.7.4 Shrikhand

A large number of dairy and non-dairy fermented foods are consumed in India (Prajapati & Nair, 2003; see also Özer, 2005). Shrikhand is a sweet, often flavoured, yoghurt-like product eaten during a meal or as a dessert in western India and Pakistan. Most shrikhand is produced in small-scale dairies or in the home from chakka.

Commercially, shrikhand is produced from milk (cow, buffalo or mixed) that is pasteurised at 71°C for 10 min, cooled to room temperature, and then inoculated with lactic culture (1–3 mL 100 mL\(^{-1}\)). Incubation is carried out at 28–30°C for 15–16 h. The fermented mixture (solids and liquid) is called dahi (FAO, 2005). Dahi has a total solids content of 13 g 100 g\(^{-1}\) and a pH of 4.5 (Younus et al., 2002). The solid curd is broken up and separated from whey by suspending in a muslin cloth. Hydro extractors or quark separators may also be used. The solid remaining, called chakka or maska, is mixed with sugar (40 g 100 g\(^{-1}\)) to produce shrikhand (Indiaagronet, 2005).

Indian standards state that \( \text{Lac. lactis subsp. lactis} \), \( \text{Lac. lactis subsp. lactis biovar diacetylactis} \) and \( \text{Lac. lactis subsp. cremoris} \) (these micro-organisms used to be known as \( \text{Streptococcus lactis} \), \( \text{Streptococcus diacetylactis} \) and \( \text{Streptococcus cremoris} \), respectively) alone or in combination with or without \( \text{Leuconostoc} \) spp. with \( \text{Lb. delbrueckii subsp. bulgaricus} \) and/or \( \text{S. thermophilus} \) are to be used to produce sour dahi (Anon., 1976).

### 6.8 Frozen yoghurt and related products

According to Lyck et al. (2006) frozen yoghurt and related products are made using different methods: (1) the base mix containing high amounts of yoghurt (40–80%) with live starter culture; (2) the base mix is similar to (1) but the amount of yoghurt used is < 40%; (3) a process known as ‘direct method’ where the mix containing yoghurt is heat-treated before aging and freezing, and the frozen product does not contain the live organisms of the starter culture; (4) adding a starter culture to the base mix, fermenting and freezing; and (5)
unfermented method where the base mix is mixed with the starter culture before freezing. The same authors have provided different recipes for the manufacture of these products.

Sample preparations for microscopic analysis of frozen products are detailed in Chapter 9. For frozen yoghurt, low-temperature SEM has been used and, according to Smith (personal communication) the sample was prepared as follows. (1) The chunks of frozen yoghurt were removed from the tub and broken into suitable size under liquid nitrogen. The frozen sample was mounted into the clamping device under liquid nitrogen (–196°C). Two samples of frozen yoghurt (3 mm³) were mounted on a copper holder designed for the Emitech K1250X cryo preparation unit (Ashford, Kent, UK). The holders were designed specifically for frozen samples with clamps to hold the sample pieces firmly. (2) The frozen holder was pulled into the transfer device, and immediately placed under vacuum. The samples were transferred frozen and under vacuum into the preparation chamber of the cryo unit where the frozen

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**Fig. 6.19 (a–d)** Low-temperature SEM micrographs of frozen yoghurt at different magnifications after 10 minutes of sublimation at –80°C. Note (A) an air bubble, (C) ice crystals, and (B) starter culture (arrows) in the serum phase. Bar sizes: (a) 30 μm; (b) 6.0 μm; (c) 60 μm; (d) 0.3 mm. Reproduced by permission of A. Smith (personal communication) of Guelph University, Ontario, Canada.
yoghurt was fractured to expose a surface for sublimation. (3) The yoghurt was sublimated for 10 min at –80°C, a standard freeze-drying temperature. This process removed enough ice from the ice crystal sockets to better identify the structures. (4) When sublimation was complete, the samples were coated with 30 nm of gold. The thin coat of this high atomic number element provides conductivity to prevent the sample from absorbing the electron beam, and also results in more secondary electrons being created. (5) The holders were then transferred, frozen and under vacuum, into the SEM (Hitachi S-570; Tokyo, Japan) where they were held on the cold stage at temperatures < –130°C. (6) Images were captured digitally using Quartz PCI imaging software (Quartz Imaging Corp., Vancouver, Canada).

By using the above-mentioned method of sample preparation, the microstructure of yoghurt (Figs 6.19a–d) was shown to consist of air bubble, ice crystals and starter cultures; however, no fat globules were evident as the frozen product was made from low-fat yoghurt.

6.9 Dried fermented milk products

Traditionally, plain/natural and low-fat yoghurt is concentrated, shaped into flat rolls and sun-dried (Kurmann et al., 1992; Tamime & Robinson, 1999). Desert dwellers, for example, utilise dried yoghurt in the preparation of food dishes and soups and even consume it like biscuits with tea. To our knowledge, the microstructure of commercially available dried or instant yoghurt has been reported by Kaláb (1992); however, the manufacturing stages using two-stage driers (the first is a spray-drier while the second is a fluid-bed drier) have been reported by Tamime (2003).

*Kishk* is the name for dried yoghurt mixed with cereal (or other additives), which is made traditionally throughout the region between the eastern Mediterranean and the Indian subcontinent. Many traditional names have been applied to these dried fermented milks (Kurmann et al., 1992; Tamime & O’Connor, 1995) and, depending on the ingredients used, it is possible to classify them into three categories: (1) products containing parboiled cracked wheat; (2) products containing vegetables, herbs and/or spices; and (3) products containing other types of cereals, such as barley, oats, sorghum, rice, maize or pearl millet, and chick pea (Tamime & Robinson, 1999).

A survey of 25 commercial samples of Lebanese kishk was carried out in the 1990s, and the gross chemical composition, nutritional value and microbiological quality were reported by Tamime & McNulty (1999) and Tamime et al. (1999a,b,c). These kishk products were from different regions of Lebanon, and made by dairy companies, at home, in granaries or supermarkets using different blends of mammalian milks (Tamime et al., 1999a,b). According to Lewis (personal communication), the general microstructure of the kishk samples consisted of aggregates of dairy components and cereal (Fig. 6.20a). Figure 6.20(b) shows smooth starch grains, Fig. 6.20(c) shows cereal cellular material including starch, and Fig. 6.20(d) shows a relatively smooth particle surface, presumably from an outer closed surface.

It is possible to suggest that these differences in the structure between the kishk samples are attributable to (1) shape of the particle (i.e. overall and ‘roundness’ of edges), (2) compactness of packing, and (3) relative distribution of cereal in the dairy structure. The
Fig. 6.20 (a–d) General views of the SEM structure of Lebanese kishk samples. A = aggregates of dairy ingredients and starch, S = smooth starch granule, CW = cell wall. Reproduced by permission of D. Lewis (personal communication).
SEM images suggest that kishk sample 1 (Fig. 6.21a; supermarket) has a reasonable range of particle size (5–100 μm dimension), the particle shapes tend to have slightly rounded corners, and reasonably dense packing in the aggregates. Kishk sample 7 (Fig. 6.21b; bought in a supermarket, unknown manufacturer) had a particle size distribution similar to sample 1 with possibly more particles at the small end of the size range; also, the particles had a mixture of shapes, i.e. some with rounded edges and some more regular, while in parts, there were more separate areas of cereals and dairy structures. The particle size distribution of kishk sample 20 (Fig. 6.21c; dairy company) were more possibly weighted towards the > 50 μm end of the sizes of kishk in sample 1; in addition, there were possibly more flake-shaped particles than in samples 1 or 2, and the aggregates were slightly more open structured. In comparison, kishk sample 21 (Fig 6.21d; dairy company) had the range of particle sizes similar to sample 1, and had some isolated cereal particles but more aggregates and fewer flakes than kishk sample 20; however, the aggregates were fairly tightly packed. While the particle size distribution of kishk sample 8 (Fig. 6.21e; granary) was similar to sample 7, they were weighted towards the < 20 μm end of the range; fewer flakes were observed compared to kishk sample 20, and the cereal structures in the aggregates were less pronounced than in the other samples. Lastly, kishk sample 10 (Fig. 6.21f; bought in a supermarket, but unknown manufacturer) had the coarsest size distribution and few flakes could be seen; the aggregates had a fairly open structure, presumably due to the dairy element tending towards a ‘stringy’ link structure rather than a coherent matrix. In conclusion, Fig. 6.22 shows the main structure of kishk (i.e. starch granules and aggregates of dairy ingredients at high magnifications.

Fig. 6.21 (a–f) SEM micrographs of Lebanese kishk samples showing the differences in their structure. Reproduced by permission of D. Lewis (personal communication). (Continued.)
Fig. 6.21 (a–f) (Continued.)
6.10 Conclusions

It is evident that microscopical studies of fermented milks and related products have been expanding rapidly for the past three decades, and new techniques have been adopted from other disciplines or developed specifically for the products reviewed in this chapter (see Kaláb, 1992, 1993). Fermented milks have become very popular with consumers worldwide, and researchers have modified the milk bases of these products over the years to ensure consumer acceptability. However, microscopy techniques have been used to improve our understanding of the physicochemical behaviours of the ingredients used, processing conditions applied and the use of different starter cultures, and the knowledge gained will ultimately assist researchers to develop new fermented milk products. In addition, SEM and TEM techniques were very popular until the early part of the 1990s and provided in-depth knowledge of the interactions that take place between the milk components (mainly the proteins and/or fat) during micellar matrix formation but, as can be seen, CSLM has become very popular in recent years, and will ultimately become a routine procedure in structure analysis. This is due to the fact that this microscopic technique is easy to use and has the ability to examine the samples without fixation. In addition, such a technique can be used to examine samples at various depths under the surface, and also to provide three-dimensional images.

References


USFDA (2003) Section 131.200 Yogurt, Subpart B Requirements for Specific Standardized Milk and Cream, Part 131 Milk and Cream Table of Contents, Chapter 1, Food and Drug Administration, Department of Health and Human Services, Title 21 Food and Drugs (21CFR131.200) pp. 304–305.


7 Microstructure of Natural Cheeses

D.W. Everett

7.1 Introduction

Milk is a low-acid and high-moisture food product and, as a consequence, deteriorates rapidly unless preservation measures are taken. The manufacture of cheese is one method to prolong the shelf-life of milk, employing several techniques to limit unwanted microbial growth, such as heating (pasteurisation), culturing, direct acidification, dehydration, refrigeration and packaging.

The microstructure of cheese consists of a complex arrangement of fat and protein aqueous phases. Natural cheeses are produced by coagulation of milk, usually through the enzymatic action of chymosin in a coagulant solution, or in some cases through the isoelectric precipitation of caseins by the action of lactic acid bacterial cultures or direct addition of acid. Enzymatically coagulated cheeses usually have higher pH and lower moisture content than acid cheeses, and are matured for a longer period of time. Acid cheeses are usually consumed fresh or after a short period of maturation. These two types of cheese have different protein structures; chymosin or other coagulant gels are characterised by thin protein fibres whereas acid-heat gels contain thicker fibres (Kaláb & Harwalkar, 1973).

Early research on a wide variety of cheeses employed scanning and thin-sectioning electron microscopy (SEM – scanning electron microscopy and TEM – transmission electron microscopy) and light microscopy to examine the microstructure. This early work provided a solid foundation upon which to examine the physicochemical interactions of cheese components in later years. Since the early 1990s, investigations into cheese microstructure have employed the newer technique of confocal laser scanning microscopy (CLSM) (Heertje et al., 1987) to complement SEM and TEM. Other more indirect techniques have also been employed, such as measurement of free oil formation, the state of water, colour determination, texture profile analysis and dynamic oscillatory rheometry. One consequence of the growth of the pizza and prepared frozen foods industries in the 1990s is the increase in production of Mozzarella cheese, primarily intended as an ingredient in prepared foods. There has subsequently been an increase in research funds available from the dairy industry to investigate Mozzarella functionality, leading to a large increase in the number of published articles on this type of cheese (Kindstedt, 2004).

It has been stated that poor-textured cheese has poor flavour, but that a well-textured cheese does not always have good flavour (Lawrence et al., 1983). Understanding cheese microstructure is not only important for creating desirable texture from the perspective of the consumer, but also for creating an environment where good flavour is not precluded.

Cheese is stated to have a macrostructure, which includes the curd granule structure, and a microstructure consisting of those structural elements that are only visible by microscopy (Kaláb, 1979b). This chapter will review the microstructural elements, in particular, the fat and proteins, and the physicochemical mechanisms that dictate their interactions in cheese. Some microstructural aspects of curd granule junction zones will also be discussed.
7.2 Manufacturing processes

A manufacturing schedule outlining the main stages in the production of Cheddar cheese is shown in Fig. 7.1. Two optional steps are included: (1) the addition of annatto dye to give a more intense yellow/orange colour, and calcium chloride to produce a firmer cheese; (2) heating and stretching of curd for the production of cheese varieties such as Mozzarella. This last step is referred to as the *pasta filata* process. More details about the processes involved in practical cheesemaking are provided by Kosikowski & Mistry (1997a,b).

Starter cultures are added to the cheese milk and allowed to grow during the initial ripening stage to start the process of metabolism of lactose to lactic acid, which ultimately

![Generic cheese manufacturing schedule. Addition of annatto dye (to produce a more intense yellow/orange colour) or calcium chloride (to increase curd firmness) are optional steps; the stretching step in hot brine is used for the manufacture of pasta filata cheeses, such as Mozzarella.](image)
reduces the pH of milk (around 6.7) to that of cheese (pH 4.6 to 6 for most varieties). An alternative method, sometimes used in Mozzarella cheese manufacture, is to add acid directly to the milk to rapidly attain a pH equal to that of the finished product. This is referred to as directly acidified cheese. Ripened milk is coagulated over a period of about 30 min by the addition of acid or coagulants, commonly at an elevated temperature of ~30°C, to produce a weak gel structure.

The coagulated milk gel is cut into small curd particles using wire grids with 6–15 mm spacing (referred to as cheese knives), and allowed to heal for 10–15 min to form a dehydrated skin on the particle surface to prevent further curd shattering during subsequent steps. The temperature is increased with concomitant slow stirring during the cooking stage, primarily intended to expel moisture (whey) from the curd in a process termed syneresis. The cooking takes place at about 38°C over a 35–45-min period for Cheddar cheese. Both time and temperature can be increased to drive out more moisture from the curd, or conversely reduced to retain more moisture in the curd. An alternative method to increase moisture content in cheese is to use cheese knives with larger spaced wires. During the cooking stage, fat globules aggregate; however, the milk fat globule membrane surrounding the globules remains largely intact (Kimber et al., 1974). Most factors in cheese manufacture will have some impact on the texture of the final cheese. For example, an increase in the set temperature or the amount of coagulant used will increase the size of casein aggregates and produce a harder cheese (Euston et al., 2002).

Whey is drained from the curds once a particular pH has been reached. This pH is a critical point in cheese manufacture because it dictates the amount of lactose remaining in the curd, and therefore the amount of lactic acid and the final pH of the product. More chymosin and less plasmin will be retained in the curd at lower pH, and this will have implications for the extent of proteolysis during the maturation of the cheese. In addition, the final calcium content of the cheese is fixed at the point of draining, as most of the soluble calcium at this pH is partitioned into the whey. Thus, the pH at the draining stage is an important factor in dictating the final structure of the cheese (Lawrence et al., 1983).

The curds are allowed to mat together after draining, and then cut into blocks, which are regularly turned in what is known as the cheddaring stage. The cheese at the end of the cheddaring stage is sometimes referred to as having a chicken breast texture, as the protein shows a string-like appearance when the curd is torn apart (Kalb & Emmons, 1978). Events that occur during this process include fusion of caseins into thicker fibres with a resultant loss of micelle identity, and a partial loss of fat globule membrane, although individual fat globules do not coalesce completely and can still be seen (Kimber et al., 1974). Cheddaring allows the continued growth of lactic acid bacteria in the warm curd, reducing the pH, and thus allowing the curd to flow and close up any gaps in the curd macrostructure. Maximum curd fusion occurs at pH 5.2 whereupon the curd has good stretchability (Lucey & Fox, 1993).

Cheddar cheese loses the ability to stretch satisfactorily over time due to excessive proteolysis, whereas Mozzarella cheese at the same pH, with less proteolysis, stretches well. The growth of lactic acid bacterial cells during cheddaring also minimises the growth of gas-producing coliforms. Moisture in the curd is reduced by frequent turning of the curd blocks or by cheddaring for a longer period of time. The development of the cheddaring process historically allowed for longer storage of a microbiologically safer cheese, and is one reason
why Cheddar cheese became a dominant cheese in England, and thus in English-speaking countries around the world.

To facilitate salt adsorption, the curd blocks are milled into small *fingers* or *chips* (~5 cm × 1 cm × 1 cm), and dry salt is sprinkled onto the curd pieces. This method of salting is a quicker way to establish salt equilibrium in the cheese compared to immersion of freshly made curd into a cold brine solution. Furthermore, dry-salting is common with British regional cheeses (e.g. Cheshire, Derbyshire, Double Gloucester or Dunlop), whereas brining is more often used for continental European cheeses. In some instances, such as pizza cheese, a combination of the two salting methods is employed.

The final stages of most varieties of cheese manufacture involve pressing the milled curd particles to assist in further whey removal and to promote a more homogeneous curd mass, followed by packaging and maturation. Some varieties of cheese, notably those that require the growth of mould on the surface, are matured under controlled temperature and humidity conditions before packaging. Cheddar, by comparison, is often vacuum-sealed in plastic bags impermeable to moisture and oxygen prior to maturation.

Pressing the curd particles is sometimes done under a partial vacuum to assist in the process of curd fusion and to remove unwanted gas holes. This creates curd granules, which are often visible without the aid of a microscope. The curd granules are slightly elongated in Mozzarella cheese due to the stretching process, unlike Gouda and Edam where the curds do not undergo the pasta filata process (Kaláb, 1977). Two types of junction zones are evident in Cheddar: (1) a thinner boundary formed from fused curd particles prior to cheddaring; (2) a thicker boundary that is deficient in fat globules from fused milled curd during the pressing stage (Kaláb, 1979b).

### 7.2.1 High-pressure processing

High-pressure processing at sufficiently high pressures, microbial cells can be ruptured and enzymes denatured and inactivated. This process can be employed to reduce the viability of unwanted microbial cells in milk. A further use is to accelerate cheese maturation by exposing the substrates in the cheese to enzymes within the lysed culture cells, assuming the pressure is not sufficient to denature the enzymes located within the cell.

Globular whey proteins can denature and casein micelles can be disrupted under high-pressure conditions (Needs *et al.*, 2000). The denatured whey proteins can complex with κ-casein on the micelle surface, potentially hindering the clotting reaction in cheese manufacture. A gel formed from smaller, disrupted casein micelles may produce a stronger gel (if the clotting reaction is not hindered) due to a greater number of micelle junction zones per unit volume within the cheese protein matrix.

High-pressure processing at 500 MPa of brined goat’s milk cheese closes up the protein matrix, with a more homogeneous structure as shown by CLSM, and with smaller and more uniform fat globule size (Buffa *et al.*, 2001). Repeated 200 MPa applications of pressure to milk prior to manufacture of Cheddar cheese results in a more compact protein matrix with smaller fat globules of ~400 nm in size. These fat globules are more evenly distributed in the protein matrix. By comparison, high-pressure processing of skimmed milk with subsequent addition of unpressurised cream has no effect on the
cheese microstructure compared to a product made from unpressurised milk; however, the casein micelles are reduced in size from 200–300 nm to 125–150 nm (Kheadr et al., 2002). High-pressure processing of Gouda cheese up to 400 MPa has been shown to disrupt the casein matrix and yield a cheese that more easily flows, at least up until 42 days of maturation at which point the differences in comparison to unpressurised Gouda are negligible (Messens et al., 2000).

7.2.2 Cheese manufactured from concentrated milk

Ultrafiltered milk

Cheese manufactured from unconcentrated cow’s milk yields about 10% of the weight of the milk as cheese. The remainder of the milk (about 90% of the original volume) is removed as whey and processed further into whey cheeses, or more commonly, whey powders. To reduce the volume of whey, and to incorporate more of the whey proteins into the cheese, milk can first be ultrafiltered to a solids content approaching that of cheese (Maubois & Mocquot, 1975). The whey proteins do not contribute directly to the protein network structure, but instead act as an inert filler material that binds additional water within the cheese, contributing to a smoother texture. Cheese manufactured from UF (ultrafiltered) milk differs from its traditional counterpart, and arguments have been put forward for the merits of using this technology (Lawrence, 1989).

There is a practical limit to whole milk concentrated by ultrafiltration by about five-fold. Lactose is partially concentrated during ultrafiltration of milk, leading to a higher concentration in cheese which can result in too much lactic acid being produced during cheese manufacture. A common method to circumvent this problem is to add water to the retentate during ultrafiltration to reduce the lactose content in the concentrated milk, effectively washing the milk, in a process known as diafiltration (Lawrence et al., 1983).

Softer cheeses, such as Camembert and Feta, have been made successfully using UF milk; however, hard cheeses such as Cheddar have proved to be more difficult due to firmer texture and impaired maturation (Green et al., 1981b; Green, 1985). Other researchers have showed that Cheddar cheese is softer when made from UF milk, possibly due to the inert whey proteins disrupting the casein matrix (Everett & Jameson, 1993).

The higher protein content of concentrated milk produces a faster rate of coagulation (assuming that a constant amount of coagulant has been added) compared to unconcentrated milk, increased fat losses into the whey, and a higher protein and moisture content of the finished Cheddar cheese (Green et al., 1981a). One consequence of this faster rate of coagulation is a coarser protein network and reduced fat retention in the finished cheese (Green, 1987). As the concentration ratio of UF milk increases, the protein network becomes coarser, with a corresponding decrease in fat–protein surface area (Green et al., 1986). The protein network in Cheddar cheese manufactured from UF milk is exemplified by thicker protein fibres, and contains more segregated fat globules compared to conventionally made Cheddar (Green et al., 1981b). These authors argued that the basic structure of cheese develops at the milk coagulation stage. The coarseness of the protein network can be reduced by decreasing the coagulation temperature of milk, the addition of coagulants to cold UF milk, or acidifying the milk prior to coagulation (Green, 1987).
The coarse protein network of cheese made from UF milk was also observed in Urfa, a white-brined cheese from Turkey (Özer et al., 2003). Feta cheese manufactured from UF milk also shows a coarser protein network when the amount of chymosin, the coagulation temperature or the coagulation time is increased (Min & Qvist, 1998; Min et al., 2003). Presumably the protein network was able to form thicker protein fibres at a faster rate at the higher temperature. Higher amounts of chymosin will coagulate the milk at a higher pH during the slow acidification process, thus enabling more protein rearrangements to take place before the final pH 4.6 is reached. Feta cheese manufactured from milk protein concentrate showed larger void spaces in the protein matrix, which was corrected by the addition of more lactose (Kuo & Harper, 2003). In contrast, Twarog and Domiati cheese manufactured from UF milk and examined by TEM and SEM showed no difference in protein microstructure compared to the conventional counterpart (Omar & Hosaja, 1986; Omar, 1987, 1988). An unspecified white-brined cheese made from UF milk showed a weaker structure at a higher level of chymosin (Al-Otaibi & Wilbey, 2005).

During ultrafiltration, milk is pumped across a membrane under turbulent conditions with a partial homogenising effect. This has the potential to reduce the size of fat globules, and has been observed in Cheddar cheese made from UF milk (Green et al., 1983). The extra surface area generated by homogenisation is coated with casein micelles, and this has the potential to alter the structure of the cheese by allowing the fat globule surfaces to interact with the surrounding casein matrix (see section 7.4).

Evaporated milk

Milk can also be concentrated by evaporation at ~70°C under vacuum. Evaporating milk concentrates the lactose and soluble minerals, whereas these components are partially removed by the ultrafiltration process. As the concentration factor of evaporated milk increases, the microstructure of reduced-fat Cheddar cheese becomes rough and rippled, and the cheese texture becomes more crumbly, presumably due to incomplete curd fusion (Anderson & Mistry, 1994).

7.2.3 Freezing

Freezing Mozzarella cheese weakens the protein matrix as water expands to form ice crystals, thus creating a more porous structure and softer cheese (Graiver et al., 2004). This effect is only significant if there is sufficient bulk water that can be frozen, that is, immediately after Mozzarella manufacture and before the water is absorbed by the swelling protein phase (see section 7.7.2). If the freezing rate of Mozzarella cheese is sufficiently fast, such that large ice crystals do not form, the protein matrix may not be sufficiently disrupted to impact upon texture (Cervantes et al., 1983). A slower rate of freezing increases the meltability of low-moisture Mozzarella, possibly due to disruption of the protein matrix by the formation of large ice crystals (Oberg et al., 1992). By this same mechanism, freezing of semi-hard sheep’s milk cheese produced a softer product compared to cheese that is more rapidly frozen (Fontecha et al., 1994). Frozen storage temperature and rate of freezing have been shown to affect free oil formation in low-moisture Mozzarella cheese in different ways, depending on the age of the cheese before freezing (Bertola et al., 1996).
7.3 Compositional parameters

7.3.1 Calcium and pH

Calcium is one of the major compositional variables affecting cheese microstructure. As the pH of cheese curd decreases below 6 towards 5.4, the curd becomes more flowable concomitant with an increase in the ratio of soluble to bound calcium (see Fig. 7.2a). Citrate will complex soluble calcium ions and prevent them from facilitating protein–protein interactions, thus expanding the protein phase in cheese. This is shown in SEM of Cheddar cheese with added citrate, where the area occupied by the protein phase increases (Pastorino et al., 2003c). The proportion of soluble calcium in Cheddar cheese decreases during maturation of the product from around 72% to 57% over a 9-month period, although the pH of the cheese does not change to a large extent (Lucey et al., 2005). This slow solubilisation is difficult to explain, as the partitioning of calcium between bound and soluble forms should be a much more rapid occurrence.

The maximum of cheese flowability is reached at around pH 5.2 to 5.4. Below pH 5.2 there is a net reduction in casein charge due to shielding by protons and calcium ions. Proteins, therefore, associate through hydrophobic interaction to an increasing degree as pH is reduced. This parallels an increase in the brittleness of the cheese, particularly below pH 5, as exemplified by crumbly Cheshire cheese with a pH of 4.8.

Lowering the pH of Cheddar cheese from 5.3 to 4.7 results in a contraction of the protein matrix and a decrease in the size of casein substructural units from 10 nm to 2 nm (Pastorino et al., 2003b). Figure 7.2(b) illustrates the decrease in the size of these units as pH decreases, along with the subsequent change in cheese texture. At pH below 4.8 the casein subunits are

Fig. 7.2 (a) Curd flowability as a function of pH, showing total calcium and the proportion of calcium in the serum phase. Source: Lawrence et al. (1987) Journal of Dairy Science, 70, 1748–1760. Reproduced by permission of the American Dairy Science Association and Journal of Dairy Science.
small, elongated, and of the same dimensions as an unfolded casein molecule (Lawrence et al., 1987). At higher pH, protein–protein interactions are mediated by calcium rather than through hydrophobic interaction, and cheese is more fluid and protein rearrangements are more likely to take place (Euston et al., 2002). This increase in fluidity also takes place at higher temperatures. Higher pH cheeses tend to be more elastic, such as Swiss-style cheeses with pH around 5.4. At an intermediate pH of 5, protein–protein interactions are at a minimum.

### 7.3.2 Salt

A higher salt concentration causes the protein phase in cheese to swell and absorb water, thus enhancing protein–water interactions. The role of salt in cheese maturation has been reviewed by Guinee (2004). Vacuum-brining of Manchego cheese in a pressurised brine solution results in a more homogeneous protein network with smaller and more dispersed fat globules, presumably through a partial homogenisation effect from the vacuum-brining process (Pavia et al., 1999). By comparison, regular brining of Manchego cheese yields an irregular protein matrix with large pores and large aggregated fat globules. A higher salt level in Muenster increases solvation and swelling of the protein matrix (Pastorino et al., 2003a).

### 7.3.3 Fat replacers

Fat can be replaced in cheese by fat mimetics based on proteins, carbohydrates or modified triacylglycerides, such as sucrose polyesters (see review by Tamime et al., 1994). Protein and carbohydrate mimetics serve to open up the protein matrix and allow greater moisture retention. Microparticulated whey protein particles (0.1–1.5 μm in size) have been shown to associate with both the water phase (Armbruster et al., 1995) and the protein and fat phase of Cheddar cheese (Aryana & Haque, 2001). These particles are believed to be non-interacting with the surrounding protein matrix and serve as protein structure-breakers (Mackey & Desai, 1995). Reducing the level of fat will have some impact on the protein phase, such as the observed increase in the size of protein aggregates in Feta cheese (Sipahioglu et al., 1999).

The addition of low-methoxy pectin, microparticulate whey proteins or whey protein concentrate to Manchego cheese gives a more homogeneous and compact protein matrix, as observed by SEM, compared to the full-fat cheese (Lobato-Calleros et al., 2001). A fibrous web-like structure is evident in cheese containing microparticulated whey proteins, which may represent aggregated whey proteins. Calcium pectate crystals are evident when low-methoxy pectin is used as a fat replacer (Lobato-Calleros et al., 2001). The addition of fat mimetics made from modified tapioca starch or lecithin produced a similar microstructure to full-fat Feta. Web-like structures are evident in reduced-fat Cheddar cheese with lecithin added as a fat mimetic, possibly representing protein–phospholipid aggregates (Drake et al., 1996). The addition of granular lecithin as a fat mimic to reduced-fat Cheddar yields a more open protein matrix (Drake et al., 1998).

Modified triacylglyceride fat replacers incorporated into reduced-fat Mozzarella cheese results in higher moisture, less free oil, but similar melt properties to a full-fat cheese (Rudan
et al., 1998b). In reduced-fat Mozzarella, homogenisation with fat substitutes decreased the amount of fat lost into the whey (Rudan et al., 1998b). Low-fat Cheddar containing sucrose polyesters of milk fatty acids has smaller fat globules that are more uniform in size (Crites et al., 1997), possibly as the globules are more likely to shatter during the manufacturing process. These fat substitutes are believed to follow a filled-gel composite model where there is no interaction between the fat globules and the surrounding casein matrix. In most studies where the fat substitute is lipid-based, the fat must first be homogenised with an emulsifier, usually milk protein-based, before addition back to the skimmed or reduced-fat milk. As a consequence, there is a confounding effect on the interpretation of results due to the homogenisation step. It is often not clear whether changes to microstructure and functionality are due to the different fat types or to the process of homogenisation.

7.3.4 Milk from different mammalian species

The differences in the casein content of milk from different species would be expected to have some impact on protein structure of cheese. Feta cheese made from a higher ratio of goat’s to sheep’s milk mixture was firmer, and showed a more compact and less porous protein structure by cryo-SEM (Tsigkros et al., 2003). Higher homogenisation pressure (100 MPa) of goat’s milk gave a more homogeneous protein microstructure consisting of thinner chains of protein aggregates, and also resulted in more moisture retention in the product (Guerzoni et al., 1999). Cow’s milk Mozzarella has more free oil formation upon heating of the cheese compared to the same cheese made from goat’s milk (Imm et al., 2003).

7.4 Fat globule microstructure

The presence of fat in cheese is necessary, in most cases, to develop the characteristic flavour profile and mouth-feel. Fat globules also have an impact on texture by partially disrupting the casein fibrous matrix to soften the texture. Reduction of fat by more than one-third in Cheddar cheese often results in poor flavour and a texture that is too firm (Banks et al., 1989) or uncharacteristic flavour and bitterness. Jameson (1990) reported that Mozzarella cheese has been successfully produced with less fat, unlike most other varieties of cheeses, perhaps due to this cheese being consumed more for its textural than its flavour properties.

There is some debate about whether fat globules participate directly in cheese microstructure by binding to the casein matrix, or act as inert filler material by partially disrupting the casein matrix. Undoubtedly, both mechanisms occur to some extent, as large fat globules are more likely to disrupt the matrix compared to the smaller globules occluded within the protein void spaces. Large fat globules are more likely to be distorted, and perhaps exist as free fat trapped within the protein void spaces compared to small fat globules (Michalski et al., 2004). In a study of model systems, Green et al. (1990) observed that hydrophobically coated glass spheres had a strengthening effect on the surrounding protein matrix, indicating that hydrophobic association may be important for holding fat globules into place in a Cheddar cheese casein matrix. Recombined fat globules coated with casein have also been shown to interact strongly with an acid casein matrix (van Net & Dentener-Kikkert, 1982).
Fat globules in Cheddar cheese aggregate during the maturation process, with starter culture bacterial cells located near the fat–water interface (Dean et al., 1959; Kimber et al., 1974). Aggregation of fat globules in low-fat Mozzarella cheese has been observed by SEM (Tunick et al., 1993); however, the globules in Meshanger cheese remain dispersed during the maturation period (de Jong, 1978). This is likely due to the high-temperature cooking and stretching process in Mozzarella cheese manufacture that imposes large stresses on the heated fat globules, promoting globule coalescence.

Reduction of fat in Cheddar cheese yields a higher volume fraction of the protein phase and less fat globule aggregation, with a corresponding increase in viscosity, decrease in meltability and increase in free water that can be removed by centrifugation (Guinee et al., 2000a). This free water is termed expressible serum. Fat globules size is more uniform in lower-fat Cheddar, and the globules are more dispersed compared to the full-fat cheese (Mistry & Anderson, 1993). Aggregation of fat globules may be due to shear-induced processes during the manufacture of the cheese, which would be compounded at a higher fat level.

### 7.4.1 Homogenisation

Homogenisation of the cheese milk can be employed to reduce the size of fat globules, to create a whiter cheese, and to increase the amount of retained moisture in the cheese (Metzger & Mistry, 1994; Rudan et al., 1998a). A review of the advantages and disadvantages of manufacturing cheese from homogenised milk is provided by Jana & Upadhyay (1992).

Small discrete fat globules as well as larger, more irregularly shaped areas of fat are evident in Cheddar manufactured from homogenised milk (Guinee et al., 2000b). These larger fat areas form as a consequence of the shearing of milk during processing, as well as by pressures imposed upon the fat globules by the shrinking casein matrix during cheese manufacture, and by rupture of the native milk fat globule membrane with subsequent coalescence. Homogenisation of cream will have less impact on globule size as the volume of the serum phase is less than for skimmed milk, so there is less protein available to coat the increased surface area. Despite this, there is still a reduction in fat globule size, albeit smaller than the reduction when skimmed milk is homogenised. The smaller-size fat globules from homogenised milk or cream carry through into the structure of the finished cheese (Rudan et al., 1998a).

The extra surface area of the fat–water interface produced by homogenisation is coated primarily with casein micelles and micelle fragments (Cano-Ruiz & Richter, 1997). This may allow the fat globules to participate in the casein matrix as copolymers (Guinee et al., 2000b). Fat globules coated with adsorbed casein micelles (shown as rough particles at the fat–water interface) and sodium caseinate (a monolayer coverage shown with a smooth interface) are shown in Fig. 7.3. A consequence of casein adsorption onto the fat globule surface is that the density of micellar junction zones in the cheese is reduced, therefore the casein matrix does not compact as readily, and the cheese retains more moisture (Green et al., 1983).

Fat globules in reduced-fat Cheddar cheese manufactured from homogenised milk are small and form clusters, which are evenly distributed in the protein matrix (Metzger & Mistry, 1995; Rudan et al., 1998a). However, homogenisation has been observed to decrease fat globule clustering in reduced-fat Cheddar cheese (Emmons et al., 1980). Heating cheese
induces fat globule coalescence in Cheddar, whereas heating of milk in combination with homogenisation prior to cheese manufacture has little effect, presumably as the newly formed membrane after homogenisation is heat-stable (Guinee et al., 2000b). Other consequences of homogenisation include a slower rate of aggregation of casein micelles during the coagulation stage of the milk, poorer curd fusion with a weaker curd structure, and a reduction in the amount of whey (Green et al., 1983).

7.4.2 Recombined milk

Although it is far more common to make cheese from fresh milk, pasteurised or unpasteurised, it is possible to reconstitute whole milk powder for cheese manufacture. The composition of the reconstituted milk may not differ from that of the fresh milk; however, the drying process has some impact on protein conformation and perhaps the mineral balance as well. These differences may induce structural changes in cheese.

Recombined milk is produced by emulsifying milk fat with an aqueous suspension of dairy proteins by homogenisation, and mixing this emulsion back into the skimmed milk to achieve the same protein and fat composition as natural milk. Better emulsifying proteins (such as Na-β-caseinate) form more spherical fat globules that do not aggregate over time in recombined milk cheese, whereas poorer emulsifiers (such as α-lactalbumin and Na-α₂-caseinate) yield cheese containing globules (~2 μm in size), as well as larger fat structures (10–50 μm in size) aligned in the protein matrix that may be pools of free oil (Everett et al., 1995; Everett & Olson, 2003). Fat globules coated with caseinate, rather than the native milk fat globule membrane, tend to be more circular and dispersed when observed by CLSM in Cheddar cheese (Everett & Olson, 2003). Cheese containing globules coated with Na-α₂-caseinate is oily and crumbly, indicating that the globules are not integrating sufficiently into the casein matrix and maintaining globule integrity. Perhaps the more phosphorylated

![Fig. 7.3](image-url) Freeze-etching of fat globules (a) homogenised in skimmed milk, and (b) in milk homogenised with sodium caseinate. Bar = 1 μm. Source: Schmidt & Büchheim (1992) *Journal of Microscopy*, 167, 105–121. Reproduced by permission of Blackwell Publishing, Oxford, UK.
Na-\(\alpha_\text{S_2}\)-caseinate does not exhibit sufficient hydrophobicity when adsorbed onto fat globule surfaces to allow the globules to integrate into the protein matrix by hydrophobic association, thus reducing cheese firmness.

Curd granule junctions in Cheddar cheese made from homogenised milk are not easily visible, as the small fat globules are continuous across the junction zones, whereas in cheese made from non-homogenised milk the junction zones are more easily distinguished from the surrounding curd matrix due to localised depletion of the larger globules (Emmons et al., 1980). A soft brine cheese made from reconstituted whole milk powder has been shown to have an aggregated protein structure with more open pores within the casein matrix, with smaller fat globules, compared to a control cheese manufactured from raw milk (Omar & Bühheim, 1983). The cheese milk was reconstituted using homogenisation, which will have a confounding effect on the microstructure, although no adsorption of casein micelles to the fat globule surface was observed.

### 7.4.3 Free oil formation

The origins of free oil formation are still not conclusively known, despite the wealth of information on cheese microstructure. It is clear, however, that Mozzarella cheese with higher fat content has more free oil upon heating (Kindstedt & Rippe, 1990). Fat globule clustering may be another factor in inducing globule rupture, or perhaps simply it is an indicator that rupture is more likely (Rudan et al., 1998a). Full-fat Mozzarella has more free oil when heated, and also more fat and extensive fat globule clustering. A higher fat content Cheddar will have more free oil upon heating, as also observed in Mozzarella (Guinee et al., 2000a). Homogenisation will hinder fat globule coalescence and rupture in the cheese and result in less free oil; however it will also reduce the stretching and flowing properties due to a greater number of smaller and more highly emulsified fat globules interacting with the casein matrix (Lelievre et al., 1990; Metzger & Mistry, 1995). Although milk fat globules are encapsulated by a native membrane in fresh milk, this may change as other proteinaceous species adsorb to the fat globule surface during maturation of the cheese with some impact on the propensity for globule rupture.

Examination of Cheddar cheese by CLSM shows that fat globules are irregularly shaped (average size 2.4 \(\mu\)m) and may have aggregated over time or ruptured to form pools of oil. This fat globule clustering has been observed in Cheddar cheese along the direction of the protein fibres (Kalb, 1977), and some elongation of the fat areas (that are not necessarily emulsified globules) is evident (Kalb, 1979b). Large areas of fat are less circular than smaller areas, indicating that perhaps the large areas consist of either globule clusters or pools of free oil (Everett & Olson, 2003).

Void spaces within the protein matrix, containing fat globules, in reduced-fat Cheddar cheese made from evaporated milk become more elongated during the maturation period (Anderson & Mistry, 1994). Measurement of the deviation from sphericity of fat globules is one possible predictive mechanism to examine the putative transition from emulsified to pools of free fat. This built-in obsolescence of fat globules could be employed to produce a softer and more melttable cheese. Although some of the larger fat areas in cheese are possibly pools of free fat with a high degree of deviation from circularity as observed by CLSM, a conclusive relationship between cheese circularity and free oil formation in Cheddar or
Mozzarella cheese has not yet been established (Everett & Olson, 2003; Rowney et al., 2003b).

The forces imposed by the protein matrix during cheddaring and pressing may rupture the fat globules to form pools of free fat trapped within the protein matrix (McMahon & Oberg, 1999; Everett & Olson, 2003). These forces are capable of distorting the fat globules, which can resume a more spherical shape once the pressure from cheddaring has ceased (Kalb & Emmons, 1978). By comparison, fat globules in Gouda and Edam, which are not cheddared, are more spherical and dispersed (Kalb, 1977).

7.4.4 Meltability

Meltability increases at higher temperatures due to a lessening of protein–protein interactions (Lucey et al., 2005). The melting point of fat within the globules will have some impact on texture and meltability. Higher-melting-point fats strengthen a composite food gel, although when fat globules are bound to the surrounding protein matrix there is no observed effect of the melting point of the fat (Green et al., 1990). Small casein-coated globules bound to the casein matrix containing a high-melting-point fat fraction may increase the firmness of the cheese. However, the reverse would likely occur if the globules were larger and the temperature was higher, thus the fat globules and the cheese would be more easily deformed.

7.5 Protein microstructure

Immuno-labelling techniques in conjunction with TEM have shown that the casein micelle surface consists of κ-casein (Schmidt & Büchheim, 1992); however, there is some controversy over the substructure of the micelle in fresh milk (McMahon & McManus, 1998; Walstra, 1999). One hypothesis is that the casein micelles are assembled from sub-micelles, of the order of 20 nm, which are depleted of κ-casein, and that associate by interaction with colloidal calcium phosphate (Knoop et al., 1973; Åström & Jenness, 1984). A more recent modification to this theory states that casein molecules assemble around colloidal calcium phosphate to form nano-clusters (Holt & Horne, 1996). In either case, there is some substructure that is evident even after cheese manufacture.

The transition from milk to gel to cheese curd is defined in part by the association of casein micelles to form a fibrillar, and eventually over time a more amorphous, casein network (King & Czulak, 1958; Kimber et al., 1974). Fused protein structural units of dimensions 1–5 μm are evident in Cheddar cheese, with openings in the network of around 1.5 μm in size containing fat globules which do not appear to participate in the protein network (Eino et al., 1976a). Reducing the level of fat in Cheddar cheese results in smaller and less numerous cavities within a thicker protein matrix (Bryant et al., 1995; Drake et al., 1996). Protein fibres in Cheddar cheese begin to thicken noticeably 20 h after cheddaring (Kalb & Emmons, 1978), and appear orientated in a parallel fashion, particularly with cheeses that are heated and stretched, such as Mozzarella cheese, with serum channels containing aggregated fat globules located between the fibres (Guinee et al., 1999). This is less evident in Cheddar cheese as the forces imposed during the cheddaring process are not as severe as those that occur during the pasta filata process of Mozzarella curd manufacture.
This orientation of the protein fibres in Cheddar cheese decreases over time (Kalh, 1977). The openings in the protein network that contain the serum phase decrease in volume over time. This is evident by a reduction in expressible serum from Cheddar cheese during the maturation period (Guinee et al., 2000a), and is most likely due to increased binding of water by the protein phase.

Meshanger is a traditional high-moisture Dutch cheese that was redeveloped in the 1970s after knowledge of the manufacturing process was lost for several decades. The cheese is characterised by rapid softening during the first two weeks of maturation. Although surface yeasts and moulds grow, these do not contribute significantly to the ripening process, and the cheese is therefore said to ripen from the inside out (Noomen & Mulder, 1976). The protein fibres in Meshanger cheese become more dense with larger spaces between the fibres, of between 0.3 and 5 μm in size, as the cheese ages, ostensibly contributing to the rapid change in texture (de Jong, 1978). This cheese softens rapidly as a result of intense chymosin activity in the two weeks after manufacture, particularly at the centre of the cheese where the pH is slightly lower at 5.0 compared to the surface at 5.3 (Noomen, 1977).

7.5.1 Protein hydrolysis

Chymosin is a preferred source of milk coagulant due to its specificity of hydrolysis of the Phe105–Met106 bond in κ-casein. In the absence of cow’s calf chymosin, proteases from other sources may be used for the coagulation of milk. These may include pepsin from various sources (such as from cows, pigs and chickens), as well as proteinaceous extracts from plants, bacteria and fungi. There is some impact by the coagulant on cheese microstructure. Cheddar cheese made from milk coagulated with cow’s calf chymosin has a more compact sponge-like protein matrix compared to cheese made with pepsin, where there are larger cavities and a more fibrous protein network (Eino et al., 1976b). This may explain the softer curd and lower yield of pepsin cheese.

During maturation of the cheese, proteolysis takes place through action by enzymes present in the milk, the bacterial cultures, and from residual chymosin. This is part of the mechanism contributing to cheese softening during maturation. The number of soluble hydrophilic peptides in Emmental cheese increases over time, leading to the conclusion that the remaining insoluble casein network is becoming more hydrophobic as the cheese ages (Gagnaire et al., 2001). As casein fibres associate by hydrophobic interaction (among other physicochemical interactions), this implies that the matrix may be rearranging over time and becoming more compact.

7.5.2 Casein substructural units

Colloidal calcium phosphate binds to phosphate groups esterified to the serine amino acids on caseins within the micelle structure, so it would be intuitive to state that a reduction in calcium would likely disrupt the micelle into smaller fragments. Casein substructural units appear to be smaller in size when the pH of cheese is lower, i.e. 10–15 nm for Gouda at pH 5.3 and 3–4 nm for Cheshire at pH 4.6 (Hall & Creamer, 1972). This pH dependency has also been observed in pasta filata string cheese (Taneya et al., 1992) and soft cheeses (Knoop & Bühheim, 1980). Freshly made Domiati cheese shows large aggregated casein
particles, which become smaller after four weeks in salted whey, possibly by calcium displacement by sodium ions (Abd El-Salam & El-Shibiny, 1973). Electron-dense regions, as observed by TEM, are the size of the putative casein micelle substructural units, and appear to grow in size from 12 to 23 nm over six weeks in low-fat Mozzarella cheese during maturation, with larger spacings in between (Cooke et al., 1995). The increase in size between the larger protein units over time may perhaps facilitate the increase in melting and softening.

Softening of cheese during the maturation period has long been thought to be initiated by the hydrolysis of the Phe\textsuperscript{23}–Phe\textsuperscript{24} bond in $\alpha$\textsubscript{s1}-casein, leading to the hypothesis that this casein associates by hydrophobic interaction and is the structure-forming protein in cheese (Creamer & Olson, 1982; Creamer et al., 1982; Lawrence et al., 1983; Charalambides et al., 1995), and most notably with high-moisture cheeses such as Meshanger (de Jong, 1977). In addition, the application of urea as a dissociating agent to Emmental cheese has led to the conclusion that $\alpha$\textsubscript{s1}-casein, rather than $\beta$-casein, is the structural backbone of cheese (Gagnaire et al., 2002). More recent work has shown that cheese softening is a more complex issue. Cheese will still soften if hydrolysis of $\alpha$\textsubscript{s1}-casein is inhibited, and this softening is likely to be caused by a shift in the ratio of soluble to insoluble calcium (O’Mahoney et al., 2005). The loss of calcium in its bound form may also increase the susceptibility of proteins to hydrolysis, contributing to the softening process (Lucey et al., 2005).

7.6 Minerals

Milk contains a relatively large amount of calcium phosphate, a water-insoluble mineral that increases in solubility if either temperature or pH is decreased. Calcium phosphate crystals (20–30 μm in size) have been found in Cheddar cheese along curd particle fusion lines, perhaps crystallised from pockets of entrapped whey (Brooker et al., 1975). These crystals will become more soluble at lower cheese pH. Crystals of calcium orthophosphate dihydrate (CaHPO\textsubscript{4}.2H\textsubscript{2}O) were found in the seams of cheese, surrounded by a 20-μm-thick compacted protein layer devoid of crystals (Conochie & Sutherland, 1965). Crystal growth can be controlled either by washing the milled curd particles either with water or with a solution of calcium chloride to remove excess phosphate.

Large calcium lactate crystals (around 80 μm in size) have been found in cultured cheese, but not in directly acidified cheese (Brooker et al., 1975). These white calcium D(−)-lactate crystals are often found on the surface of cultured ripened cheese and are thought to be caused by non-starter lactic acid bacteria racemising lactic acid to the D(−) and L(+) isoforms (Johnson et al., 1990; Chou et al., 2003).

7.7 Mozzarella and other pasta filata cheeses

7.7.1 Microstructure of Mozzarella cheese

Mozzarella cheese, traditionally made from buffalo’s milk in Italy, is now made from cow’s milk in many places, including the United States, Australia and New Zealand. The pasta filata process, whereby curd is heated and stretched, imparts desirable functional proper-
ties to the cheese (see reviews by Kindstedt, 1993; Rowney et al., 1999). Mozzarella and other pasta filata-style cheeses are usually consumed for their textural properties, such as stretching, melting, shredding, partial free oil formation and browning, rather than for their flavour.

When freshly manufactured, the cheese contains a large amount of expressible serum which prevents it from being successfully shredded for later use as a food ingredient (Guo & Kindstedt, 1995). Cheese must be aged for a period of about ten days to allow for moisture absorption by the casein matrix before shredding. During this storage time, usually at low temperature, β-casein partially disassociates from the casein matrix due to lessening of the forces of hydrophobic association and becomes the main intact casein in the serum phase. After about seven weeks post-manufacture, the amount of free oil that arises from heating the cheese increases to unacceptable levels and this, coupled with a decrease in apparent viscosity, renders the cheese unsuitable for use in heated food products, such as pizza. Generally, higher amounts of fat will produce more free oil, whereas higher levels of salt will inhibit free oil formation but produce a tougher, more elastic, but less meltable cheese (Everett et al., 2004). This leaves a short window of opportunity for the cheese to be suitable for further processing (Kindstedt, 1995). Much research activity is focused on extending this window of usability.

Mozzarella cheese is classified into four types in the United States: (1) Mozzarella, (2) low-moisture Mozzarella, (3) ‘part-skim’ Mozzarella, and (4) low-moisture ‘part-skim’ Mozzarella (Kindstedt, 1993). All four cheeses should more correctly be termed pizza cheese, as they differ considerably from the composition and manufacturing methods of traditional Mozzarella cheese. Pizza cheese is made using automated and largely enclosed cheese manufacturing equipment in countries such as Australia and New Zealand. This modified process employs dry-salting of the cheese curd, a more economical method than the traditional brining.

7.7.2 Protein microstructure

The porosity of the aggregated fibrous casein micelles in Mozzarella curd decreases during the pasta filata stretching and heating stage, with a gradual loss of micellar identity (Oberg et al., 1993). The heating and stretching of curd induces the protein fibres and serum channels to align in the direction of stretching in Mozzarella (McMahon et al., 1993) and string cheese (Taneya et al., 1992). The protein fibres are smooth after the stretching process, but become rough after cooling in brine, with indentations evident from fat globules and bacterial cells (McMahon et al., 1999). These bacterial cells are located near the interior surface of voids within the protein matrix in Mozzarella cheese, where fat globules would be expected to co-locate (see Fig. 7.4). Evidently globules do not press into the protein matrix when the cheese is hot, presumably as the globules are more easily deformed at higher temperatures.

Serum channels are located in the interstitial protein fibre region, and contain water, residual serum proteins, minerals, bacterial cells and fat globules. During cheese ripening, intact caseins and peptides produced by hydrolysis are transferred to the serum phase (Guo & Kindstedt, 1995; Kindstedt & Guo, 1998; Rowney et al., 2004). This has profound implications for the microstructure of Mozzarella cheese, as discussed below.
A sequence of events takes place during the maturation of Mozzarella cheese that contributes to changes in cheese functionality. Proteins become less aggregated and begin to associate more with the water phase, in other words, becoming more hydrated (McMahon & Oberg, 1998). This protein swelling is enhanced by high salt and low ionic calcium levels, and leads to an increased propensity for melting, presumably as the protein aggregates flow more easily due to a lessening of protein–protein interactions (McMahon & Oberg, 1999; Åshi et al., 2004). The effect is for the protein fibres to absorb moisture, as shown by the

*Fig. 7.4*  Scanning electron micrograph of (a) high fat and (b) low-fat Mozzarella cheese within one week of manufacture, showing cavities in the protein matrix and bacterial cells. Bar = 10 μm. Source: Cooke et al. (1995) Electron-density patterns in low-fat Mozzarella cheeses during refrigerated storage, in *Chemistry of Structure–Function Relationships in Cheese* (eds E.L. Malin & M.H. Tunick), Reproduced by permission of Springer Science and Business Media.
complete loss of expressible serum 10 to 20 days after cheese manufacture (McMahon et al., 1999; Guinee et al., 2002). Salt, therefore, induces a greater water-holding capacity through structural swelling of the protein matrix with concomitant solubilisation of intact caseins, a phenomenon termed peptisation (Guo et al., 1997). Lower levels of calcium will enhance the peptising action of sodium chloride, which is one reason why directly acidified Mozzarella, with less calcium, absorbs more moisture than a cultured cheese (Paulson et al., 1998).

Water in cheese is either tightly bound to the protein matrix and, therefore, unavailable as a solvent, entrapped where it is in close proximity to the protein matrix, or found as bulk water within the serum channels (McMahon et al., 1999). This bulk water can be centrifuged from Mozzarella cheese during the first few days after manufacture as expressible serum. The pasta filata process increases the proportion of bulk water in the cheese, as indicated by water being more mobile as examined by NMR compared to non-pasta filata cheese (Kuo et al., 2003). Bulk water is absorbed by the swelling protein matrix during the maturation period, and the serum channels are reduced in volume (Zisu & Shah, 2005). Heating of Mozzarella results in larger and more numerous serum pockets, caused by increased hydrophobic association of proteins whereby protein–protein interactions are favoured (Pastorino et al., 2002).

Entrapped water increases during cheese ripening whereas the level of bound water remains constant (McMahon et al., 1999). As the total moisture level should be unchanged, particularly if the cheese is sealed in water-impermeable packaging, the level of bulk water must be reduced over time. Therefore, water is transferred from the bulk to the entrapped phase during the maturation of the cheese, confirmed by the measurement of water mobility using NMR (Kuo et al., 2001).

Bound water is closely related to the protein content of cheese, whereas fat is related to the amount of bulk water (McMahon & Oberg, 1999; McMahon et al., 1999). This fat–water relationship initially seems rather peculiar; however, it arises due to fat globules being located within the serum channels. Higher levels of fat means a larger volume of bulk water serum pockets, which are absorbed into the protein matrix over time. Eventually the protein phase will expand and compress the fat globules located within the serum pockets.

7.7.3 Compositional parameters

Calcium and pH

Calcium and pH are interdependent factors that are now understood to be the major determinants of microstructure in Mozzarella cheese (Guinee et al., 2002). The pH dictates the amount of calcium that is partitioned into the curd structure at the point of draining of the whey, and also the ratio of soluble to insoluble calcium in the final cheese. Insoluble calcium, which is bound to protein in cheese directly, contributes to cheese protein microstructure (Kindstedt & Guo, 1998) as the protein fibres are more closely associated through calcium phosphate bridging. At lower pH the proportion of ionic soluble calcium rises which will assist in shielding the charges on the proteins, thus allowing association of the proteins through hydrophobic interaction. These two types of interaction are strongly pH-dependent and produce different types of cheese texture (see section 7.3.1).

Calcium has a large effect on Mozzarella cheese structure and functionality (McMahon & Oberg, 1999). Increased amounts of soluble calcium will enhance protein–protein inter-
actions, and thus decrease the association of protein with the water phase, to the detriment of meltability (Joshi et al., 2003). Lower levels of calcium result in decreased numbers of serum pockets and less expressible serum, but increased meltability and decreased firmness. Above pH 5.4, the average for this cheese, the melted structure of the cheese becomes more fibrous, whereas below pH 5 the structure consists of incompletely fused particles with poor meltability (Kindstedt et al., 2001). Cheese meltability and the proportion of soluble calcium are reversible over the relatively wide pH range of 4.8 to 6.5 (Ge et al., 2002).

Good curd flow requires sufficient casein hydration to promote interaction with the water phase. Higher levels of soluble calcium improve protein–protein interactions, reduce protein hydration, promote curd syneresis and, therefore, reduce meltability. The enhanced protein interaction is evident in low-moisture ‘part-skim’ Mozzarella where calcium induces the area occupied by the protein matrix to shrink, as observed by SEM (Pastorino et al., 2003d). This enhanced protein–protein interaction, with concomitant compacting of the protein network, may be due to increased hydrophobic association of proteins through calcium shielding of the casein charges. By this same shielding mechanism, soluble calcium reduces the extent of protein–water interactions (i.e. solvation). The proportion of soluble calcium increases as pH decreases, suggesting that at low pH the extent of protein solvation and swelling is depressed. Conversely, at high pH the protein matrix will swell and absorb more water, and serum channels will decrease in size. Depletion of calcium causes the protein matrix to become more swollen one day after manufacture (Guinee et al., 2002), indicating enhanced casein solvation. This may also facilitate increased levels of proteolysis (Joshi et al., 2003). Therefore, the level of calcium is a compromise between the desired functional properties (see Fig. 7.2a).

Unsalted directly acidified Mozzarella has poorer melt properties and a more open protein microstructure compared to salted cheese (Paulson et al., 1998). Directly acidified Mozzarella curd at pH 5.6 has good stretching properties (Kosikowski & Mistry, 1997a) as more calcium has been lost into the whey, despite the increased proportion of calcium bound to the protein matrix at the higher pH. A lesser amount of total calcium appears to be necessary to promote meltability and flow behaviour (Kindstedt & Guo, 1998).

Salt

Salt can be incorporated into cheese by direct addition of dry salt to the milled curd pieces, immersing curd blocks in cold brine (usually 8–23 g NaCl 100 g⁻¹ water), or a combination of these two processes. As Mozzarella cheese ages, the amount of expressible serum reduces to zero after about 10–20 days (Guinee et al., 1995; Everett et al., 2004). This can be shown by the closing up of voids within the protein matrix in Mozzarella cheese over time (see Figs 7.5a–d).

The rate of reduction in expressible serum over time is slower for directly acidified cheese compared to cultured cheese (Paulson et al., 1998). Increasing salt levels reduces the amount of expressible serum (Rowney et al., 2004), and this is understood to be caused by increased protein swelling by absorption of cheese moisture (Guo et al., 1997). Unsalted Mozzarella cheese has a higher level of expressible serum than salted cheese. Mozzarella cheese with no salt has a more open protein matrix with larger serum pockets compared to a salted cheese (see Fig. 7.6). Unsalted cheese with higher amounts of expressible serum will swell over time, but much more slowly than for salted cheese (Guo et al., 1997). Cooling the cheese
Fig. 7.5 Scanning electron micrograph of part-skim Mozzarella cheese after: (a) 1 day; (b) 7 days; (c) 14 days; (d) 21 days storage at 4°C, showing the closing up of the serum channels. Bar = 20 μm. Source: McMahon et al. (1999) Journal of Dairy Science, 82, 1361–1369. Reproduced by permission of the American Dairy Science Association and Journal of Dairy Science.
in a brine bath at a lower temperature results in less free oil and more expressible serum, presumably as the hydrophobic interaction responsible for protein interactions is reduced at the lower temperature (Rowney et al., 2004).

Brining of pasta filata Ragusano cheese causes a loss of moisture at the surface of the curd block with a corresponding shrinkage of the surface protein matrix with decreased protein porosity, thus creating an impediment to water movement into and out of the curd block (Melilli et al., 2003). Higher sodium chloride concentration and longer brining time also reduce protein porosity at the surface layer (Melilli et al., 2005).

Salt reduces the amount of free oil in aged Mozzarella (Everett et al., 2004), possibly by increasing the emulsifying ability of caseins (Kindstedt et al., 1992), thereby impairing meltability as there is less free oil to lubricate the protein matrix. With increasing salt, the serum pockets are reduced in size, apparent viscosity increases, but there is no effect on fat globule size or shape in Mozzarella cheese at a point one day after manufacture (Rowney et al., 2004).

Fig. 7.6 Scanning electron micrograph of (a) unsalted and (b) salted non-fat Mozzarella at 7 days post-manufacture. Salted cheese was translucent and had no expressible serum. Bar = 10 μm. Source: Paulson et al. (1998) Journal of Dairy Science, 81, 2053–2064. Reproduced by permission of the American Dairy Science Association and Journal of Dairy Science.
For one-day-old Mozzarella cheese, the increased extent of protein swelling induced by a higher salt content does not appear to impact upon free oil formation (Rowney et al., 2004). The fat globules may be squeezed by the swelling protein matrix immediately after manufacture, but the rate of fat globule coalescence and rupture must be a much slower process, therefore having no impact on free oil formation. Cold brining is more effective at causing protein swelling compared to a combination of dry and hot-brining, perhaps due to the greater osmotic pressure difference between the surface and the centre of the cheese in the cold brine bath, resulting in fat globules being squeezed by the protein matrix and more free oil being formed.

In contrast to the effect in one-day-old Mozzarella, increasing the salt level in aged Mozzarella cheese decreases free oil formation upon heating (Everett et al., 2004). Fat globules are increasingly embedded into the expanding casein matrix over time and are squeezed by the pressure imposed by the swelling casein fibres. The matrix is also undergoing proteolysis and softening over time, thus allowing fat globules to be pressed into the protein phase.

Higher levels of salt inhibit proteolysis and the cheese will be firmer, thus potentially increasing the propensity for fat globule rupture as the protein matrix expands over time. However, the age-induced softening of the casein matrix far outweighs the relatively small amount of salt-induced hardening, so increasing the level of salt will simply press and trap the fat globules into the soft protein matrix without globule rupture, and with no increase in free oil.

At low salt concentration, the lesser amount of casein swelling will not be sufficient to trap the globules in the soft casein matrix. In this situation, the increase in free oil may be due to a higher level of proteolysis-induced rupture of the globule membrane layer as the globules are not as constrained by the casein matrix. Thus, protein swelling and proteolysis of the casein matrix and the fat globule membrane are two linked factors that impact on free oil formation (Everett et al., 2004). By contrast, salt has no effect on free oil formation at one day after manufacture as very little proteolysis has taken place to induce sufficient softening of the cheese protein matrix or rupture of the membrane, and protein swelling has not reached a sufficient level to apply pressure to the fat globules (Rowney et al., 2004).

Exopolysaccharides

Exopolysaccharide-producing starter cultures can be added to milk prior to cheese manufacture to increase the ratio of water to protein in low-fat Mozzarella cheese, thus improving meltability (McMahon Øberg, 1998). These exopolysaccharide-producing bacterial cells are located within the serum channels (Bhaskaracharya & Shah, 2000). Increasing the water content is desirable to reduce the cost of the cheese (it is more profitable to sell water), but will result in a cheese that cannot be shredded satisfactorily. The exopolysaccharides are able to bind water that otherwise would be available as free solvent in the serum channels within the cheese microstructure.

Fat replacers

Carbohydrate- and protein-based fat replacers can also be used to retain more moisture in the cheese and improve meltability (Zisu & Shah, 2005). These may include whey proteins, cellulose, starches and other polysaccharides. Maltodextrin and modified potato starch replac-
ers will open up the serum channels from 5–10 μm in skimmed milk cheese to 10–50 μm, thus allowing more free moisture to be retained within the larger channels (Bhaskaracharya & Shah, 2001). Larger size fat replacer particles are more effective at allowing for greater incorporation of moisture in cheese (McMahon & Oberg, 1998). Denatured whey proteins have also been employed to open up the protein matrix to create larger serum channels; however, this also results in a weaker curd and increased protein losses into the whey, and a tough, fibrous texture with poor melting properties (Mead & Roupas, 2001). Figure 7.7 shows a protein-based fat mimetic particle located within an opening in the protein fibrous matrix.

**7.7.4 Fat globule microstructure and meltability**

The state of fat in four types of cheese is shown in Fig. 7.8. Stretching of Mozzarella curd results in fat forming into pools that are elongated along the void spaces between the protein fibres (Fig. 7.8d). By comparison, Cream cheese (Fig. 7.8a), Cheddar (Fig. 7.8b) and Processed cheese (Fig. 7.8c) do not undergo curd stretching to the same degree, so the fat particles are more spherical and are more likely to be retained within an emulsified globule structure. Processed cheese contains highly spherical and emulsified fat globules (see Chapter 8).

Fat globules in low-fat Mozzarella exist within irregular-sized protein cavities of 10–20 μm in size, as examined by SEM, and aggregate into clusters and become more spherical during the maturation period (Cooke et al., 1995). Cavities within the casein matrix of Mozzarella cheese increase in size and become more elongated during maturation (Poduval & Mistry, 1999; Imm et al., 2003; Ishi et al., 2004), indicating that fat globules are pushed
together and thus aggregate. There is evidence that the individual fat globules decrease in size during the maturation period (Guinee et al., 2002). Although the cavities containing fat globules become more elongated over time, this does not necessarily mean that the fat globules are more stretched (Everett et al., 2004). Pools of free oil are, however, likely to be elongated due to the absence of an emulsifying membrane.

The heating and stretching stage of Mozzarella cheese manufacture (the pasta filata process) induces some changes in the fat globule microstructure. The extent of curd stretching is the major factor that induces elongation of fat areas, rather than the rate of stretching or the rate or extent of compression. Curd that is stretched and heated to greater extents produces more free oil (Rowney et al., 2003a), so presumably elongated fat areas represent pools of free oil. Heating may induce coalescence and rupture of fat globules with increased free oil formation.

Fat globules in Mozzarella, and in Provolone, another pasta filata cheese, are clustered as a consequence of the heating and stretching process (Kalb, 1977). These fat globules interrupt the protein network and contribute to a softening of the matrix. Higher-fat Mozzarella contains larger and more numerous void spaces within the protein matrix compared to lower-fat cheese (see Fig. 7.4). The globules can also limit the extent of deformation of the curd and prevent further compaction, thus moisture is more difficult to retain in a low-fat cheese as there is less volume of void space to contain the water phase (McMahon et al., 2003).

**Fig. 7.8** Confocal laser scanning micrograph of different cheeses: (a) Cream cheese; (b) Cheddar cheese, curd junction shown by arrow; (c) Processed cheese; (d) Mozzarella cheese at 28 days post-manufacture. Fat is shown by the lighter shade of grey. Bar = 25 μm. Source: Auty et al. (2001) *Journal of Dairy Research, 68*, 417–427. Reproduced by permission of Cambridge University Press and *Journal of Dairy Research*. **Microstructure of Natural Cheeses** 193
There is a negligible amount of expressible serum in salted, directly acidified non-fat Mozzarella cheese after manufacture (Paulson et al., 1998), mostly likely due to the protein matrix becoming more compact as fat globules are largely absent and, therefore, not able to open up the casein structure to form serum channels. Expressible serum in full-fat Mozzarella is higher for cheese containing more fat, leading to the conclusion that fat globules serve to open up the protein matrix and allow water to accumulate in serum channels or pockets (McMahon et al., 1999). This is a similar function played by protein- and carbohydrate-based fat substitutes (see above).

Factors that affect Mozzarella cheese meltability include pH, calcium distribution, age of cheese, fat, protein, salt and moisture, although not all of these are of equal importance. Fat and unbound bulk water are reported to be important factors (Tunick et al., 1993), although it has been stated that if the pH, calcium, moisture and salt are correct, then fat is not as important for inducing good melt properties (McMahon & Oberg, 1998). Meltability of low-fat Mozzarella cheese improves with longer maturing time and higher fat and water contents (Tunick et al., 1993). Higher amounts of free oil also increase the meltability of Mozzarella (McMahon et al., 1999). The protein matrix has also been argued to be the most important factor influencing meltability, with fat playing a lubricating role (McMahon & Oberg, 1998; Guinee et al., 1999). Fat may also serve to open up the protein matrix to limit protein–protein interactions that hinder melting (McMahon & Oberg, 1999). Meltability of Mozzarella increases over time due to a transfer of water from the bulk phase to the protein matrix, thereby increasing protein–water and decreasing protein–protein interactions (McMahon et al., 1999).

Reduced-fat Mozzarella cheese is firmer, less meltable, less white and more translucent due to less light scattering by the reduced number of fat globules, and shows less free oil upon heating (Rudan et al., 1999). The reduced meltability is due to enhanced protein–protein interactions, at the expense of protein–water interactions which facilitate melting (McMahon et al., 1999). Less fat means a lower volume of bulk water phase, hence less entrapped water that is in close proximity to the protein phase.

### 7.7.5 Homogenisation

Homogenisation produces smaller fat globules that are coated with intact casein micelles and micelle fragments. These are capable of interacting with the surrounding casein matrix, thus contributing directly to protein structure. Homogenisation of the milk for Mozzarella cheese manufacture yields a more porous structure as measured by TEM (Tunick et al., 1997). Mozzarella containing fat globules coated with casein is firmer and produces less free oil, indicating that the globules integrate into the protein matrix and are less easily ruptured (Rowney et al., 2003b). Larger fat globules and pools of free oil are more evident in cheese manufactured from non-homogenised milk (see Fig. 7.9).

Increasing the temperature of homogenisation produces smaller fat globules, presumably as liquid globules at a higher temperature are more easily broken apart under turbulent conditions (Tunick et al., 2000). Homogenisation of milk or cream increases the whiteness of reduced-fat Mozzarella due to increased light scattering, and the resultant smaller globules are more dispersed in the cheese (Rudan et al., 1998a).
High-pressure homogenisation using a Microfluidizer™ creates a very small globule size with a narrow particle size distribution. This type of homogenisation is characterised by passage of a coarse emulsion, such as milk or cream, through narrow bores under high velocity, which collide and break apart fat globules by cavitation and turbulence. Mozzarella cheese produced from milk homogenised in this instrument produces smaller fat globules that aggregate in the cheese during maturation (Tunick et al., 2000), in a similar fashion to valve homogenisation, that operates at a lower pressure.

Fig. 7.9  Confocal laser scanning micrograph of Mozzarella made from (a) homogenised milk where small fat globules shown by arrow, and (b) non-homogenised milk. Arrow A shows a pool of free fat; arrow B shows a small emulsified fat globule; fat is shown by a lighter shade of grey. Bar = 10 μm. Source: Rowney et al. (2003b) *Journal of Dairy Science*, 86, 712–718. Reproduced by permission of the American Dairy Science Association and *Journal of Dairy Science*.
7.7.6 Recombined milk

Recombined milk can be manufactured with different melting point fractions of milk fat by homogenisation with skimmed milk. Fat globule size and shape in Mozzarella cheese is independent of the melting point of the fat, but a lower melting point fraction exhibits more free oil formation and lower apparent viscosity upon heating (Rowney et al., 2003b). Perhaps the lower melting point fraction, with lower viscosity, is able to permeate through the protein matrix after fat globule rupture and contribute to the improved melting properties. Higher-melting-point fraction fat, by comparison, may not be sufficiently dispersed within the protein network to increase meltability.

Buttermilk is a byproduct of the butter manufacturing process, containing a high proportion of phospholipids derived from the native milk fat globule membrane. As such, buttermilk and buttermilk powder have good emulsifying properties and may have efficacy in producing recombined milk for cheese manufacture. The enzymes within the milk fat globule membrane layer may be important for the development of cheese flavour. The fat globules in this case may be more highly emulsified and less likely to form free oil upon heating. Many studies, however, have focused on simply adding buttermilk directly to milk before cheese manufacture; thus it is not conclusively known where the buttermilk components are located in the finished cheese microstructure. The direct addition of buttermilk reduces free oil and meltability, and softens reduced-fat Mozzarella, perhaps a result of the more spongy casein matrix that has no evident orientation of protein fibres (Poduval & Mistry, 1999).

7.7.7 Light scattering by cheese constituents

Colloidal material of similar size to the wavelength of visible light within cheese, such as protein aggregates, water droplets or small fat globules, will scatter light and the cheese will appear whiter and more opaque. The lower amount of emulsified colloidal fat in reduced-fat Mozzarella cheese scatters light to a lesser extent compared to cheese with more fat globules.

Sodium chloride increases the extent of casein micelle solvation, in contrast to ionic calcium which reduces solvation (Creamer, 1985). The association between casein molecules is lessened at higher sodium chloride levels, promoting association of casein with water and thereby increasing protein solvation. The larger serum channels in unsalted cheese are ~10 μm in width, yielding a more heterogeneous microstructure (McMahon & Oberg, 1998). Salted Mozzarella contains smaller protein aggregates with a more compact protein matrix compared to the more open structure of unsalted cheese (Paulson et al., 1998). These authors observed that salted cheese was more translucent, whereas unsalted cheese was more opaque due to light scattering by the serum pockets.

The effect of salt on opacity can also be seen in brined cheeses. Increasing the level of salt applied to Gaziantep, a semi-hard unfermented Turkish cheese, yields a reduction in the level of whiteness (Kaya, 2002), possibly due to a decrease in the size of serum pockets to a size that scatters light more effectively.

A reduction in the amount of expressible serum produces a less-white cheese due to reduced light scattering by the smaller-sized serum pockets (Joshi et al., 2003). Pre-acidi-
fication of milk to lower pH values reduces the volume of expressible serum and decreases the level of whiteness of unmelted low-fat Mozzarella cheese (Metzger et al., 2001). This is presumably due to protein solubilisation and swelling from the reduced level of soluble calcium, which decreases the number and size of serum pockets.

Mozzarella becomes more opaque when heated, and this process is thermoreversible after cooling and reheating the cheese (Paulson et al., 1998). The reversibility may be due to reformulation of water pockets or protein aggregates when the cheese is reheated. The transition temperature is about 20°C for unsalted cheese and 40°C for salted (Dave et al., 2001). This heat-induced phenomenon suggests that structural units are forming at higher temperatures of similar size to the wavelength of scattered light. These structural units include protein aggregates formed through the process of hydrophobic interaction, and serum pockets that appear at higher temperatures as the protein aggregates grow in size and are spaced further apart (Pastorino et al., 2002). The protein aggregates presumably disassociate when the temperature is reduced, to account for the thermoreversibility. Decreased solubility of β-casein and subsequent formation of aggregates of this casein that scatter light at higher temperatures have been associated with increased whiteness when Mozzarella cheese is heated (Joshi et al., 2003). Reduced-fat Mozzarella that has been heated and cooled shows a thermoreversible greenish tint due to less scattering of light (Fife et al., 1996), presumably due to the coloured riboflavin molecule.

7.8 Cottage Cheeses

Cottage cheese is manufactured from skimmed milk by coagulation with lactic acid bacteria and a small amount of a coagulant to the isoelectric pH (4.6) of casein. This produces a skimmed curd that is cooked at 50°C, cooled and dressed with salted cream. The microstructure of Cottage cheese during the manufacturing process is shown in Fig. 7.10.

The cooking process would intuitively suggest that the curd particles have a skin of dehydrated protein. The outside of the curd particle has been shown to have a denser protein matrix (Kalb, 1979b), but not so thick as to prevent whey drainage from within the curd particle (Glaser et al., 1979; Kalb, 1993). The casein micelle size increases from 90 nm to around 210 nm after the cooking stage (Glaser et al., 1979), fusing to form a protein matrix that is similar to yoghurt but denser (Kalb, 1979a). Filaments from lactic acid bacteria have been observed, attached to the protein matrix (Kalb, 1980).

Fat globules are shown to be embedded into the protein matrix of Chhana, an acid-coagulated soft Cottage cheese manufactured in India (Adhikari et al., 1992). This type of cheese requires heating to above 90°C, which results in the denaturation of β-lactoglobulin and subsequent complexing with κ-casein on the surface of the casein micelle. Kariesh, an acid-coagulated skimmed milk cheese from Egypt, exhibits extensive aggregation and fusion of protein after six weeks of storage in 14 g NaCl 100 g −1 brine, with a similar protein microstructure to yoghurt (Abd El-Salam Omar, 1985). Olomouc cheese cakes, a fresh acid skimmed milk curd manufactured in Moravia, consists of chains of aggregated casein particles which fuse as the cheese ages (Kalb Palo, 1987).
Fig. 7.10  Scanning electron micrograph of cultured Cottage cheese during manufacture: (a) early gel; (b) at cutting; (c) end of healing; (d) during cooking at 40°C; (e) during cooking at 50°C; (f) end of cooking at 55°C. Bar = 1 μm. Source: Glaser et al. (1980) *Journal of Dairy Science*, 63, 37–48. Reproduced by permission of the American Dairy Science Association and *Journal of Dairy Science*. 
7.9 Cream cheeses

Cream cheeses (including Neufchatel) are manufactured from milk standardised to a higher fat content, then set to a gel at pH 4.6 by the action of lactic acid cultures. A coagulant can be added to aid in the coagulation process. Baker’s cheese is a variety of Cream cheese made from skimmed milk. Cream cheese contains a uniform protein matrix, sometimes aggregated into 20-μm clusters, and containing small fat globules (Kalb, 1985). Protein aggregates up to 1000 μm in size containing casein and heat-denatured whey proteins have been observed in Cream cheese (Sainani et al., 2004). Figure 7.11 shows a compacted protein aggregate that may contribute to the gritty mouth-feel of some Cream cheeses. As the fat content of Cream cheese increases, the size of the fat globules increases and more clustering of globules has been observed. At low salt concentration, increasing the level of salt reduces the size of fat globules; however, at higher salt concentrations the size increases, possibly due to increased fat globule clustering (Wendin et al., 2000).

Homogenisation of milk prior to double Cream cheese manufacture will allow the fat globules to interact more strongly with the protein matrix, and produces a more elastic cheese with less free oil formation. Fat globules are smaller and more dispersed compared to cheese made from unhomogenised milk, although very high pressures yield a brittle cheese with fat globules reaggregating and partially rupturing (Sanchez et al., 1994).

7.10 Mould-ripened cheeses

7.10.1 Background

Mould (white or blue) can be introduced into the cheese manufacturing process to add complexity to the texture and flavour of the matured product. Some types of cheese, such
as Camembert and Brie, are characterised by a surface flora layer that contributes to the maturation of the cheese. Such cheeses are often considered to mature from the outside in due to contributions by the external flora to texture-producing biochemical reactions.

Varieties of cheese, such as Stilton, Roquefort, Gorgonzola, and blue cheeses in general, contain a mould in the interior of the cheese that softens the texture and produces the characteristic pungent aroma and taste. During the manufacturing process, the freshly made blocks of cheeses are pierced with rods to allow oxygen to enter into the curd to facilitate mould growth. In addition, microscopic channels exist within the protein matrix of blue cheeses in the early stages of maturation, closing up as the cheese matures. These channels are less than 1 μm in size and contain much of the mould growth (Washam et al., 1979).

7.10.2 Camembert cheese

Camembert cheese is characterised by a complex multilayer growth of surface flora during the maturation process. In the initial few days of maturation, yeasts appear on the surface. A 200–400 μm layer of *Geotrichum candidum* then colonises the surface of the cheese, followed within three weeks by a third layer, *Penicillium camemberti* (previously known as *P. caseicolum*, *P. caseicola*, *P. candidum* and *P. album*), 450–600 μm thick (Rousseau, 1984). Later on in the maturation process, micrococci and corynebacteria appear on the surface.

The presence of surface mould and bacteria on Camembert cheese produces a complex cascade of reactions that soften the cheese over time (Karahadian & Lindsay, 1987). The surface flora produce ammonia from the catabolism of proteins after the supply of lactate is exhausted. Production of ammonia and carbon dioxide raises the pH of the exterior layers of the cheese, and a calcium ion gradient is created as a consequence of the precipitation of calcium phosphate at the surface (Brooker, 1987). Calcium ions then migrate from the interior outwards, and this contributes to the softening of the cheese by reducing the extent of casein crosslinking. The overall increase in pH in Camembert during the maturation period increases the rate of plasmin-induced proteolysis that also contributes to cheese softening. Thus, the maturation process is driven by the development of pH and calcium gradients in the cheese, rather than putative migration of enzymes from the surface of the cheese to the interior, an event that is unlikely to occur (Noomen, 1983).

The manufacturing process has some impact upon the protein structure in Camembert cheese. An increase in the acidity of curd during Camembert manufacture or an increase in the storage temperature of the cheese within the first 20 h results in a coarsening of the protein matrix (Knoop & Peters, 1972). Cheese manufacture with reconstituted milk powder yields a more porous protein structure with poor aggregation of casein particles (Peters & Knoop, 1974). The use of homogenised milk in cheesemaking results in partially ruptured fat globules embedded in the curd coagulum, whereas partially homogenised milk enhances fat globule clustering (Knoop & Peters, 1972).

Fat globules can both interact with the surrounding protein matrix or serve as protein matrix breakers. Larger fat globules in Camembert cheese tend to be more aggregated and are surrounded by thick protein strands, whereas smaller globules are enveloped by thinner protein strands (Michalski et al., 2003). The higher total surface area of smaller fat globules may allow greater water binding and moisture retention in the cheese. In addition, small
globules are associated with small voids in the protein matrix that more effectively trap water, resulting in a higher-moisture cheese.

7.11 Conclusions

Natural fermented cheeses quite clearly have an immensely complex microstructure. Many techniques have been developed to probe cheese microstructure, including direct methods of observation such as TEM, SEM, CLSM and other light microscopy, and other more indirect methods such as laser light particle size analysis, rheology, and measurement of free oil formation and expressible serum. An understanding of microstructure provides knowledge of how texture develops in cheese during ripening, and allows changes to the manufacturing process with more predictable results for the quality of the finished cheese.

The state of water (bound, entrapped or bulk), the state of fat (globular or pools trapped within voids in the protein matrix), the extent of protein association (through calcium phosphate bonds or hydrophobic interaction), the pH, and the mineral and ionic balance (especially sodium chloride and calcium) all play important roles in developing cheese microstructure. An additional layer of complexity is the fermentation process, which produces biochemical reactions in cheese that add to the inhomogeneity of the microstructure. The transformation of milk – an aqueous suspension of fat globules, casein micelles, lactose, whey proteins and minerals – into the partially dehydrated dairy product with constituents interacting in an intricate fashion, produces the tremendous variety of cheeses that we know and enjoy today.

References


8 Processed Cheese and Cheese Analogues

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8.1 Introduction

Processed cheese, a dairy product usually made from natural cheeses, emulsifying salts and, eventually, other dairy or non-dairy ingredients has a history dating back to the early part of the 20th century. In search of new strategies for a sophisticated manufacture of cheese with an extended shelf-life, bypassing traditional methods such as air-drying or smoking, Jan Hendrikzoon established a heat-treatment procedure for canned Gouda cheese in 1899 (Kammerlehner, 2003). As pasteurisation led to insufficient results in the case of Emmental cheese, the Swiss researchers Walter Gerber and Fritz Stettler closely followed traditional fondue preparation and, after some experiments with tartaric acid, added sodium citrate prior to heat processing of the comminuted raw material to achieve a stable but modified product. Later on, it was noticed that other salts may also be used and, due to their function, these were referred to as ‘melting salts’ or ‘emulsifiers’ (Kammerlehner, 2003). An up-to-date definition of processed cheese is

‘a cheese-based product, made by blending mainly hard and semi-hard, natural cheeses, coagulated by rennet, at different stages of maturation, with surface-active salts to generate a smooth and homogeneous blend by the action of heat and shear’.

The image of processed cheese has changed significantly over the years, as it developed from a product enhancing the marketing value of cheeses with unsatisfactory quality to a product which is tailor-made with respect to its functional properties (Walstra et al., 1999). Such functional properties include meltability, flowability, stretchability, or susceptibility to oiling off, which largely depend on the application or anticipated use (Guinee et al., 2000) and are responsible for the large increase in consumption (Sörensen, 1997).

Processed cheese varieties are usually distinguished by texture – comprising a broad range from firm and sliceable products usually distributed in blocks, slices or in grated form, to creamy processed cheese spreads – and also by composition. In its narrow sense, this is reflected by regional legislation; for example, in Germany, > 50 g 100 g⁻¹ of the total solids of processed cheese must be from cheese, and the addition of other dairy ingredients such as milk fat, milk protein fractions and whey powder, and the use of stabilisers is allowed (http://bundesrecht.juris.de/bundesrecht/k_sev/index.html). In processed cheese preparations, the addition of non-dairy ingredients such as flavours, natural and artificial colourants, meat products, spices and herbs, and functional ingredients is permitted.
The use and consumption of processed cheese products varies in regional markets. The industry for processed cheese products reached a global value of ~€7.2 billion in 2001, with a total sales volume of 1.4 billion kg (http://www.market-research-report.com/datamonitor/DMCM0353.htm). Between 1999 and 2003, annual production in the 15 member states of the European Union (EU) varied between 525 000 tonnes and 545 000 tonnes. About one-third of this production comes from Germany, ~25% from France, and Belgium accounts for ~8% (ZMP; Zentrale Markt- und Preisberichtsstelle in Berlin, personal communication). In central European countries in 2003, the proportion of cheese produced as processed cheese was 8.6% of 1941 000 tonnes (Germany), 12.1% of 160 400 tonnes (Switzerland), and 19.7% of 151 400 tonnes (Austria) (http://www.milch-markt.de/de/index.html; http://www.kos-net.ch/pdf/geschaeftsbericht_2002_d.pdf). The German annual per capita consumption was 21.8 kg cheese, comprising 1.6 kg of processed cheese in 2003 (http://www.milch-makt.de/de/milchactuell/branchenzahlen_actuell/milchaktuell_zahlen_daten.html). The corresponding consumption figures for cheese and processed cheese in the United Kingdom in 2003 were 5.2 kg and 0.62 kg per head per year, respectively (http://www.statistics.defra.gov.uk/esg/publications/efs/2003/chapter5.pdf).

During the manufacture of natural cheeses noticeable changes in the structure of the product are evident, and this fact results in the development of new structures in processed cheeses, which have frequently been described as concentrated emulsions of individual, spherical fat droplets of a size of 1–10 μm, embedded in a hydrated protein matrix (Taneya et al., 1980; Heertje et al., 1981; Lee et al., 1981; Kaláb et al., 1987; Klostermeyer & Büchheim, 1988; Berger et al., 1989; Savello et al., 1989; Auty et al., 2001; Schäffer et al., 2001). The fat globules decrease in size as emulsification intensity increases (Carić et al., 1985), and as processing conditions become more vigorous (Kaláb et al., 1987). Paracaseinate-coated fat globules attach to casein strands, thus contributing to the continuity of the matrix (Guinee et al., 2004). The microstructure of processed cheese and cheese analogues has frequently been visualised using optical microscopy (Kaláb et al., 1987; Awad et al., 2002), electron microscopy (Heertje et al., 1981; Lee et al., 1981; Kaláb et al., 1987; Savello et al., 1989; Awad et al., 2002; Abou El-Nour, 2003; Lee et al., 2003; Hennelly et al., 2005), and confocal laser scanning microscopy (Auty et al., 2001; Lee et al., 2003).

Cheese analogues are processed cheese substitutes solely made on the basis of dairy ingredients, or products in which dairy components are partly or entirely replaced by non-dairy ingredients, mainly of vegetable origin (Shaw, 1984). The main market for cheese analogues is in the United States, with an annual production of ~300 000 tonnes (Guinee, 2002b), whereas the cheese analogue market in Europe is almost non-existent (Hoogenkamp, 1996). The sales increase of cheese analogues closely reflects the increase in sales of convenience food, with a number of products being created specifically for this purpose (Anon., 1999). Cheese analogues are cost-effective to produce due to the incorporation of cheaper ingredients and the simple manufacturing technology (Eymer & Pangborn, 1988), while nutritional demands by the consumer (regarding, for example, fat content, fatty acid composition or cholesterol) and demands from the industry regarding further processing of cheese analogues can be more easily fulfilled than in conventional processed cheese.
8.2 Processed cheese technology

8.2.1 Gross composition and ingredients

Natural cheese accounts for at least 51 g 100 g\(^{-1}\) and up to 95–98 g 100 g\(^{-1}\) of the composition in processed cheese preparations. Cheese as a raw material affects the character of the processed product in a significant way; the protein content and, especially, the amount of intact casein affect microstructure, rheology and texture properties after processing (Wolfschoon-Pombo, 2002). Hence, even long-term matured cheese varieties such as Emmental or Cheddar, with a ratio of soluble nitrogen (SN) to total nitrogen (TN) of \(\sim 0.15–0.25\) (Upadhyay et al., 2004), result in processed cheeses with a firm, long body and good sliceability (Meyer, 1973). Cheeses with a more intense proteolysis (SN/TN > 0.40) are used predominantly for cheese spreads.

Other dairy ingredients include milk fat (mainly butter or anhydrous milk fat) for standardisation and functional modification, milk proteins (e.g. caseins, caseinates or whey proteins) for texture modification, and lactose (whey powder) as a filler. In a comparative study, however, Muir et al. (1997) showed that the fat content mainly determines sensory texture, whereas flavour remains almost unaffected. Apart from the emulsifying salts, the non-dairy ingredients comprise flavours and flavour enhancers, acidifying and sweetening agents, colourants and preservatives. In line with some compositional specifications, these materials have recently been reviewed by Guinee et al. (2004).

With regard to cheese analogues and the origin of their ingredients, Shaw (1984) distinguished three major groups, with the second group being economically the most important.

(1) In dairy-based cheese analogues, milk fat and milk proteins, mainly casein, caseinates, or blends thereof, are used. Numerous studies have been carried out to investigate the influence of the base ingredients, especially of the protein fraction, on the functional properties of cheese analogues (Chen et al., 1979; Fleming et al., 1985; Cavalier-Salou & Cheftel, 1991; Abou El-Nour et al., 1996; Mleko & Foegeding, 2000).

(2) In cheese analogues with dairy components partly replaced, it is mainly the milk fat which is substituted by vegetable oils and, frequently, this replacement is justified by functional benefits or by health claims; however, it may simply be cheaper (Keane & Glaeser, 1990; Bachmann, 2001). A number of studies conducted using vegetable oils, such as soy bean oil, peanut oil, palm kernel oil, corn oil, or coconut oil, have been reported (Chen et al., 1979; Marshall, 1990; Lobato-Calleros et al., 1997, 1998; Jaros et al., 2001). It is a common result of these studies that the effect of the origin of the fat can be related to its physicochemical properties which, in turn, depend on the fatty acid composition.

(3) In non-dairy cheese analogues, both the protein and the fat phase are of vegetable origin (Guirguis et al., 1985; Santos et al., 1989; Kim et al., 1992; Verma et al., 2005). These products are, however, commercially unimportant, as are cheese analogues wherein protein or fat (Kiely et al., 1991; Mounsey & O’Riordan, 1999; Muir et al., 1999) is completely replaced by other ingredients.
8.2.2  Emulsifying salts

Among the most important ingredients used in processed cheese manufacture are emulsifying salts which, depending on the nature of the salt, are added in an amount ranging from 1 to 3 g 100 g⁻¹. The salts are incorporated to prevent oiling off and moisture exudation during manufacture and cooling, as they enhance protein hydration and the emulsification of free fat. In combination with the supply of heat and the action of shearing these salts which, in the narrow sense, cannot be used for the preparation of oil-in-water or water-in-oil emulsions, convert insoluble para-casein into hydrated sodium para-caseinate, showing a much higher water-binding capacity (Carić et al., 1985). Para-caseinate emulsifies the dispersed oil droplets during processing and contributes to the formation of a stable oil-in-water emulsion by reducing the free interfacial energy of the fat phase (Guinee, 2002b). Additional effects, which are caused by the addition of the emulsifying salts, are calcium masking (or sequestration), hydration and swelling of para-casein, pH increase and buffering, stabilization of the oil-in-water emulsion, and structure formation (Cavalier-Salou & Cheftel, 1991; Carić & Kaláb, 1993; Marchesseau et al., 1997). Finally, the addition of the salts results in a smooth and homogeneous processed cheese mass.

It is generally accepted that the most important function of the emulsifying salts is the masking of calcium (Fig. 8.1). Mainly the sodium salts of phosphoric acid and citric acid, including sodium citrates, sodium ortho-, pyro- and polyphosphates as well as various phosphate blends, are used; hence this masking is based on the exchange of divalent Ca²⁺ ions attached to the casein for the monovalent Na⁺ of the emulsifying salt. This particular ion exchange reduces the extent of calcium bridging that crosslinks and aggregates the para-casein molecules and disrupts the structural integrity of the casein. The calcium masking results in a partial hydration of the insoluble para-casein, thus leading to a conversion into sodium phosphate para-caseinate (Carić et al., 1985; Marchesseau et al., 1997). As a consequence, the solubilised caseinate is able to interact with the water and oil interfaces in the processed cheese mass.

Fig. 8.1 Effectivity of emulsifying salts using Na⁺HPO₄ as an example. Adapted from Berger et al. (1989).
Applying an appropriate mixture of emulsifying salts causes an increase in pH of the cheese blend from 5.0–5.5 up to 5.6–5.9 and, furthermore, contributes to pH stabilisation due to its buffering capacity (Gupta et al., 1984; Cavalier-Salou & Cheftel, 1991; Marchesseau et al., 1997). Due to this particular pH increase, the calcium-masking ability of the emulsifying salts is augmented by the negative charges on the para-caseinate, thus promoting a concomitant increase in hydration and solubility of para-casein and the formation of a stable product. During processing, the hydrated para-caseinate in the aqueous phase starts to coat the surface of dispersed free oil droplets, leading to their emulsification by interface formation (Cavalier-Salou & Cheftel, 1991; Carič & Kaláb, 1993; Bowland & Foeedging, 1999, 2001). It is clear that both emulsifying salt concentration and the shearing intensity determine oil distribution and oil droplet size and, hence, the microstructure and texture of the processed cheese. The increase of the water-binding capacity of para-caseinate leads to some immobilisation of water and, consequently, to a sufficient increase of the apparent viscosity forced by continuous agitation at high temperature (i.e. ‘creaming’) (Fox et al., 1996; Guinee, 2002a,b; Guinee et al., 2004). Upon cooling, the dispersed phase undergoes significant changes as liquid oil partly crystallises, causing a large increase in viscosity. Processed cheese at low temperature can be regarded as a composite material with the dispersed fat globules, which are linked to the protein network, acting as a filler (Carič & Kaláb, 1993; Jaros et al., 2001).

In many cases, the best effects can be obtained using emulsifying salts with a monovalent cation, mainly sodium, combined with polyvalent anions. As polyvalent anions have a higher water-absorption ability than monovalent anions, the effectiveness of the emulsifying salts increases with increasing valence of the anion. In addition, the buffering capacity of phosphate depends on the chain length: mono- and diphosphates have a high buffering capacity, thus being able to act as pH-correcting agent, and the higher the chain length, the lower the buffering capacity (Schär & Bosset, 2002; Guinee et al., 2004). As some of the emulsifying salts perform better than others, they are usually applied in combination. The main factors determining the blend composition are base material properties, the processing regime, and the type of cheese or analogue to be produced. As regards phosphate-based emulsifying salts, trisodium phosphate and linear condensed phosphates such as pyrophosphates and polyphosphates are mainly used (Table 8.1). In the case of salts of citric acid, the trisodium salt is most commonly used due to its high buffering capacity in a pH range between 5.3 and 6.0. Also, these types of salt are described as having a ‘clean’ flavour. Monosodium and disodium citrate show poor emulsification properties, causing oiling off and water separation, and lead to a relatively high acidity (Carič & Kaláb, 1993).

Trisodium citrate and sodium phosphates readily bind calcium and are, therefore, mainly applied in the production of soft, easily melting cheeses. An increased concentration of trisodium citrate, however, may result in decreased meltability due to a higher level of emulsification, but improves the firmness of processed cheese (Gupta & Reuter, 1993). The application of trisodium phosphate is usually accompanied by an increase in meltability (Kaláb et al., 1991; Pal, 2002). The excessive use of phosphates may, however, lead to casein degradation and the development of a bitter off-flavour (Mayer, 2001). Citrates and monophosphates have a relatively low effect on emulsification, creaming and viscosity increase as compared to di- and tripolyphosphates. Awad et al. (2002) reported a decreased number of fat globules in processed cheese made with citrates or monophosphates.
Table 8.1 Sodium salts used in processed cheese manufacture.

<table>
<thead>
<tr>
<th>Group</th>
<th>Emulsifying salt</th>
<th>Example with formula and structure</th>
<th>Buffering capacity</th>
<th>Emulsification</th>
<th>Ion exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrates</td>
<td>Mono-, di- or tri-sodium citrate</td>
<td>Trisodium citrate (Na₃C₆H₅O₇)</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Citrate structure" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orthophosphates</td>
<td>Mono-, di- or trisodium phosphate</td>
<td>Trisodium phosphate (Na₃PO₄)</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Orthophosphate structure" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrophosphates</td>
<td>Di-, tri- or tetrasodium</td>
<td>Tri-sodium diphosphate (Na₃HP₂O₇)</td>
<td>Moderate</td>
<td>Very high</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>diphosphate</td>
<td><img src="image" alt="Pyrophosphate structure" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyphosphates</td>
<td>Pentasodium tripoly-</td>
<td>Sodium polyphosphate (Graham’s salt: NaₙP₆O₁₆)</td>
<td>Low to very low</td>
<td>Very high $(n = 3–10)$ to low</td>
<td>High to very high</td>
</tr>
<tr>
<td></td>
<td>hexaphosphate; Sodium tetrapoly-</td>
<td><img src="image" alt="Polyphosphate structure" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>hexametaphosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data compiled from Berger *et al.* (1989) and Fox *et al.* (1996).
The size of fat globules in processed cheese is determined by both the intensity of the shearing action and the type of emulsifying salt. For trisodium citrate, a higher fat globule diameter and enhanced melting properties were observed than for disodium phosphate or tetrasodium diphosphate. Firmness and, partly, elasticity of the cheeses are affected in the opposite direction (Guinee, 2002a). Cavalier-Salou & Cheftel (1991) also observed a significant inverse relationship between meltability of processed cheese and fat emulsification, and between meltability and firmness; in addition, the same authors linked the meltability of the product to a high degree of casein dissociation and to a high pH of the end product. Dimitreli et al. (2005) observed an increased peptisation coefficient for processed cheeses with increased pH and moisture content.

An enhanced addition of phosphates usually increases the firmness of processed cheese. Mleko & Foegeding (2000) explained this fact by the high calcium-complexing ability of the polyphosphates, thus leading to a disruption of casein micellar structure and to a higher availability of casein molecules for network formation. Sodium polyphosphates \( (\text{Na}_{k+2} \text{P}_k \text{O}_{3k+1}) \) are usually applied in the production of firm, non-melting cheeses. The high degree of firmness and the reduced melting index is related to enhanced interactions in the protein matrix, as polyphosphates exhibit a more intense crosslinking than citrate and are, therefore, responsible for the development of the internal structure of the protein matrix (Gupta et al., 1984; Guinee et al., 2004). When storing processed cheese, polyphosphates are hydrolysed to tri- and diphosphates and then, more slowly, to monophosphates, which are excellent pH buffers.

### 8.2.3 Manufacturing process

The basic steps in processed cheese manufacture (in traditional cooking and in UHT processing) comprise size reduction and comminution of the base cheeses, the mixing with other ingredients and, in traditional processing, batch cooking at temperatures of ~ 80–110°C for 5–20 min (Fig. 8.2). After the base cheeses have passed quality control (regional regulations for products used for processing include criteria such as mycotoxins; Kammerlehner, 2003), the surface should be cleaned, thus eliminating undesired contamination such as moulds. Pre-breakers, mincing machines and, mainly in case of rind material, counter-rotating roller mills are used to comminute the cheese base into small particles (Kessler, 2002). The increase in surface area allows better interactions with added ingredients or water, and heating in the cooking unit is, therefore, more effective and uniform. Mixing units (Fig. 8.3), which usually have a higher capacity than the cheese cookers, allow the production of homogeneous and uniform blends by virtue of balancing potential differences in raw material composition and processing.

In batch processing, the finely ground mass, which contains all the ingredients, is transferred into cookers with vessel sizes up to 2000 kg and, hence, a processing capacity up to 12 000 kg h\(^{-1}\) (Fig. 8.4). Basically, these systems also allow blend processing without pre-mixing, i.e. the ingredients are directly added into the vessel. This processing regime, however, has some serious disadvantages where the total processing time is longer, and batch-by-batch mixing results in an increased influence of the composition of the batch. The cookers are jacketed kettles with heavy lids, withstanding an internal pressure of up to 1 MPa. While continuously stirring at ~ 120 revolutions per minute (rpm), steam heating of the kettle and direct steam injection is applied to rapidly heat the mixture to the desired
Fig. 8.2  Flow chart for the processing of traditional (left) and UHT processed cheese (right).

Fig. 8.3  Mixing and buffering unit for blending raw materials during processed cheese production. Reproduced by permission of Karl Schnell GmbH & Co., Winterbach, Germany (http://www.karlschnell.de).
temperature (usually 105–110°C). After several minutes of holding time, the mixture is cooled to ~ 85–90°C by addition of a defined amount of water, external cooling and pressure reduction, which is also advantageous for the removal of undesired odours. In this particular production step, the blend thickens and viscosity increases by several magnitudes (Kirchmeier et al., 1980; Dimitreli & Thomareis, 2004).

Both the demands for continuous processing and enhanced shelf-life, achievable by a sufficient inactivation of bacterial spores, have led to various modifications of blend processing. In continuous cooking, the minced cheese base is mixed with the added ingredients and agitated in a screw mixer. The blend is then transferred into cylinders, which ensure a proper agitation and mixing by rotating propeller shafts with scrapers. Heating to 110–125°C is partially done by water jacketing, but mainly by steam infusion (~ 0.6 MPa pressure), which is responsible for a high heating rate. After maintaining the desired temperature for an appropriate period of time (20–200 s) in holding tubes, the product is cooled in scraped surface heat exchangers and then transferred to the filling machines. When combined with the use of proper raw materials, hot filling and cool storage, this technology ensures a shelf-life of several months.

Products with further increased shelf-life result from the application of ultra-high temperature treatment (Fig. 8.5) or sterilisation. Subsequent to the holding tubes following the continuous cooker (~ 90°C), the blend is transferred into another scraped heat exchanger wherein the cheese mass is, within seconds, heated to ~ 140°C by steam infusion. After a holding time of a few seconds, the product is flash-cooled to 80–90°C. Homogenisation is optional, but has the advantage of better emulsification (smaller oil droplets) and may reduce the amount of undissolved particles. Generally, high-temperature treatment is only suitable for spreadable processed cheese, whereas much lower temperatures should be applied for block-type or processed cheese slices (Kammerlehner, 2003). In the case of sterilised

Fig. 8.4  Batch unit for processed cheese production. Reproduced by permission of Karl Schnell GmbH & Co., Winterbach, Germany (http://www.karlschnell.de).
products with a largely extended shelf-life, amino acid degradation and browning is likely to occur (Kristensen et al., 2001; Bunka et al., 2004).

8.3 Rheology and structure of processed cheese

8.3.1 Background

Processed cheese is a complex system consisting of protein, fat, salts, water and other ingredients. Various properties of processed cheese products are affected by the composition and nature of the raw natural cheese, the nature and amount of the emulsifying salts, and the manufacturing process. According to Heertje et al. (1981), the microstructure develops in two different stages: (1) the casein matrix of the raw material is disaggregated into subunits; and (2) the gelation stage is responsible for the formation of string-like structures consisting of dissociated protein fragments (Carić et al., 1985). Both optical and confocal laser scanning microscopy (CLSM), in line with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), have been used in several studies. These studies generally show a distribution of spherical fat droplets, their size ranging between 1 and 10 μm, which are embedded in a continuous protein matrix (Taneya et al., 1980; Heertje et al., 1981; Lee et al., 1981; Kaláb et al., 1987, 1991; Klostermeyer & Büchheim, 1988; Berger et al., 1989; Savello et al., 1989; Marshall, 1990; Kaláb, 1995; Marchesseau et al., 1997; Auty et al., 2001; Schäffer et al., 2001; Awad et al., 2002; Lee et al., 2003; Hennelly et al., 2005). The fat and para-caseinate in processed cheese are distributed more homogeneously than in natural cheese, and there is significantly less coalescence of fat globules. The mean fat globule size varies greatly, depending on type and concentration of the emulsifying salts used as well as on processing conditions.
Natural cheeses are made by pressing curd grains into a uniform mass, in which the points of contact are called curd granule junctions (Carić et al., 1985). When natural cheese is melted without any additives, a heterogeneous, gummy and pudding-like mass with extensive oiling off and moisture exudation, particularly on cooling, is generated. During processing, the three-dimensional calcium para-casein network is partially disintegrated, forming a sodium para-caseinate dispersion with appropriate emulsifying and water-binding properties. However, the intensity of thermal treatment affects the emulsifying capacity of the proteins and, therefore, the ability of the cheese proteins to keep the fat globules in the dispersed state within the protein matrix. Emulsifying salts restore it by binding calcium, which is present in the caseins, promote emulsification of free fat and dehydration of protein, and thus largely contribute to the formation of a smooth, homogeneous and stable product. The fat droplets in the continuous matrix are of varying size (Taneya et al., 1980; Heertje et al., 1981; Lee et al., 1981; Kaláb et al., 1987; Savello et al., 1989; Carić & Kaláb, 1993), but smaller than that in natural cheese. The protein matrix is less compact and fused than in natural cheeses and, as has been shown by high-resolution TEM, appears in short strands of para-caseinate, which are finer than those in natural cheese (Fox & McSweeney, 2003). The para-caseinate particles may correspond to casein sub-micelles, which are released from the cheese matrix due to calcium chelation induced by the addition of emulsifying salts (Taneya et al., 1980; Heertje et al., 1981). The para-caseinate membrane, which covers the surface of the dispersed free fat droplets, appears to connect to the matrix strands, thus being responsible for the continuity of the gel network structure. Auty et al. (2001) visualised the microstructure of various cheese types using CLSM and observed a continuous protein matrix containing irregularly shaped fat globules in Cheddar cheese, protein and fat almost linearly aligned in Mozzarella cheese, and an emulsion-like system in processed cheese, i.e. a homogeneous protein phase containing spherical fat droplets of 2–25 μm in size.

In block-type and sliceable processed cheeses, the protein strands in the matrix are longer (up to 100 μm long and ~ 20 nm wide), and inter-strand connections are more numerous than in spreadable processed cheese products, where the matrix-forming para-caseinate aggregates (elongated particles of ~ 25 nm in diameter) are more dispersed and consist predominantly of individual particles (Taneya et al., 1980; Heertje et al., 1981; Carić et al., 1985).

The extent of emulsification is related to the mean fat globule size, which is reduced as emulsification advances, as well as to the microstructure of the protein matrix, as string-like structures are formed (Carić et al., 1985). Klostermeyer & Büchheim (1988), who studied the relationship between the microstructure of protein matrices and creaming, found areas of lower protein concentration (diameter ~ 1–2 μm) in uncreamed processed cheese products. After moderate creaming, the dimensions of these areas decreased to ~ 0.5 μm in diameter and, by producing a homogeneous protein matrix, disappeared completely at optimal creaming.

8.3.2 Influence of blending ingredients

Apart from natural cheeses, various other dairy and non-dairy ingredients are used in the manufacture of processed cheese and cheese analogues, and some examples include cheese rework, milk protein ingredients such as skimmed milk powder or whey powder, whey protein
concentrate or co-precipitates (Fox, 2001), milk fat and/or vegetable oil, emulsifying salts, colouring and flavouring agents, thickeners (locust bean gum, pectin, starch) and spices. These ingredients largely influence the development of microstructure and, consequently, processed cheese texture and functional properties, such as meltability.

The raw material for processed cheese production is mainly natural cheese, made from milk clotted by rennet. During cheesemaking, and induced by the addition of chymosin, casein micelles aggregate and tend to form a loose network with embedded fat globules. Shrinkage and syneresis induced by mechanical agitation and heat cause a significant fusion within the matrix of para-casein aggregates (Walstra & van Vliet, 1986). On a macromolecular basis, this matrix consists of large casein fragments. Residual activities of the clotting enzyme, plasmin and, mainly, the proteolytic activity of starter micro-organisms or, in case of raw milk cheeses, the indigenous microflora result in a significant breakdown of the protein. The degree of maturation found in the base cheeses greatly affects the microstructure, rheology and texture properties of the processed product (Carić & Kaláb, 1993). In young cheeses or in cheeses with a low degree of proteolysis the caseins, which are characterised by a high emulsifying capacity (Dickinson, 2003), appear almost unchanged (Chambre & Daurelles, 1997). Cheeses showing a more intense proteolysis are characterised by casein breakdown products which, due to a decreased length of the individual casein units, account for an increased softness in processed cheeses. In the manufacture of block processed cheese, mainly young cheeses with 75–90 g 100 g⁻¹ intact casein are used, whereas for spreadable products, medium-aged cheeses with around 60–70 g 100 g⁻¹ intact casein are required (Carić & Kaláb, 1993).

The use of young cheeses reduces raw material costs and results in stable emulsions with high water-absorbing capacity and processed cheeses with a firm body and good slicing properties which, however, show a tendency to harden during storage. On the other hand, full flavour, good flow characteristics and enhanced meltability but low emulsion stability and a soft consistency can be achieved by processing mature cheeses. Piska & Štětina (2004) observed that, when increasing the proportion of mature cheese in the raw material, the complex modulus, the yield stress and hardness, adhesiveness and gumminess as measured by instrumental texture profile analysis decreased. Viscoelasticity was not affected. At the extreme, cheeses with a very low level of intact casein tend to result in poor emulsification (Fox et al., 1996).

Besides economic constraints, cheese rework is frequently incorporated into the base mix to affect the structure and texture of processed cheese products (Berger et al., 1989). According to Fox et al. (1996), an addition of up to ~ 20 g 100 g⁻¹ rework enhanced the creaming effect during manufacturing, especially in blends with a high moisture content (e.g. cheese spreads) and/or with a high content of mature cheese. Depending on the type and amount of the rework, apparent viscosity increased in line with the added amount, and Kaláb et al. (1987) also observed a positive correlation between apparent viscosity of the cooked emulsion and the firmness of the final product. In addition, the meltability tends to decrease in products containing rework.

When adding skimmed milk powder or lactose at levels of approx. 2–5 g 100 g⁻¹ to the blend, the processed products usually exhibit an enhanced susceptibility towards non-enzymatic browning during storage (Kombila-Moundounga & Lacroix, 1991; Fox et al., 1996). Skimmed milk powder improves the spreadability and stability of processed cheese,
whereas lactose usually leads to lower spreadability and lower water activity (Berger \textit{et al.}, 1989; Carić \& Kaláb, 1993). However, an excess of lactose may also increase the tendency to form mixed crystals during storage.

Cheese base, usually produced by acidification of ultrafiltered milk, pasteurisation and further concentration up to a dry matter content of \(~ 60\ \text{g} \ 100\ \text{g}^{-1}\), is frequently introduced as a cheese substitute due to a more consistent quality and lower cost. As the casein is almost intact, an increasing level of substrate leads to processed products which are firmer, less elastic, and show a meltability reduction which, in turn, is caused by whey protein denaturation during heat treatment (Tamime \textit{et al.}, 1990; Younis \textit{et al.}, 1991).

Apart from cost advantages and compared with intact casein, acid and rennet caseins or caseinates are characterised by, for example, superior emulsifying capacity or enhanced stretching properties. Sodium caseinates are widely used in spreadable cheese analogue manufacture, with their high water-binding capacity and good emulsification properties promoting creaming, whereas rennet casein is primarily used in the manufacture of cheese analogues (Marshall, 1990; McCarthy, 1990; Fox \textit{et al.}, 1996; Guinee, 2000b). Solubility, hydration behaviour and functional performance of rennet caseins are affected by the raw material and casein processing (Ennis \& Mulvihill, 2001), by processing temperature during cheese analogue preparation, and by both type and concentration of the emulsifying salt (Ennis \textit{et al.}, 1998; Ennis \& Mulvihill, 1999; Abou El-Nour, 2003). According to Savello \textit{et al.} (1989), the meltability of processed cheeses depends on the type of the casein used in manufacture. Kwak \textit{et al.} (2002) used hydrolysed sodium caseinate as an emulsifier in processed cheese manufacture and observed improved emulsifying activity of casein after 4 h of hydrolysis, therefore allowing the replacement of phosphates.

In order to adjust the fat content of processed cheese during manufacture, some butter or anhydrous milk fat is added to the premix. Vegetable oils are only used in cheese analogues (Carić \& Kaláb, 1993). Marshall (1990) observed a finer protein matrix in processed cheese analogues when the fat content was increased, and TEM micrographs also showed a finer dispersion of fat and, consequently, an increase in the fat/protein interfacial area. Jaros \textit{et al.} (2001) demonstrated that fat composition significantly affects the rheological properties of cheese analogues, with a lower modulus of elasticity in processed cheese analogues with softer fats. Tamime \textit{et al.} (1999) substituted milk fat with starch or particulated whey protein and found that cheese analogues made with anhydrous milk fat contained a higher amount of fat globules compared to those made with fat substitutes.

Hydrocolloids, including locust bean gum, guar gum, carrageenan and alginates, pectins and carboxymethylcellulose, are allowed in pasteurised processed cheese spreads at a maximum level of 0.8 g 100 g\(^{-1}\) (Fox \textit{et al.}, 1996). Schäffer \textit{et al.} (1999) studied the differences between processed cheeses made with 2 g 100 g\(^{-1}\) emulsifying salts or with 2 g 100 g\(^{-1}\) stabilisers. The initial temperature for gel–sol transformation was significantly lower in the case of samples made with hydrocolloids, and the products were more inhomogeneous and exhibited a grainy structure (Schäffer \textit{et al.}, 2001). After adding hydrocolloids to fat-free processed cheese spreads, Swenson \textit{et al.} (2000) found an increase in firmness and a decrease in meltability as compared to control samples. Guar gum produced the softest texture, and gelatin exhibited the largest effects on meltability. Lee \textit{et al.} (1996) investigated how low-molecular-weight emulsifying salts act during cheese analogue production and showed that cationic emulsifying salts resulted in hard and elastic products, probably
due to the positive charges on the emulsifying salt responsible for a reduction of the net negative charges, thus leading to a denser protein network structure. The incorporation of an anionic emulsifier (sodium dodecyl sulphate) increased net negative charges and led to very soft and inelastic cheeses. Drake et al. (1999) added soy lecithin during processing of reduced-fat cheeses and observed that, as regards texture, the cheeses containing lecithin were more similar to the full-fat control cheeses than were reduced-fat cheeses without lecithin. Cheeses containing lecithin were less firm, more cohesive, smoother and showed no negative effects on acceptability.

In addition to the replacement of milk protein by vegetable proteins (El-Sayed, 1997; Ortega-Fleitas et al., 2001), a number of attempts have been made to replace protein with various types of starch. Mounsey & O’Riordan (1999) produced imitation cheeses containing various amounts of pre-gelatinised maize starch and observed a decrease in meltability with increasing levels of starch. When using various types of native starch, fat globules were smaller than in the control (Mounsey & O’Riordan, 2001), indicating a more intense fat emulsification (Savello et al., 1989). Apart from meltability, all starch types reduced cohesiveness of the imitation cheeses due to changes in the fat globule size distribution and disruption of the protein matrix. Hardness was increased by wheat, potato or maize starch, but reduced by waxy maize or rice starch, and rice starch appeared most capable for substituting part of the casein in imitation cheese.

As native whey proteins usually do not interact with emulsifying salts, they do not participate in the reversible gel–sol–gel transformation, but merely act as fillers. Nevertheless, the addition of whey proteins shows a particular influence on swelling behaviour and flowability (Berger et al., 1989). Savello et al. (1989) reported that processed cheeses made from acid casein and whey proteins did not exhibit any abnormal physical structure and were well emulsified with uniform, small fat globules. When using rennet casein, whey proteins did not affect the degree of emulsification during manufacture. The substitution of cheese base or casein by whey proteins resulted in an increased firmness, and in a decrease in meltability of heated cheeses, presumably attributable to the appearance of a fibrous structure (Savello et al., 1989; Hill & Smith, 1992; Gupta & Reuter, 1993; Mleko & Foegeding, 2000, 2001). In general, gels containing higher proportions of β-lactoglobulin were less easy to break (Green et al., 1986), and the increased firmness can be attributed to the unfolding of whey protein molecules during heat treatment. Hydrophobic groups join the oil–water interface, thus enhancing emulsion stability (Modler, 1985; Abou El-Nour et al., 1996). The decrease in meltability was explained by improved emulsification in samples containing higher fractions of whey protein concentrate (Gupta & Reuter, 1993).

In mixed rennet casein gels, Mleko & Foegeding (2000) observed an increase in fracture stress, fracture strain and water-holding capacity with increased whey protein concentration, presumably due to improved hydration properties (Mangino, 1984). The substitution of casein with whey protein isolate increased yield stress but decreased meltability. When substituting casein with whey protein polymers in cheese analogues, the yield stress increased and meltability decreased; however, only the concentration of whey protein polymers and not the state of aggregation of the polymers was responsible for meltability. French et al. (2002) observed similar effects, with an additional dependency on the type and ratio of emulsifying salts.
In contrast to the above-mentioned studies, the meltability of processed cheese spreads made from ras cheese and quark increased with the addition of whey protein concentrate and emulsifying salts, but slightly decreased during storage (Abd El-Salam et al., 1996; Al-Khamy et al., 1997). Kaminarides & Stachtiaris (2000) reported that the replacement of Kasseri cheese by whey protein concentrate and soybean oil caused a decrease of the hardness of cheese analogues. Cheeses made with highest amounts of whey protein and soybean oil were softest and showed lowest elasticity. Hill & Smith (1992) analysed processed cheese spreads made with whey protein precipitates of reconstituted whey protein concentrate 35 g 100 g\(^{-1}\) and whey protein precipitates of reconstituted whey. The samples made from whey protein concentrate were firm and showed a grainy structure, whereas soft and smooth spreads were obtained when using precipitates from reconstituted whey. Thapa & Gupta (1992) investigated changes in sensory and texture parameters during storage of processed cheese foods with added whey protein concentrates and reported an increase in hardness and a decrease in cohesiveness, springiness and adhesiveness as a result of reduced pH. Browning was also observed in all samples as a consequence of Maillard reactions.

### 8.3.3 Effects of moisture and pH

During processed cheese manufacture, water is used to dissolve the emulsifying salts, hydrate the proteins and disperse the components as well as to adjust particular product properties, such as softness and spreadability in cheese spreads or meltability in block processed cheeses. In general, water is known to decrease the firmness of processed cheeses and to improve meltability (Gupta & Reuter, 1993; Lee et al., 2004; Hennelly et al., 2005). In dynamic testing, cheese analogues showed a response typical for weak gels, and firmness expressed in terms of storage modulus were twice as high for cheeses with a moisture content of ~ 53 g 100 g\(^{-1}\) than for cheeses with 58 g 100 g\(^{-1}\) moisture (Pereira et al., 2001). The reduced firmness caused by an increase in the moisture content can be attributed to improved hydration and the resulting weakness of the casein network, and also to the reduction of the protein-to-water ratio (Fox et al., 1996; Ennis et al., 1998). Additionally, it was observed in a number of studies that fat droplet size usually decreases with decreasing moisture content and, hence, their number increases (Pereira et al., 2001; Lee et al., 2004; Hennelly et al., 2005). The increase in interface area is due to the high shearing forces, which are necessary to process a cheese mass with a low moisture content (Fig. 8.6) and, therefore, high viscosity. More proteins can be bound to the interface, and coated droplets assist in matrix formation (Jost et al., 1986; Aguilera & Kinsella, 1991; Pal, 1996). Since dispersed fat globules in processed cheese systems act as an active filler (Carić & Kaláb, 1993; Jaros et al., 2001), the stability of the system and the elasticity of the product increase. Additionally, samples with a low moisture content (< 48 g 100 g\(^{-1}\)) showed a high tendency towards oiling off, which results from a low emulsion stability caused by an insufficient hydration of the proteins in the system (Lee et al., 2004; Hennelly et al., 2005). Dimitreli et al. (2005) observed water separation in processed Gouda cheese with high water content (> 53 g 100 g\(^{-1}\)) when using polyphosphates, and oiling off in processed cheeses with reduced moisture content (< 42.5 g 100 g\(^{-1}\)) when using tetrasodium pyrophosphate.

The pH influences rheology, texture and microstructure of processed cheeses and cheese analogues due to its effects on protein–protein interactions and on casein hydration, as well
as the effects on calcium masking caused by the emulsifying salts. Product pH is related to the pH of the emulsifying salts and increases linearly with the amount of added salts (Gupta et al., 1984; Cavalier-Salou & Cheftel, 1991; Abd El-Salam et al., 1996), thus enhancing the negative charge of the para-caseinate (Marchesseau et al., 1997; Guinee, 2002a; Dimitreli et al., 2005). A low pH (4.8–5.2) usually yields short, dry, firm and granular cheeses with high susceptibility to oiling off whereas, at high pH > 6.0, processed cheese is very soft and moist with an excessive flow on heating (Gupta et al., 1984; Cavalier-Salou & Cheftel, 1991; Lee et al., 1996; Marchesseau et al., 1997; Awad et al., 2002). pH-induced changes in the flow properties may be a consequence of enhanced charge repulsion between proteins (Lee & Klostermeyer, 2001). As the pH increases, the compactness of the protein microstructure is reduced, leading to decreasing interactions between proteins and a weaker protein network (Lee et al., 1996; Awad et al., 2002). Apart from flowability, an increase in pH also leads to enhanced meltability (Savello et al., 1989; Swenson et al., 2000). Literature results describing the impact of pH on texture and rheology of processed cheese are summarised in Table 8.2.

8.3.4 Processing conditions

The microstructure of processed cheese products is also affected by processing conditions, especially cooking time and temperature. The most important factors are the physicochemical imbalance, which is reached after treatment, and changes in the water-binding capacity of proteins, lactose and salts (Berger et al., 1989). As induced by heating, a homogeneous, smooth mass is achieved at ~ 70–75°C, the creaming reaction, leading to a sufficient viscosity change, starts at around 80–90°C, and product shelf-life is improved (70–140°C) (Carić & Kaláb, 1993; Fox et al., 1996; Schäffer et al., 1999). Calcium para-caseinate is converted into hydrated sodium para-caseinate, and the charged and reactive molecules emulsify the oil droplets and, concomitantly, bind water. The interactions between proteins are responsible
for the formation of a crosslinked matrix during cooling of the oil-in-water emulsion, and the viscosity increases (Lee et al., 2003; Dimitreli & Thomareis, 2004). Marchesseau & Cuq (1995) analysed the influence of heat treatment on the water-binding properties of processed cheese gels and found that enhanced temperatures caused a reduction of the water-holding capacity, accompanied by an increased susceptibility to exudation.

Generally, temperature as well as duration and speed of agitation during processing increase the creaming reaction with a concomitant increase in firmness and decrease in spreadability and meltability. An increase in processing time resulted in a progressive increase in elasticity, firmness and viscosity of processed cheese (Kaláb et al., 1987; Mleko & Foegeding, 2000; Stellrecht, 2000; Lee et al., 2003), whereas Swenson et al. (2000) observed that an increase in processing time caused a significant decrease of firmness and increased meltability. These results point to the low structure-forming ability of fat-free

<table>
<thead>
<tr>
<th>Product</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed cheese and spreads</td>
<td>Low pH: mealy, dry, crumbly cheese with high oiling-off</td>
<td>Gupta et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>High pH: soft, high flowability when heated</td>
<td></td>
</tr>
<tr>
<td>Processed cheese (model)</td>
<td>Increasing pH increased meltability</td>
<td>Savello et al. (1989)</td>
</tr>
<tr>
<td>Cheese analogues</td>
<td>Increasing pH caused a decrease in firmness and viscosity</td>
<td>Cavalier-Salou &amp; Cheftel (1991)</td>
</tr>
<tr>
<td>Processed cheese (model)</td>
<td>Low pH: syneresis, firmer than high-pH cheeses</td>
<td>Lee et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>Decreasing the pH resulted in increased ( G' ) and firmness</td>
<td></td>
</tr>
<tr>
<td>Processed cheese spreads</td>
<td>Slightly increased pH with increasing emulsifier content</td>
<td>Abd El-Salam et al. (1996)</td>
</tr>
<tr>
<td>Processed cheese</td>
<td>Low pH: granular samples, emulsification defects</td>
<td>Marchesseau et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>High pH: elastic, softer cheese</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increasing pH from 5.7 to 6.7, and decreasing pH from 5.7 to 5.2, resulted in decreased firmness</td>
<td></td>
</tr>
<tr>
<td>Fat-free processed cheese spreads</td>
<td>Low pH: cheese is more spreadable</td>
<td>Swenson et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>High pH: cheese melts more readily</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increasing the pH resulted in increased firmness of the cheese</td>
<td></td>
</tr>
<tr>
<td>Reduced-fat processed cheese spreads (model)</td>
<td>Low pH: soft cheese</td>
<td>Lee &amp; Klostermeyer (2001)</td>
</tr>
<tr>
<td></td>
<td>High pH: sticky cheese</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreasing the pH resulted in increasing firmness and viscosity</td>
<td></td>
</tr>
<tr>
<td>Block-type processed cheese</td>
<td>Decreasing the pH resulted in increasing firmness</td>
<td>Awad et al. (2002)</td>
</tr>
</tbody>
</table>
products caused by limited possibilities for protein–protein interactions. Lee et al. (2003) observed similar trends in viscosity profiles for processed cheese and fat-free processed cheese, and concluded that the creaming reaction is primarily a protein-based reaction, not necessarily requiring the presence of fat.

As has been shown in electron micrographs, the proteins exist as discrete particles at the beginning of the cooking process and, at maximum viscosity, begin to form a network-like structure which becomes compact when cooking is continued (Lee et al., 2003). During the initial phase of cooking, the casein strands are dispersed by virtue of the action of mechanical shear and emulsifying salts, and calcium-phosphate bridges are broken. The proteins are then reassociated to form a network structure, responsible for the increase in apparent viscosity. The decrease in viscosity with further cooking was explained by a collapse of the gel-like network structure (Fig. 8.7). In overcreamed processed cheese, interactions between proteins cause a too-intense, compact structure, associated with product defects described as grainy, dry and brittle (Kaláb et al., 1987; Berger et al., 1989; Lee et al., 2003).

Fig. 8.7 TEM images of freeze-fractured model processed cheese samples. Cooking time: (a) 2 min; (b) 32 min; (c) 44 min. Bar = 0.2 μm. Source: Lee et al. (2003) Food Science and Technology, 36, 339–345. Reproduced by permission from Elsevier Academic Press, Oxford, UK.

Fig. 8.8 Effects of cooking temperature (75°C) and effective cooking time (5 min) on physical properties of fat-free cheese spreads. Cooking time: white symbols, 0 min; grey symbols, 5 min; black symbols, 20 min. Adapted from Swenson et al. (2000).
The increase in processing temperature leads to a significant reduction in viscosity of the cheese mass (Dimitreli & Thomareis, 2004) and, in the final product, usually results in reduced fat globule diameter, accompanied by an increase in firmness (Lee et al., 1981; Kaláb et al., 1987). Processed cheese, in which the fat occurs as large particles, is softer than products containing small fat globules (Carić and Kaláb, 1993). In fat-free processed cheese spreads, however, increasing the processing temperature from 60 to 80°C resulted in a significant decrease in firmness (Fig. 8.8) and, in a temperature range from 60 to 90°C, in an increase in meltability and spreadability (Swenson et al., 2000).

8.3.5 Changes during cooling and storage

When processed cheese is cooled, the homogeneous and viscous mass sets to form a characteristic body, which may vary from a soft and spreadable to a firm and sliceable texture. The crosslinked matrix is a consequence of interactions between proteins, and interactions between para-caseinate-coated fat globules and the dispersed para-caseinate (Fox et al., 1996). Block-type processed cheeses exhibit a high proportion of long protein strands, and are made up from para-casein aggregates, whereas soft products are characterised by the absence of long protein strands and consist predominantly of individual particles (Taneya et al., 1980; Heertje et al., 1981; Carić & Kaláb, 1993; Fox et al., 1996). The emulsified fat globules (~ 0.3–5 μm) including the crystallised fat are dispersed in the protein network, thus contributing to structure formation.

Physical properties and final texture of the product are influenced by, among other factors, cooling conditions, storage time and storage temperature of the processed cheese (Marchesseau & Cuq, 1995). The changes result from loss of water, hydrolysis of polyphosphates, changes in the ionic equilibrium, crystal formation, oxidation and non-enzymatic browning, enzyme activity and interaction with packaging materials, and are influenced by product composition, processing, packaging and storage conditions (Schär & Bosset, 2002), which can be summarised as follows:

- Loss of water depends on storage temperature and time as well as the packaging material used, and leads to a firmer structure.
- Hydrolysis of polyphosphates to tri- and diphosphates, and then, more slowly, to monophosphates, causes a significant hardening (Ney, 1988, 1989; Berger et al., 1989; Carić & Kaláb, 1993).
- The loss of water and polyphosphate hydrolysis affect the ionic equilibrium and, therefore, induce a decrease in pH. Interactions between proteins, emulsifying salts and calcium change the water-binding capacity and, consequently, texture, and the products become firmer (Awad et al., 2002; Schär & Bosset, 2002).
- According to Berger et al. (1989) and Carić & Kaláb (1993), an excess of emulsifying salts or lactose, a high calcium content in natural cheeses, and a high pH or prolonged storage time may lead to the formation of crystals (Fig. 8.9).
- Some enzymes, still active after processing, may cause significant proteolysis and lipolysis during storage.
- Maillard reactions between reducing sugars and amino acids cause a brown colour and off-flavour, especially when there is a high lactose content (Berger et al., 1989; Carić & Kaláb, 1993).
Reactions induced by light and oxygen presumably causing colour changes and flavour degradation are relevant for samples wrapped in transparent packaging material(s) and for products stored in brightly lit rooms.

Migration of packaging components as well as corrosion, caused by salts and acids present in the processed cheese, may lead to product deterioration.

Piska & Štětina (2004) studied the influence of the cooling rate on rheological and texture properties of processed cheese. Rapid cooling led to a decrease of the complex modulus and yield stress and, additionally, to a decrease in the parameters of hardness, adhesiveness and gumminess obtained in instrumental texture profile analysis, along with improved spreadability and increased stickiness. Quickly cooled samples were more viscous and soft (Carić & Kaláb, 1993; Piska & Štětina, 2004). Rapid cooling is especially important when producing processed cheese spreads, whereas slow cooling is recommended for block processed cheese (Carić & Kaláb, 1993).

According to Awad et al. (2002), texture profile analysis parameters such as hardness, gumminess or chewiness increase with increasing storage time, presumably attributable to the decomposition of emulsifying salts, which decrease the pH. Cohesiveness decreased during storage. Younis et al. (1991) found similar results, and also a reduction in elasticity after six months of storage. In processed cheese spread, meltability tends to decrease with storage time (Younis et al., 1991; Abd El-Salam et al., 1996; Al-Khamy et al., 1997). In addition, the sensory properties are influenced by the storage time (Muir et al., 1999), as is colour development due to the formation of Maillard reaction products (Younis et al., 1991; Carić & Kaláb, 1993).

![Fig. 8.9](image_url) Crystals of insoluble calcium phosphate (P) and sodium citrate (C), and fat globules (F) and bacteria (b) dispersed in the protein matrix (M) of processed cheese. Source: Guinee et al. (2004) Pasteurized processed cheese and substitute/imitation cheese products, in cheese: Chemistry, Physics and Microbiology, Vol. 2 (eds. P.F. Fox, P.L.H. McSweeney, T.M. Cogan & T.P. Guinee), Reproduced by permission of Elsevier Academic Press, Oxford, UK.
8.4 Conclusion

This chapter provides a general overview of some chemical and physicochemical aspects of processed cheese product manufacture, and the impact of interactions between raw materials, emulsifying salts and processing conditions (temperature, time and shear) on the structure and microstructure of these products. When adding emulsifying salts, heating of the natural cheeses is accompanied by continuous agitation, leading to a smooth, homogeneous and stable processed cheese. The physicochemical changes which occur during the manufacture of processed cheese include calcium release, hydration and swelling of para-casein, pH buffering and emulsification of non-globular fat as a result of the decomposition of large fat globules into smaller droplets, and these interactions lead to the stabilisation of the oil-in-water emulsion and, finally, structure formation.

Processed cheeses are used in several forms, including slices, blocks, shredded products and sauces, and as an ingredient in numerous convenience products or ready-to-use food preparations. Consequently, the product characteristics, including texture and melting properties, are tailored for each type of processed cheese and application. For example, tailoring of the product starts with the selection of the raw material (cheese type and the degree of proteolysis), and the amounts and types of emulsifying salts. In turn, these salts affect the pH of the blend and, in addition, the final properties may be affected by the intensity of the mechanical treatment, cooking time and temperature, and storage conditions.

During the past century, the image of processed cheese products has changed significantly. A couple of decades ago, processed cheeses were mainly made to utilise raw materials of low or inferior quality but, nowadays, these products are highly functionalised to fulfil both the requirements of the industrial user as well as the demands of the consumer. In the case of traditional processed cheese, future trends may comprise, for example, the development of new dairy protein-based emulsifiers for partial replacement of the conventional emulsifying salts, thus leading to enhanced consumer acceptance. In addition, the manufacture of different processed cheese products with improved convenience properties, or the development of products with improved nutritional properties, is a permanent source of innovation. Finally, the world economic value of processed cheese will, obviously, depend on the ability of manufacturers to provide the consumer with high-quality products and to follow the trends required for snack foods, which are become increasingly important in our hectic lifestyles.

References


9 Microstructure of Frozen and Dairy-Based Confectionery Products

D.F. Lewis

9.1 Introduction

Milk, with its combination of protein, lipid and carbohydrate, underpins a wide range of products and its functionality introduces a wide range of properties to foods. This chapter examines how microscopy studies can help us understand the use of dairy ingredients in frozen products, typified by ice cream, and confectionery products typified by toffee-type formulations, chocolates and couvertures. As with most branches of food production, technology tends to lead the science – in other words the food technologists tend to work out how to make the products and then the food scientists tell them why it works. The knowing why, however, is important, particularly when natural variations in ingredients or relatively slight changes in formulation or process cause a previously stable product to become unstable. The product range in this chapter requires the technologist to manage fats, sugars, proteins and, in the case of frozen desserts, ice and water to produce attractive and stable products. The microscopist’s task is equally challenging – to visualise all these elements in a way that still bears some relationship to the real world of the product.

9.2 Technological issues

9.2.1 Ice cream

While frozen dairy-based desserts have probably been around since Roman times, the first documented recipes for products resembling modern ice cream appeared in the 1600s. McGee (2004) reports a recipe for ‘neige de fleurs d’orange’ from Nouveau Confiturier in 1682, which includes mixing sweet cream, sugar and petals of orange flowers, freezing in a wine cooler using layers of ice and salt and shaking from time to time over a period of two hours. On the face of it, a simple process, and yet one which now embraces subtle variations to produce a range of products while maintaining underlying principles to retain stability.

One of the features controlling the variety of ice cream is the amount of air that is incorporated into the mix. The level of air in an ice cream is calculated as overrun, and is described as the volume of air incorporated as a percentage of the initial mix volume. Generally more expensive ice creams have a lower overrun. The ability of a mixture to incorporate and retain air during beating is obviously an important economic consideration, and the mechanism of air stabilisation is therefore of significance. Air is generally incorporated into ice cream during the freezing step by the use of beaters within the freezing chamber.
Ice crystal growth and the stability of ice crystals is critical to the mouth-feel of ice cream. Generally, faster freezing rates will give smaller crystals, and physical disruption by using a scraped cooling surface will further reduce the ice crystal size and hence improve the smoothness of the product. During the initial freezing of the ice-cream mix, ice crystal formation is incomplete and a period of hardening at low temperature is required to allow full development. In any frozen mixture of solutes, ice formation results in two phases: a crystalline ice phase and an ‘amorphous’ eutectic phase. The balance between ice and eutectic varies according to the concentration of solutes in solution and the temperature of storage. As crystallisation occurs the eutectic phase becomes more concentrated and consequently the viscosity of the eutectic phase increases. The increased viscosity in the eutectic phase slows down the rate of ice crystal formation and, for this reason, ice formation during the initial freezing is incomplete and the hardening stage is required to allow equilibrium to be reached between ice and eutectic.

Ice cream should normally be stored at –18°C or below and, to achieve the optimum ice to eutectic balance in these products, the total soluble solids in the ice-cream mix should be in the region of 24–27 g 100 g⁻¹. These solids are generally derived from sugars, milk solids-not-fat (SNF) and additional functional ingredients, such as stabilisers, emulsifiers and freezing modifiers. This combination of solutes represents the balance necessary in an ice-cream formulation. The milk SNF comprises proteins and lactose along with mineral salts and vitamins. In determining the ratio of milk SNF and sugars in the mixture, the problem of lactose crystallisation needs to be considered. Lactose tends to crystallise more readily than most sugars and can cause problems of ‘sandiness’ in the final ice cream.

In addition to achieving the balance of solutes in the ice-cream mix, it is also necessary to achieve a balance between sugar and fat in the mixture. Recommended fat and sugar contents of ice cream vary according to the method of freezing, and Table 9.1 illustrates the recommendations of Rothwell (1985) as reported by the Ice-Cream Alliance’s Technical Fact Sheet No. 4.

Consumer preference studies by Guinard *et al.* (1996) indicated that changing the sugar content between 8 and 16 g 100 g⁻¹ was the main determinant of preference with an optimum

<table>
<thead>
<tr>
<th>Freezer type</th>
<th>Fat</th>
<th>Sugar</th>
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<tbody>
<tr>
<td>Vertical (batch)</td>
<td>6</td>
<td>12.0</td>
</tr>
<tr>
<td>Vertical (batch)</td>
<td>7</td>
<td>12.5</td>
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<tr>
<td>Vertical (batch)</td>
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</tr>
<tr>
<td>Continuous</td>
<td>12</td>
<td>15.0</td>
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around 14 g 100 g⁻¹, but changes in fat content between 8 and 16 g 100 g⁻¹ had less effect on preference – again the optimum was around 14 g 100 g⁻¹.

The emulsification of the fat in the recipe is important for two reasons. (1) Non-emulsified fat in a mixture can interfere with air incorporation into a mixture and can also create an unpleasant, greasy mouth-feel. It is particularly important that the fat is well emulsified in the ice-cream premix before freezing. (2) High-pressure homogenisation is used along with emulsifiers to achieve this emulsification. However, the fat is a major contributor to flavour in the ice cream and, if it is too completely emulsified in the final product, then there will be a blandness to the product. Hence, a small amount of destabilisation during the freezing stage can lead to a preferable product by allowing better flavour release in the mouth. During the freezing stage of the manufacture, the fat should be as far as possible in the crystalline state. Fat crystallisation tends to develop slowly and so an ‘ageing’ step where the mix is held at 4°C for several hours is included in the process before the freezing step.

Once the ice cream is frozen, it will normally be deposited into containers before it is hardened. One of the causes in deterioration of ice cream is ice crystal growth. If temperature fluctuations are allowed to occur during storage, then there will be a process of partial melting and recrystallisation of ice. This process is known as Ostwald ripening, which tends to allow the larger ice crystals to grow at the expense of smaller ones. Eventually, this process will lead to shrinkage and collapse of the ice-cream structure as the ice distorts the air cells. Figure 9.1 illustrates a schematic diagram of the ice-cream process, and Table 9.2 details the ingredients used in ice-cream making including their effect on the product.

9.2.2 Chocolate and couvertures

Chocolate and couvertures are regarded as continuous fat phases incorporating solid inclusions of sugar, cocoa plant tissue and milk solids. Important characteristics of the products are appearance, flavour and texture. In terms of appearance, the chocolate is generally expected to have a shiny surface, and the important texture characteristics are the ‘snap’ when the bar is broken or bitten into and the ‘melt in the mouth’ effect on eating. These properties are dependent on fat crystallisation and this is controlled by tempering, which allows the cocoa butter to crystallise into the correct polymorphic form. Incorrectly tempered chocolate will be susceptible to bloom – a dull powdery deposit on the surface of the bar. In manufacturing terms, it is also important that the viscosity of the molten chocolate is controlled to allow depositing into moulds, pumping along pipelines and obtaining an even coating in enrobed products. Consequently, controlling the properties of chocolate and couvertures involves an understanding of fat crystallisation along with the complex interactions between proteins, sugars and fats.

Processing milk chocolate is normally by one of two processes:

(1) The crumb process involves heating and condensing cocoa liquor, sugar and milk and drying the concentrated mixture to produce a hard, aerated chocolate crumb; this crumb is then combined with more cocoa butter and sugar to produce chocolate with a distinctive flavour and texture.
Milk powder can also be mixed directly with cocoa liquor and sugar to produce a powder-based milk chocolate. Couvertures where the cocoa butter is replaced with other vegetable fats are also produced by direct addition of milk powder. The breakdown of the milk particles influences the viscosity of the molten couverture.

The manufacture of crumb chocolate is described in Fig. 9.2.

### 9.2.3 Caramel and toffee

Caramels and toffees are generally non-crystalline sweets that contain milk fat and milk solids. Traditionally, sweetened and condensed milk is used as the source of the milk components though milk and/or modified powders can be used to control the properties of the toffee. Toffees are normally chewy and sticky, and the butterfat gives a creamy taste and mouth-feel. Depending largely on the boiling temperature and the blend of sugars, the texture of the product can vary from a soft centre in a chocolate bar to a hard chew product. In addition to flavour related to the fat component, a characteristic flavour is developed by interaction between the proteins and sugars during the cooking process. As with ice cream,
Table 9.2  Main ingredients used in frozen ice cream and desserts.

<table>
<thead>
<tr>
<th>Component</th>
<th>Comments/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fat</strong></td>
<td>Contributes to smooth texture of ice cream, assists processing by lubrication during freezing</td>
</tr>
<tr>
<td>Dairy fat</td>
<td>Legally required for dairy ice cream, gives distinctive flavour and creamy mouth-feel</td>
</tr>
<tr>
<td>Vegetable fat</td>
<td>Used in economy ice creams and for ice creams produced to meet specific dietary needs</td>
</tr>
<tr>
<td><strong>Milk solids-not-fat (SNF)</strong></td>
<td>Contribute to texture and mouth-feel and encourage incorporation of air</td>
</tr>
<tr>
<td>Lactose</td>
<td>Contributes to viscosity of eutectic between ice crystals; can cause ‘sandiness’ problems by crystallising</td>
</tr>
<tr>
<td>Caseins</td>
<td>Help control formation and stability of fat droplets in emulsified state</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>Help in controlling emulsification, aeration and iciness</td>
</tr>
<tr>
<td>Minerals</td>
<td>Source of calcium and magnesium; citrates and phosphates influence functionality of proteins</td>
</tr>
<tr>
<td><strong>Sweeteners</strong></td>
<td>Provide sweet flavour of ice cream and contribute to viscosity of eutectic and hence texture</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Most commonly used because of price and flavour</td>
</tr>
<tr>
<td>Invert syrup</td>
<td>Provides increased sweetness</td>
</tr>
<tr>
<td>Trehalose</td>
<td>Reduced sweetness, less carcinogenic and possibly lower insulin response (Tharp, 2004)</td>
</tr>
<tr>
<td><strong>Stabilisers</strong></td>
<td>Used to improve melting and storage properties</td>
</tr>
<tr>
<td>Locust bean and guar gums</td>
<td>Gums derived from plant sources</td>
</tr>
<tr>
<td>Carboxymethyl cellulose (CMC)</td>
<td>Chemically treated cellulose to provide solubility</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>Extract of seaweed</td>
</tr>
<tr>
<td>Carageenan</td>
<td>Moss or algal extract used in combination with other gums</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Animal gel now largely replaced by plant gums</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Modifies ice formation used for ‘soft scoop’ properties</td>
</tr>
<tr>
<td><strong>Emulsifiers</strong></td>
<td>Promotes emulsification of fat in pre-mix</td>
</tr>
<tr>
<td>Egg yolk or lecithin</td>
<td>Traditional emulsification agent in ice cream</td>
</tr>
<tr>
<td>Glycerides</td>
<td>Reduce surface tension between fat and water phases</td>
</tr>
<tr>
<td>Polysorbate</td>
<td>Also known as Tween; sorbitol fatty acid complex</td>
</tr>
<tr>
<td>Condensed buttermilk</td>
<td>Byproduct of butter manufacture source of phospholipids</td>
</tr>
</tbody>
</table>

Note: information derived largely from the website of the University of Guelph, Ontario, Canada (www.foodsci.uoguelph.ca/dairyedu/icingr.html).
emulsification of butterfat is a crucial factor, and a small amount of free fat will improve the flavour release in the mouth, while too much free fat will give a greasy appearance and mouth-feel. The droplet size of the fat also affects the viscosity of the toffee, which is important for the handling of the product.

Stability in the final product is generally achieved by limiting the crystallisation of sugars within the matrix of the toffee. In some products, sugar crystallisation is encouraged to produce fudge or ‘Scottish tablet’ type products. In smooth caramel, the crystallisation of lactose can produce grittiness, and poor dispersion of milk protein can also produce an uneven texture. In essential terms, the process is shown in Fig. 9.3.

**Fig. 9.2** The manufacturing stages of crumb chocolate.
9.3 Microscopical issues

Air, fat, sugar, protein and water make confectionery products a difficult proposition for the microscopist, and few of the well-established methods of the biological microscopist can be used without considerable adaptation. Invariably, techniques that preserve one element are detrimental to another. For example, long-term osmium tetroxide fixation will preserve lipids through associating with the unsaturated bonds, but this process damages the proteins. Any aqueous fixative will dissolve sugar, but solvent-based fixation may cause additional sugar crystallisation. Rapid freezing can cause damage by ice crystal formation; higher sugar levels may protect against this damage, but can interfere with the sectioning of frozen material. Consequently, it is rare to find a single microscopy technique that will provide all the information needed in an indisputable form and, generally, multiple methods are required.

9.3.1 Ice cream

Ice cream is a good example of a product that requires several microscopical approaches to fully understand its structure, and the influence that changes in that structure have on the properties of the product. The key structural elements in ice-cream production are ice crystals, air cells, fat droplets and casein micelles.

Normally, the observation of ice crystals requires the sample to be frozen at the time of examination. Berger & White (1979) reported a squash mount of ice cream in an amyl alcohol and kerosene mixture at –14.4°C for studying ice crystal size, and Fig. 9.4(a) shows this type of preparation. Light microscopy allows distinguishing of ice crystals from sugar crystallisation, e.g. lactose, by the use of polarised light. Chang & Hartel (2002) also used this technique but, by allowing the preparation to warm to –6°C, were able to allow air cells to rise to the top of the slide and be distinguished by focusing. Ice cream can be sectioned in a cryostat and viewed using a cold stage on the microscope; this approach is in some ways more controllable than simple squash mounts, but there are some indications that ice crystals may fracture during sectioning. Figure 9.4(b) shows a cryostat section of ice cream.

Cold-stage scanning electron microscopy (SEM) or cryo-SEM can also be used to examine ice cream, and has become one of the most common approaches to study the ice-cream microstructure. Figure 9.5 shows a typical micrograph produced by cryo-SEM; the
air cells and ice crystals can be recognised, and additional information on the ice crystal matrix can be obtained by allowing the ice to sublime before coating and examination. The distribution of lipid droplets can be studied through the manufacturing process by light microscopy, and in this way the effects of different homogenisation pressures, emulsifiers and stabilisers can be studied. Figure 9.6 shows a typical light microscopy view of an ice-cream emulsion.

However, Berger & White (1979) showed that a significant proportion of fat droplets in ice cream were smaller than can be visualised by light microscopy, and they employed
Fig. 9.5  General view of ice cream examined by cryo-SEM. ‘i’ shows an area of bridging between ice crystals; white arrow points at a bridge of ice crystals; black arrow shows small ice crystals.

Fig. 9.6  Light microscopy of an emulsified mix. Reproduced by permission from the late G. White.
electron microscopy to study this detail of the distribution. In this case, freeze etching was used to prepare replicas for examination by transmission electron microscopy (TEM); this approach allows both small fat droplets and casein micelles to be visualised, and Fig. 9.7 shows a typical freeze-etched micrograph.

Fig. 9.7 Transmission electron microscopy (TEM) from freeze-etched preparation of ice cream. (a) General view showing ice crystals ‘i’ and air cells ‘a’; (b) detailed view showing the inside surface of an air cell with fat droplet ‘d’, smeared fat ‘f’, and casein micelles in eutectic phase ‘c’.
Another approach to preparing samples for TEM is thin sectioning and, in the case of ice cream, this requires the use of freeze-substitution where the specimen is slowly dehydrated and infiltrated with resin while being maintained at low temperatures (see Fig. 9.8). Generally, the process takes several days with the sample being slowly warmed from –20°C to 0°C. The technique is described by Goff (Goff et al., 1999; Goff, 2000), and was also used by Garcia-Nevarez (1996) to study the use of ultrafiltered (UF) milk in ice-cream manufacture. The technique is particularly useful for visualising casein micelles and the nature of the air/eutectic interface.

Fig. 9.8  Transmission electron microscopy (TEM) of thin-sectioned freeze-substituted ice cream. (a) General view showing air cells ‘A’, ice crystals ‘I’ and protein inclusion ‘P’; (b) detailed view showing casein micelles ‘c’ and fat globule ‘f’.
The freeze-substitution technique can also be used to prepare sections for light microscopy (Fig. 9.9), and Lewis (1993) described how infiltrating the specimen with a fluorescent dye during freeze-substitution and examination of the resin-embedded sample by confocal light microscopy can give three-dimensional views of ice cream and be particularly useful in determining linkages between ice crystals as the ice cream ages. In addition, a more detailed account of microscopy techniques for ice cream has been recently reported by Smith et al. (2004).

Using the techniques described above, considerable input into the development of ice-cream technology has been assisted. General accounts of ice-cream structure and function can be found in the reviews by Marshall & Arbuckle (1996) and Marshall et al. (2003) among others.

Food microscopy, in general, became established in the early years of the 20th century, and early studies in ice cream explored the relationship between ice crystal growth and organoleptic assessment of ice-cream quality. In general terms, the fluctuation of the temperatures during storage of the ice cream leads to ice crystal growth and, as larger ice crystals are produced, so the ‘iciness’ of the product increases. The effect of stabilisers on this process has been studied by observing ice crystal growth, and a recent example of this has been reported by Barford (2004). A closer look at cryo-SEM suggests that the intermediate process for crystal growth may be more complex, and may involve the development of a ‘sintered’ structure where ice crystals form a network through the ice cream by ‘spot welds’ linking individual ice crystals. Figure 9.10 illustrates these features. The growth of ice crystals eventually leads to shrinkage and a collapse of the air cell structure, and Fig. 9.11 shows a cryo-SEM of a collapsed ice cream.

![Image of sectioned freeze-substituted ice cream](image.png)

**Fig. 9.9** Light microscopy of sectioned freeze-substituted ice cream. Note enlarged ice crystal (arrow) and general distribution of ice crystals; examining the sections by light microscopy or examining the sample en bloc by confocal microscopy usefully places the transmission electron microscopy (TEM) of these samples in a wider context.
Fig. 9.10  (a) Cryo-SEM and (b) confocal views of ice cream showing bridging between ice crystals ‘i’. Note ‘a’ is an air cell; confocal imaging en bloc allows 3D representation that cannot be achieved with monochrome 2D images; in 3D it is possible to visualise the 3D matrix of the connected ice crystals.

Fig. 9.11  Cryo-SEM view of a collapsed ice cream. Note the large and irregular void spaces ‘v’.
Air cell stabilisation is another area where microscopy can aid the control of ice-cream functionality. In general, the proteins adsorb to air interfaces to encourage and maintain foam formation. Zhang & Goff (2004) used immuno-gold labelling to follow the fate of β-casein and β-lactoglobulin at air interfaces, and evaluated the effect of EDTA in disaggregating casein micelles; these authors also investigated competitive displacement of protein from the interface by emulsifiers. Low levels of fat are well known as disrupters of protein foams, and so the stability of ice-cream air cells is dependent on the emulsification of fat in the mixture. A study of freeze-etched samples indicated the importance of fat droplets and free fat associated with the air interface in ice cream, and Fig. 9.7 shows fat droplets and free fat areas at an air cell surface (Berger & White, 1979). The same authors suggested that some fat destabilisation improved flavour release, although too much would result in a greasy mouth-feel.

Ice cream is not perceived as a healthy food, either in terms of obesity or of tooth decay, and low-fat versions of ‘ice cream’ have been developed. Some replacement sugars are available (Tharp, 2004). Confocal microscopy on freeze-substitution prepared samples has been used to study the effects of replacing sucrose with trehalose (Bannatyne, 2001). Figure 9.12 shows a compilation of micrographs illustrating the structural changes produced at 50% and 100% replacement of sucrose. These images suggest that trehalose replacement produces a structure where the eutectic phase is more disrupted by ice crystals, and this relates to load/penetration tests carried out at varying temperatures between –20°C and –2°C, which show the sucrose-based ice cream softening more rapidly on warming than the trehalose replacement samples.

Ice cream represents a challenge for the food microscopist – both in terms of producing appropriate samples, and then interpreting those samples and relating them to processing, chemical composition and organoleptic properties. However, for the best part of the last century, microscopy has been one of the leading investigative approaches for understanding ice cream, and ongoing developments in microscopy indicate that this will continue to be the case.

Fig. 9.12 Confocal views of freeze-substituted ice cream made with (a) trehalose, (b) 50 : 50 mixture of trehalose and sucrose; (c) sucrose. There is more extensive disruption of the eutectic as the trehalose content is increased.
9.3.2 Toffee formulations

In the scientific literature, the microscopy of toffee manufacture is relatively unreported, and most of the information that is available relates to studies carried out at Leatherhead Food International – previously known as the Leatherhead Food Research Association (LFRA), and the British Food Manufacturing Industries Research Association (BFMIRA). Grover (1937) examined the emulsification process in toffee manufacture by devising a method of gently dispersing toffee in warm water and mixing the solution with a warm gelatin solution. A drop of the resultant solution was placed on a microscope slide with the gelatin setting to prevent Brownian movement from blurring the photography of fine fat droplets. Elson & Cruickshank (1979) used freeze fracture techniques and TEM to study the effect of variations in condensed milks on toffee behaviour, and Dodson et al. (1984a,b) adapted Grover’s technique by replacing gelatin with agar and embedding for thin sectioning to study a range of variables in the milk component of toffee. These reports are summarised by Lewis (1988, 1995). Similar studies using confocal microscopy have been reported by Keogh et al. (2001).

Just as ice cream comprises a sugar/water matrix with fat droplets, ice crystals and air dispersed through it, so toffee is a dispersion; in this case, the fat droplets are dispersed through a sugar-based matrix. In normal toffee, the degree of emulsification is controlled by the interactions between whey proteins and casein. Whey proteins appears to form a membrane around the fat droplets and, during cooking, the casein micelles attach themselves to this membrane. The attachment of casein micelles to the whey membrane can lead to a loss of flexibility in the membrane and this, in turn, can cause a breakdown of the emulsion and some fat instability. As with ice cream, a small amount of free fat adds to the flavour release in the mouth, but too much leads to an unsatisfactory result, i.e. a greasy product. The extent to which casein attaches to the membranes, and consequently the degree of breakdown during the cooking stage, is influenced by a number of factors including the casein to whey ratio, mineral balance in the milk protein and the degree of heat treatment given to the liquid milk prior to manufacture.

Figure 9.13 shows phase-contrast microscopy of ‘normal’ toffee at the premix, half-cooked and finished stages of processing, and some breakdown of the emulsion is apparent. However, Fig. 9.14 shows how this breakdown pattern changes as the calcium level in the milk is reduced, and here the emulsion tends to remain relatively intact.

Similarly, Fig. 9.15 shows the pattern for a toffee manufactured with whey alone. Figure 9.16 shows TEM micrographs of different toffees, i.e. normal, calcium-reduced and whey-only, illustrating the lower degree of association of casein with the membrane in the calcium-reduced and whey-only toffees.

In toffees made from powdered milk rather than condensed milk there is often a roughness caused by poor dispersion of the milk powder; Fig. 9.17 shows this effect by light and scanning electron microscopy. The effect is most marked in powders where the milk has undergone high-heat treatment before drying. Sugars can sometimes crystallise out to give grittiness, and with whey powders this may be lactose; hydrolysing the whey reduces the risk of lactose crystallisation, but sometimes dextrose can crystallise.
**Fig. 9.13** Phase-contrast images of toffee made with skimmed milk powder: (a) at pre-mix stage; (b) at half-cooked stage; (c) as a finished product; also note the evidence of a large fat area.

**Fig. 9.14** Phase-contrast images of toffee made with calcium-reduced milk powder: (a) at pre-mix stage; (b) at half-cooked stage; (c) as a finished product; also note some clumping but little breakdown in fat emulsion.
Fig. 9.15 Phase-contrast images of toffee made with calcium-reduced milk powder: (a) at pre-mix stage; (b) at half-cooked stage; (c) as a finished product. There is little breakdown in fat emulsion during cooking.

Fig. 9.16 Transmission electron microscopy of toffee made with (a) skimmed milk powder, (b) calcium-reduced powder, and (c) the finished product made with demineralised whey powder. Sections are of the finished toffees. Note aggregation of casein micelles at the fat membrane in (a), smaller casein micelles in (b), and intact whey membranes in (c).
9.3.3 Chocolate and couvertures

As with ice cream, microscopy has been used for almost a century to explain the behaviour of chocolate. Campbell & Clothier (1921) studied the polymorphic changes in cocoa butter, and these studies led to the concept that cocoa butter could exist in stable or unstable crystal forms. In turn, this concept considered that ‘bloom’ on chocolate was caused by the unstable crystals reverting to a stable form in an uncontrolled way, and that led to the concept of tempering where, by controlled cooling and shearing, crystallisation could be made to occur in a stable form. This allowed the larger-scale production of chocolate products and underpinned the development in the 1930s of many of today’s popular chocolate bars.

With the invention of the electron microscope, bloom on chocolate was revisited by Jewell (1972) and, in order to carry out this work, it was necessary to develop a technique for producing frozen replicas of chocolate surfaces. The results of Jewell’s work, along with X-ray diffraction studies, revealed that cocoa butter could exist in six polymorphic crystal forms. Four of these forms are rather unstable at room temperature and a fifth form is unstable at temperatures a little above normal room temperature. The conversion of this fifth form of cocoa butter to the sixth, i.e. most stable form, is associated with the formation of bloom on the surface of chocolate. These observations allowed a deeper understanding of the tempering process and suggested conditions that would lead to better packing of crystals and, this in turn, has resulted in bloom becoming less of a problem to the chocolate industry. Bloom problems are now more often related to incompatibility of fats migrating from the centre of a composite chocolate bar; Fig. 9.18 illustrates some of the crystalline forms of cocoa butter.

In the UK, milk protein is generally manufactured by the crumb process described earlier. In other parts of the world, milk chocolate is often made directly from milk powders. The two types of chocolate tend to differ in flavour and mouth-feel. Dodson et al. (1984c) and Pepper & Holgate (1985) studied the use of various milk-derived powders in chocolate and chocolate-flavoured coatings (couvertures). Similar studies using confocal microscopy have been reported by Keogh et al. (2001).

Fig. 9.17 Poorly dispersed protein in toffee as viewed by (a) light phase-contrast microscopy and (b) scanning electron microscopy.
An important characteristic of chocolate and chocolate-flavoured coatings used to enrobe products is the viscosity of the molten coating. If the coating is too low in viscosity, then the coating will tend to flow off the centre, whereas too-viscous a coating may not cover the centre adequately. The viscosity depends partly on the presence of an emulsifier such as lecithin to reduce the surface tension between the lipid continuous phase and the sugar and protein dispersed through it. It also depends on the particle size of the protein and sugar particles included in the blend. Very small particles will tend to give rise to a very viscous product and, conversely, coarse particles will produce a more runny coating. When milk powders are used, the final particle size depends on the extent to which the milk powder particles break down during the chocolate or couverture mixing and refining process. Some particles, such as calcium caseinate, are densely packed and lack air inclusions – these tend to survive processing and produce a low-viscosity product; other powders, such as ultrafiltered and calcium-reduced powders, tend to be highly aerated and break down easily to produce many fine particles and a more viscous product.

Sections of coatings can be prepared for light microscopy and stained with eosin in ethanol (sugar-saturated to avoid dissolving sugar) and, when viewed with polarised light, this allows the identification of sugar and protein. Dried powders can be directly mounted and coated for SEM, and resin-embedded samples of coating can be prepared for TEM. Chocolate crumb tends to show a relatively fine distribution of protein, and has a relatively high viscosity compared with non-cocoa butter couvertures. Figures 9.19 and 9.20 illustrate some of the powders and coatings, respectively.

### 9.4 Conclusions

Microscopy is in essence the most fundamental way of investigating the reasons for products behaving as they do – seeing is believing. Microscopy extends the power of the human eye, allowing us to see the invisible. For confectionery and frozen products containing milk products, this has helped to understand and develop the technology behind their use. As microscopy techniques develop and as the demands on the technologist become more creative, the insights provided by microscopy in its many forms will remain important.
Fig. 9.19 Scanning electron microscopy of (a) calcium caseinate powder, (b) calcium-reduced milk powder and (c) demineralised whey powder. Note the solid particles in (a), fragile aerated particles in (b) and intermediate particle in (c) (see also Chapter 5).

Fig. 9.20 Transmission electron microscopy of chocolate-flavoured coating made with (a) calcium caseinate powder, (b) calcium-reduced milk powder and (c) demineralised whey powder. Note the solid caseinate particle in (a), aerated fragment (b), and small fragments and membranous structures in (c).
References


10 The Microscope in Troubleshooting

D.F. Lewis

10.1 Introduction

Anyone using a microscope in a food manufacturing environment will sooner or later be presented with a sample accompanied by a list of questions along the following lines:

(1) What is it?
(2) How did it happen?
(3) When did it happen?
(4) Why did it happen to me?
(5) Who is to blame?
(6) Where did it come from?
(7) What should I do next?

In general the problems will boil down to three types:

(1) Extraneous matter: first, requiring identification of the offending item, and second, an explanation of the consequences and the likely cause.
(2) Plant malfunction caused by fouling or microscopical damage.
(3) Technological issues, such as: Why has the product become unstable? Does this product infringe my patent? Why is their product cheaper or better than mine?

The first step in approaching these problems is to document the sample well – this is imperative if there is any possibility that a legal process will follow. Where a contamination issue is involved then as much information as possible should be obtained by non-destructive techniques before undertaking procedures that will alter the object. At this stage a thorough examination in a stereo microscope with appropriate photographic records is essential. The importance of accurate documentation can be illustrated by a court case where the local authority complaint of glass pieces in a canned product failed because the public analyst’s report indicated that the product had been in a jar, had a different reference number and was received on a different day than the sample described by the local authority as being in a can. The irony was that in this case the glass had quite definitely derived from the manufacturer’s factory!

In addition to ensuring the security of the product, there will be cases when the safety of the microscopist is important. In the early days of AIDS, when little was known about its properties and when only a very few laboratories were capable of analysing for its presence, a product containing a condom and a piece of paper indicating that the product might
have been contaminated with AIDS was received. The issues were: Did the product contain human semen? Had the condom been heat processed with the product or introduced after processing? Was there any evidence of HIV in the product?

The answers (in those days) required several specialist laboratories, and each had to use secure and safe facilities. Similar issues arise where hypodermic needles or parts of them are found in food products.

During the initial assessment, making sure that the history of the sample is correct will save a lot of time in the long run. Products rarely become unstable without changes in the process or the ingredients – the problem is more often that a whole draft of changes has occurred, mostly undocumented. Time spent on reconnaissance is rarely wasted.

10.2 Extraneous matter

In recent years, considerable advances have been made in forensic analysis, but the microscope remains the front-line tool for examination of foreign matter in foods. A publication that provided the ‘philosophical approach’ to foreign body investigation was produced by Smith in 1983. This approach was illustrated by Lewis (1993), and it was adopted and extended by Edwards & Redpath (1995).

In broad terms, the approach to identifying foreign matter involves making an initial categorisation of the object, and then applying specific tests to confirm and refine the categorisation until an identification is made. If the initial categorisation is not confirmed then it can be reassigned in the light of the new findings. Smith (1983) proposed the following eleven categories for the initial classification.

- **Recognisable objects**: This would include readily identifiable objects, such as staples, string and screws. Within this category, it may be possible to make more specific identification, but the main challenge is to identify how and when the contamination occurred.
- **Metal items**: While it may not be possible to always identify the object, it will often be possible to know that the item is made of a metal. Scanning electron microscopy with X-ray microanalysis will quickly and, relatively non-destructively, give a composition for the metal; microscopy will often give clues as to its source and processing history based on surface wear and adhering debris.
- **Metal/non-metal composites**: Some items will consist of metallic and non-metallic components, for example electronics, plastic/foil packaging or insulated wiring. The confirmation and elaboration of this categorisation requires identification of the separate parts.
- **Biological materials (animal origin)**: This fairly general group includes anything of animal origin from skin fragments to whole organisms. Standard keys are available for insects; hairs can sometimes be identified based on reference works and general animal parts can be referred to histology textbooks.
- **Biological materials (plant origin)**: Plant-based foreign bodies also cover a wide range of objects from wood splinters to seed pods. As with animal products a range of keys are available to assist with precise identification.
• **Crystalline materials:** These can be recognised by their angular form, often with flat faces and well-defined angles. Many crystals are anisotropic and show brightly in polarised light microscopy; a few crystals are isotropic (e.g. salt). *The Particle Atlas* (McCrone & Delly, 1973) will often help with the identification of crystalline materials.

• **Fibrous materials:** Some materials that are not readily identifiable will, nevertheless, have characteristics that allow them to be classified, and some materials are clearly fibrous in nature. Fibrous materials can be animal, vegetable, mineral or synthetic in origin. Polarised light microscopy coupled with staining will often allow samples to be placed into one of these types.

• **Laminates:** Just as some materials can be seen to be fibrous, others can be seen to be laminates with different layers of material held together. Flexible laminates will often be packaging materials, and more rigid laminates suggest fabrication materials.

• **Amorphous hard material:** Sometimes items have little to classify them other than that they are hard. Glass, stone fragments and hard plastics may be initially classified in this group. In food contamination terms, glass is probably the most significant contaminant. Lewis (1984, 1986) gives a detailed account of methods available for glass typing.

• **Amorphous soft material:** As with amorphous hard material, some contaminants can only be classified as soft initially. Oily and greasy deposits may come into this category or they may be simply discoloured areas of the product.

• **Composites:** If none of the above categories represents the object then it may be necessary to simply consider the item as a composite of materials and start identifying the individual components.

The process of categorisation and identification is aided by the use of checklists and, as a starting point, Smith (1983) lists the following:

**Metal checklist**

- Nuts, bolts, washers, rivets, and roves from machines or maintenance activity
- Welding spelter, welding scale, welding rod from maintenance activity
- Filings and swarf due to maintenance activity or machinery wear
- Rust flakes
- Detached flakes of chrome plating
- Fragments broken from machines, especially cutter blades and stirrers
- Trunking and fittings
- Tools and implements
- Thermocouple wires and probes
- Sieve wires
- Flexible metal hose and metal-reinforced rubber/plastic hose
- Wire brush bristles
- Braided wire-covered hose or electrical cable
- Wire wool securers
- Carton staples, metal reinforcements and banding
- Nails and screws (grub-screws)
- Tramp metal from raw materials
- Caps, lids, and other closures
• Fragments from can opening
• Can pull-rings
• Solder from cans or maintenance activity
• Essence and flavour containers
• Meat tags and pins
• Fish hooks in salmon
• Lead shot in game
• Metal foil and foil/plastic laminates
• Process instrument parts
• Light fittings
• Electrical wiring and fuses
• Piping and conduit
• Mercury from switchgear and thermometers
• Needles, pins and safety pins
• Clothing buttons, button stalks, hooks and eyes, popper fasteners or zips
• Hair clips and slides
• Jewellery, necklaces, rings, earrings, bracelets, wrist and neck chains
• Spectacle fittings, e.g. hinge pins or screws
• Wrist watch and cigarette lighter parts
• Coins
• Badges and identity tags
• Pen and pencil parts
• Keys
• Dental fillings, white amalgam or gold
• Dental screws and parts of dental braces
• Paper clips, spring clips, and staples
• Drawing pins

Wood checklist
• Fragments from boxes or pallets
• Cutting and chopping work surfaces
• Stirring paddles
• Pencils
• Baskets
• Punnets and chips
• Match-sticks and match boxes
• Material from maintenance activity

Glass checklist
• Fragments from jars and bottles broken in process
• Fragments from imperfect jars and bottles (i.e. blisters)
• Light bulbs and fluorescent tubes
• Sight tubes and protective plate glasses
• Inspection ports
• Gauge dial glasses
• Instrument case glazed doors
• Glazed cupboard doors
• Windows, external and in partitions, and doors
• First aid bottles
• Milk, beer, lemonade bottles used for refreshment
• Glass thermometers
• Containers used for sampling
• Spectacle lenses and contact lenses
• Watch dial glasses
• Necklaces and ring stones
• Refractometers and other optical test equipment
• Glass-fibre insulation
• Glass-reinforced plastics

Rubber and plastics checklist
• Packaging films and bags, including metallised plastics
• Plastic spoons and working implements
• Flashings from the moulding of plastic containers
• Plastic containers, trays, cups and tubs, liners and seals
• Brushes with synthetic bristles and also plastic stocks
• Stoppers, caps and other closures
• Moulded plastic separators from cartons
• Rubber or plastic moulds
• Plastic work surfaces
• Scraper blades on stirring plant
• Conveyor belts
• Hermetic seals on plant and control instruments
• Hose, plain or corrugates, rubber or plastic, with or without metal/textile reinforcement
• Tool handles
• ‘Squeegee’ blades
• Protective gloves, rubber or plastic
• Rubber finger stalls and wound plasters
• Sack ties
• Cable insulation
• Light diffusers and shades, light switches and fittings
• Plastic electrical conduits and fittings
• Safety glasses or face shields
• Ear plugs and ear protectors
• Spectacle frames and plastic contact lenses
• Synthetic fibre clothing
• Clothing buttons
• Hair combs and fasteners or hair-nets
• Finger nails, natural or artificial
• Erasers and eraser-tipped pencils
• Pens and propelling pencils
• Chewing gum
• Rubber footwear
• Rubber or plastic protective clothing
• Gaskets and seals, washers on taps and ballcocks
• Elastic bands
• Rubber or plastic mats
• Composition flooring
• Foamed plastic, soft or rigid, for insulation
• Plastic ceiling and wall tiles
• Plastic-cased instruments
• Clear plastic windows in instruments
• Instrument and control panel switches and knobs
• Protective plastic sheeting and covers
• Drive belts
• Synthetic strings and ropes
• Plastic tags, labels and Dymo tapes
• Clear plastic safety guards or spray hoods on machines
• Cellulose tape and insulating tape
• Synthetic filter fabrics
• Synthetic fibre cloths and swabs
• Synthetic foam sponges
• Paint flakes

Hair and fibres checklist

• Human hairs
• Meat animal hairs in meat products
• Rodent infestation hairs
• Wild animal hairs in field crops
• Feathers and feather barbules in fowl products and as a contaminant of field crops
• Fine fish bones in fish products
• Brush bristles (synthetic or natural)
• Natural bristles (squirrel, badger) may be mistaken for rodent contamination
• Filter cloths and mats, and filter aids
• Reinforcement fabrics in conveyor belts, drive belts and hoses
• Fabric-backed plastic sheets
• Fabric-lined rubber/plastic gloves or boots
• Rope, string, cotton and thread (natural or synthetic)
• Sacking (natural or synthetic)
• Clothing (natural or synthetic)
Cloths and swabs
Paper and packaging materials of laid fibre construction
Wound dressings
Braided covers on flexible wiring and hoses
Mould growths
False eyelashes or hairpieces

Extraneous vegetable matter checklist

- Twigs
- Stalks
- Leaves
- Calices
- Fruit stones and pips
- Nut shells
- Husks and bran
- Bark
- Peel and skin
- Bird, rodent, and insect nests
- Foreign vegetable species (e.g. grass, straw, leaves, weeds), and general cross-contamination
- Cigarette ends and constituent parts (tobacco, cigarette paper, filter and cork tip)

Animals or insects

Contamination by animals or insects can occur at any point along the route to the consumer; the entry points can be resolved into six main areas: (1) raw materials, contaminated before delivery to the factory; (2) raw materials store in the factory; (3) factory processing area; (4) finished product store in the factory; (5) wholesale/retail selling chain; and (6) consumer’s premises.

The risk of contamination is generally highest for raw materials. Most are derived from living material produced outdoors, often in foreign countries, where they are subject not only to parasitic pests but also to adventitious animal contamination. Further opportunity for entry of foreign animal matter can occur during bulk handling and the transport of raw materials. The risk is lower when the materials reach the planned, protective environment of the food factory. The main risk here is from well-recognised storage pests, against which continuous sanitary measures are taken. The chance of contamination decreases as material passes along the factory line to the product packaging stage. Infection in the selling chain and the consumer’s premises depends on the degree of care exercised and certainly cannot be ruled out.

The contaminating foreign matter derives from three main sources: (1) parasites which use the raw material or products as host; (2) predatory animal forms which seek the raw material or product as food; and (3) animal forms which coexist in the environment and cause accidental contamination.
10.3 Quasi foreign materials associated with specific products

Some products are particularly prone to specific defects, which give rise to consumer complaints. In some cases, the cause of complaint is not a true foreign material, but a normal constituent of the product which has become obtrusive by crystallising. Examples of problems associated with dairy products are: (1) cheese products, such as glass-like crystals of calcium phosphate or granules of lipoprotein; and (2) condensed milk and ice cream (i.e. lactose crystals).

Consider a glass complaint to see how the approach would operate – the complaint is glass in ice cream and two glass pieces are received. The pieces have a ‘rolled top’, as might be expected, from the top of a glass dessert bowl or drinking glass. The diameter of the rim is estimated by fitting against curves to be about 12 cm. There are wear marks on the concave surface of the rim, but the pieces are relatively clean. A small sliver of the glass is analysed by X-ray microanalysis, and the analysis is consistent with non-heat-resistant domestic (kitchen/tableware) glass. The sample is reported as probably from a dessert bowl. The local authority visits the home and inspects the kitchen where two further fragments are discovered in a cupboard. The two extra samples are compared with the original complaint samples, and are found to match analytically, but also can be fitted together like a jigsaw as shown in Fig. 10.1. The contamination was clearly a result of an accident in the kitchen, which had gone unnoticed. The case illustrates the problem of glass breakage producing fragments that are not recovered, and can later find their way into food products.

Sometimes the contamination can be deliberate. Over a period of three months, about ten glass samples were supplied by a low-fat spread manufacturer. All of the samples were crescent-shaped pieces, and analysis showed them to be container glass (bottle/jar), but probably from different manufacturers. The convex surfaces had a moulded pattern, and the small area or original concave surface was smooth – consistent with a blow-moulded container. Closer examination of the smooth concave surface showed metal flecks, and it was discovered that this type of piece could be produced by jabbing the base of a jar with a metallic object such as a screwdriver. Clearly, the company was being targeted by a malicious person, possibly an employee.

Fig. 10.1 Stereo light microscopy view of glass fragments recovered from ice cream and from a kitchen cupboard, showing good fit.
Sometimes teenage pranks can result in contamination complaints – another glass complaint was reported in yoghurt. Examination of the pot in which the complaint had been found showed that it had been opened and resealed. When confronted, the teenage boy in the household admitted fabricating the complaint. Perhaps a teenager was also responsible for a live codling moth larva found in a soft cheese container. The larva did not survive very long, and is normally associated with apple trees so had almost certainly been inserted after the product left the factory.

There is often a temptation for manufacturers to use cheaper ingredients than specified for the product, and sometimes the microscopist can be asked to identify whether any substitution has occurred. Miloslav Kaláb, who has produced an impressive range of publications on all aspects of dairy microscopy and still contributes via his website, explored the possibilities for identifying buttermilk adulteration of skimmed milk (Kaláb, 1980). Using a combination of scanning and transmission electron microscopy, he was able to illustrate incorporated buttermilk in skimmed milk powder. Using transmission electron microscopy, buttermilk incorporation revealed milk fat globule membrane fractions among the casein micelles, while scanning electron microscopy revealed differences in surface characteristics and differences in the susceptibility to lactose crystallisation.

The application of microscopy techniques to investigate materials that should not be present is diverse and this is its strength. Specific contamination and adulteration can normally be detected more sensitively with specific chemical and biochemical tests, but nothing approaches the ability of an experienced ‘microscopical eye’ to recognise unknown additions.

10.4 Processing plant defects

Large-scale food production requires equipment to function in a controlled manner to obtain consistency in the product. Milk is now usually homogenised and, consequently, the consumer no longer sees a cream layer at the top of the milk bottle. However, it is important that the homogenisation pressure is correct, otherwise a thin line can occur in the neck of the container and this can be unsightly. Generally, a two-stage homogenisation is used where the first stage at higher pressure reduces the fat droplet size, but tends to allow some clumping of fat droplets which can then be disaggregated by the second (lower-pressure) homogenisation step. In establishing the optimum settings for the homogenisation, a microscopical examination is the quickest way to assess each stage of the process since the microscopist can assess both the size of the individual droplets and the extent to which they are aggregated. The microscope can be sited in the factory laboratory and, hence, rapid feedback can be given as changes are made to the homogenisers. Furthermore, an occasional microscopic check can be made on the process line to ensure that problems are detected early. Of course other particle sizing techniques, such as light scattering, can give more precise measurements and are useful for confirmation, but the microscopical examination on the spot is rather more immediate and versatile. A similar approach can be useful in the production of spreads, where a simple check on the emulsification status can spot problems as they arise. Van Ham and Alma of Zuid-Nederlandse BV (Lewis, 1978) describe a method for examining the state of the emulsion in butter to allow a rapid assessment of the distribution of water in the butter and, hence, act as a guide to the keeping quality of the butter.
Most processing plants are subject to fouling and these problems fall roughly into two categories: those caused by a build-up of product in the plant, and those caused by microbial growth. Both kinds of fouling can give rise to problems. Lewis (1986) and Marrs & Lewis (1986) studied the fouling of heated surfaces by proteinaceous fluids. An experimental rig was designed to maximise fouling potential, and various parameters were altered to understand their effects. A replica technique was developed for microscopical examination of the deposits, and a detachable portion was used for direct examination in the scanning electron microscope. Transmission electron microscopy and light microscopy studies were undertaken on cross-sections of the removed deposit, and X-ray microanalysis was also carried out on cross-fractures of the deposit. In addition to factors such as temperature and composition of the fluid, two unexpected variables were discovered, partly by accident. The first variable related to the effect of calcium. The work was undertaken in a hard water area where the water was softened by the ‘Clark’ process. Problems at the water treatment plant meant that the calcium content of the water varied considerably and this resulted in an unexpected variation in the level of fouling. X-ray microanalysis quickly showed that calcium was playing a major part in increasing the fouling rate – not apparently by forming limescale as such, but rather by encouraging the protein to deposit more rapidly. The second unexpected finding came as a result of examining the structure of the fouling deposit. The deposit appeared ‘lacy’ by eye, and microscopy revealed a foam-like structure. This led to the view that air bubbles were being formed at the heated surface, and that the protein denatured more rapidly at the air–water interface. The foam structure acted as a good insulation layer, and the main foaming was seen close to the heated surface. The engineering side of the project were unconvinced by the light microscopy and transmission electron microscopy sections, but were eventually convinced by three-dimensional stereo pairs from the scanning electron microscope. Experiments incorporating a deaerator into the rig and applying back-pressure to the product side considerably reduced the rate of fouling. In this case, microscopy was able to detect the unexpected changes that were not built into the experimental plan, and this indicates how microscopy is most useful when there is an input into the project management and it is not simply used as an adjunct to provide supporting images. Figure 10.2 is a scanning electron micrograph of a fouling layer.

Fig. 10.2 Scanning electron microscopy view of fouling deposit on heated surface.
Another type of fouling is the growth of biofilms, which can be a particular problem in on-farm milk storage. The problem sometimes manifests itself in phosphatase failure caused by the milk containing heat-resistant microbial phosphatase, which survives pasteurisation. The initial thought on phosphatase failure is of course to suspect the pasteurisation plant – either leakage allowing raw milk to contaminate pasteurised milk or too low a temperature or holding time, leading to under-pasteurisation. The presence of heat-resistant phosphatase can be inferred by retesting a sample after applying a second pasteurising heat treatment – if the sample remains positive, then the result is most likely due to heat-resistant microbial phosphatase. The development of a biofilm in milk storage tanks is an indication that the cleaning procedures are inadequate. The confocal microscope is useful for examining biofilm structures in situ. The ability to obtain extended focus and three-dimensional images allows a better representation of the nature of the film. Figure 10.3 shows a biofilm on a stainless steel plate; in three-dimensional representations (either as stereo pairs or rotating three-dimensional models) the bacteria can be seen to form an open spongy network. Using appropriate stains, such as acridine orange, it is also possible to detect which bacteria are still viable and which are dead.

10.5 Technological issues

Products can fail for a wide range of reasons – frequently the food technologist is able from experience to identify the cause of the problem, but sometimes, especially with a relatively newly developed product, the cause is not immediately obvious. As with foreign bodies, an initial microscopical examination will often point to the general direction in which the fault lies, and suggest the best approach for confirmation and resolution of the problem.

A problem in the dairy industry can occur with the dispersion of powders, particularly those containing fat. Van Ham and Alma of Zuid-Nederlandse BV (Lewis, 1978) described a method for determining the amount of free fat at the surface of powder particles. The method involved the use of scarlet R (Sudan IV) dissolved in propylene glycol as a stain/mountant. This method, with a little practice, enables the microscopist rapidly to determine the distribution of the lipid within a powder sample, and to see whether fat at the surface of the powder particles is causing a dispersal problem. A similar problem can occur with cocoa powder where the cocoa butter can crystallise in form VI crystals, which make the particles difficult
to disperse cold but relatively easily when hot (Fig. 10.4). Fat crystallinity is important in fats used for pastry or cake mixes, and R.J.M. Pollitt (see Lewis, 1978) describes a method based on polarised light microscopy for estimating the suitability of fats for pastry or cakes based on the fat crystals and the nature of their aggregation.

Components of products other than fat can also cause problems by crystallising, and the polarising microscope is undoubtedly the method of choice for revealing these problems. Sugars are the most obvious candidate and, in particular, lactose in dairy products. Lactose crystallisation can occur in a range of dairy products, and gives rise to ‘sandiness’. Figure 10.5 shows lactose crystals in cajeta, a South American sweetened condensed milk product (Garcia-Nevarez, 1996). Lactose crystallisation can be a problem in milk powders, particularly when stored at inappropriate temperature or humidity. In the matrix of milk powder particles, lactose is normally in an amorphous or glassy state. The effect of lactose crystallising from this glassy state is often to cause the particles to aggregate, with consequent handling problems. The lactose crystals can generally be seen by polarised light microscopy with particles mounted in liquid paraffin or a similar mountant; mounting good milk powders in proprietary aqueous mountants, such as hydromount, will often result in slow recrystallisation of lactose in the particles. Warburton & Pixton (1978) made a study of the various crystal forms of lactose in stored spray-dried milk powders. A more recent review of the stability of spray-dried milks has been reported by Thomas et al. (2004).

![Fig. 10.4](image1.png) Scanning electron microscopy view of chocolate powder showing ‘bloom’-like fat crystals protruding from surface.

![Fig. 10.5](image2.png) Lactose crystals in stored cajeta viewed by polarised light microscopy.
Calcium salts can crystallise particularly as phosphates or, in products such as fruit yoghurts, as salts of organic acids, such as oxalate. The separated crystals can be identified by X-ray microanalysis, but watching the changes in the crystals on the addition of sulphuric acid can also confirm that they are calcium-based. Calcium-based crystals will initially dissolve in sulphuric acid, but will then recrystallise as needle-shaped calcium sulphate crystals. Adding potassium permanganate to the sulphuric acid will confirm the presence of an organic acid by being decolourised around the dissolving crystals. Calcium carbonate deposits would be seen to give off carbon dioxide gas bubbles (Lewis, 1993).

Amino acids, such as creatine and arginine, can also crystallise out in some circumstances. These might be suspected where the crystals dissolve in acids and alkali but are insoluble at neutral pH, which indicates the zwitterion nature of the crystal. In these cases, the precise identity of the amino acid is best trusted to the chemist – direct probe mass spectrometry is a quick method if the facilities are available (Lewis 1993).

Milk composition seasonality can give rise to functionality issues; sometimes this simply relates to whether the animals are feeding on grass or silage with concentrates but, occasionally, problems can arise at the end of the winter period where the cows are receiving poor-quality silage – this sometimes results in milk which performs poorly in cappuccino milk steaming and foaming equipment. In this case, the changes are typically compositional rather than structural, but sometimes more subtle changes can develop. Elson & Cruickshank (1979) reported on variations in the flow behaviour of toffees made from condensed milks at different times of year and from different manufacturing sources. The results of electron microscopy on the toffees and the condensed milks suggested that variations in casein micelle size were an important factor in determining how well the fat in the toffee was emulsified and, consequently, the flow properties of the resulting toffee.

10.6 Conclusions

Microscopy is probably the most versatile tool available for the initial troubleshooting of problems. In most cases, it will at least give a pointer to the likely area in which the problem lies and indicate which more-specific tests should be tried. In many cases, it will provide the answer without recourse to further analysis. Perhaps the biggest hurdle to its application is the availability of experienced food microscopists, and perhaps a feeling that the modest polarising light microscope is passé in this modern world of sophisticated analytical techniques. The food industry would be well advised to ensure that practical food-related microscopy expertise is maintained, be it in company laboratories, food research associations or in universities – you never know when you might need it!
References


Bibliography

General/miscellaneous


**Insects and other invertebrates**


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