Three-Dimensional Characteristics of Fat Globules in Cheddar Cheese

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ABSTRACT

The microstructure of 1-mo-old Cheddar cheese samples of different fat contents was observed with confocal laser scanning microscopy. An observation depth of 40 μm was used with a 0.5-μm distance between observation planes, which resulted in 81 sequential, two-dimensional layered images of the samples. These images were digitally reconstructed using a digital image processing algorithm we developed in 1998. Both two- and three-dimensional image analyses were performed to evaluate number, size, and shape characteristics of fat globules in the samples. Because fat globules are three-dimensional, two-dimensional views are affected by the viewing direction and location of the sample. Therefore, three-dimensional analysis provided more accurate characterization of fat globule properties than did the two-dimensional analysis. Because of limited observation depth, however, many of views of the large fat globules were cropped by the image boundaries. These globules were not easily used for overall property characterization.

(**Key words:** Cheddar cheese, fat globule, image analysis, confocal laser scanning microscopy)

Abbreviation key: CLSM = confocal laser scanning microscopy, **voxel** = volume element (in a digitized image), **2-D** = two-dimensional, **3-D** = three-dimensional.

INTRODUCTION

Quality attributes of all foods are closely related to microstructure. Stanley and Tung (21) defined microstructure as a complex organization of chemical components under the influence of external and internal physical forces. Observation of cheese microstructure and changes in it with perturbations of composition or physical forces can reveal parameters directly related to texture. For example, poor texture (rubbery) and poor flavor (meaty or brothy) are two major shortcomings of low-fat cheese (13). Reduction of the fat concentration of Cheddar-type cheese below 33% of normal significantly influences the body of cheese via changes in the protein-mineral structural mesh. Therefore, a thorough understanding of structure development in cheese is essential to enhance textural characteristics of low-fat cheese.

There are several ways to study the microstructure of food materials. The method chosen depends on factors such as the nature of the food, the microscopic information of interest, and the level of resolution required (23). Electron microscopy offers the advantage of high resolution, but sample preparation procedures such as sectioning, dehydration, and chemical fixation are laborious and may lead to artifacts. Most published studies on cheese microstructure are based on two-dimensional (**2-D**) evaluations (6, 7, 14, 15, 17, 22). Because cheese microstructure is three-dimensional (**3-D**), however, we need 3-D microstructure information to evaluate microstructural characteristics accurately. Confocal laser scanning microscopy (**CLSM**) offers an alternative way to observe food structure with high resolution but without disturbing the internal structure. It is a powerful tool to penetrate the surface of a sample and to visualize thin optical sections. These thin optical sections can be used to study the layered 2-D microstructure, and a computer algorithm can also reassemble them into 3-D images for 3-D image analysis of their microstructure (3).

As rapidly as CLSM is becoming established as a valuable tool for imaging structural and chemical components in plant and animal cells, its enormous potential for studying food systems has not yet been realized (4). The applications for study of food materials are as many and varied as they are for biomedical systems and depend on the ability of CLSM to penetrate deeply but noninvasively into the specimen; to obtain large numbers of sequential, thin optical sections that may be reassembled by a computer to produce 3-D images or stereo pairs; and to identify and localize up to three or four separate chemical components (depending on the number of laser lines available on the instrument) by using specific fluorochrome labeling techniques (11).

In addition, specimen observations can be made within a plane both transverse to and along the optical axis, as compared with conventional light microscopy,

Received September 23, 1998.
Accepted May 3, 1999.

which can only make images transverse to the optical axis. Sample preparation for CLSM involves staining the lipid or aqueous protein phase of cheese with fluorescent dyes and subsequent observation after laser excitation (4). However, CLSM is limited by the maximum possible magnification, which is about 400. Vodovotz et al. (23) provide details of CLSM functioning.

Brooker (4) presented some figures of Mozzarella cheese microstructure obtained using CLSM. Hassan et al. (9) used CLSM to observe coagulum formation resulting from milk acidification. Vodovotz et al. (23) and Blonk and van Alast (2) reviewed many other CLSM applications. We recently developed a 3-D image reconstruction procedure to build a 3-D network of fat globules in cheese (5).

Computer imaging is a powerful technique to extract and quantify features for quality assessment and control. It can also help to understand mechanisms of complex processes that alter product characteristics. It offers the advantages of accurate quantification of images and rapid data handling. Almost all instruments that provide an enlarged view of food systems can be used with image analysis, either through direct interface with a light or electron microscope or by scanning outputs such as photographs or negatives (20). The quantification of microstructure is highly facilitated by computer image analysis (12). In cheese with an oriented structure of the protein matrix (Cheddar curd and Mozzarella cheese), it is important to know the orientation before taking images because longitudinal sections differ markedly from transverse sections.

In the past, several 2-D image processing techniques were applied to study the microstructure of milk and cheese (1, 10, 12, 16, 18, 19). Cheese microstructure is 3-D, however, so a 2-D image analysis often cannot provide adequate information. For example, sometimes fat globules in a 2-D image (a layer of a 3-D image) may appear quite differently in each layer or in each viewing direction. Thus, to study the microstructure of materials, such as cheese, which have nonuniform distribution of internal elements, providing information by 3-D image analysis is very important. Therefore, unlike previously published papers on CLSM applications that simply reported qualitatively on the 3-D nature of food microstructure, we have quantified some of the 3-D image attributes.

The objective of this study was to use CLSM to characterize the 3-D nature of fat globules in Cheddar cheese and to compare results of 2-D and 3-D image analysis.

MATERIALS AND METHODS

Cheese Making

Three Cheddar cheeses were made with different fat contents: full fat (34.5% fat), low fat (13.9% fat), and very low fat (3.9% fat). The cheeses were manufactured at the Dairy Plant of the University of Wisconsin-Madison with whole milk for full-fat cheese, low-fat milk (1.5% fat) for low-fat cheese, and skim milk for very low-fat cheese. The finished cheeses (4.55-kg blocks) were vacuum-sealed and stored at 8°C until experiments.

CLSM

The samples were taken 1 mo after cheese making. A plug of each cheese was taken with a 4-mm diameter cork borer, and a slice less than 1 mm was cut from the center of the plug with a razor blade. Each slice was soaked for 2 min in a 0.1% Rhodamine B solution and placed between a microscope slide and a #1.5 cover slip. Confocal images were taken using a confocal microscope (MRC-600; Bio-Rad Microscience Limited, Hercules, CA) available at the Integrated Microscopy Resource at the University of Wisconsin-Madison. The CoMOS operating system version 6.01 was used. A 568-nm, krypton-argon laser was used to excite the Rodamine B fluorescent dye used for staining. The neutral density filter on the microscope was set to 100% transmittance. Rhodamine B was selected as the fluorescence dye because it is water soluble. Thus, soaking the slices in this solution was not expected to alter the fat distribution, which might have occurred if the dye solvent were lipophilic. Then Rodamine B caused the aqueous background of the cheese to fluoresce and the fat globules to appear as nonfluorescing black holes. Any microscopic air bubbles in the slices may also appear as black or gray holes in the confocal micrographs, and data must be interpreted with this in mind. To ensure that photobleaching of the Rodamine B fluorescent dye did not become a problem, the time spent observing the samples was kept to a minimum.

An oil-immersion 40× lens, with aperture set to 1.3, was used to observe the fat globules. The separation between observation planes was kept at 0.5 μm, the smallest separation for the 40× magnification lens. For each specimen, 81 adjacent planes (layers) were observed, which resulted in a total observation depth of 40 μm. Slices were cut immediately after removing the cheese from 8°C storage to reduce the problem of smearing fat globules while separating each sample. If the lens pushed down on the cover slip and compressed the slice, an elongation of fat globules in the z-direction might appear upon reconstruction of the images in the analysis software, which was not observed to occur. The 40× lens was retractable into the main part of the lens and was not observed to move when the lens was focused up and down at the point of image.

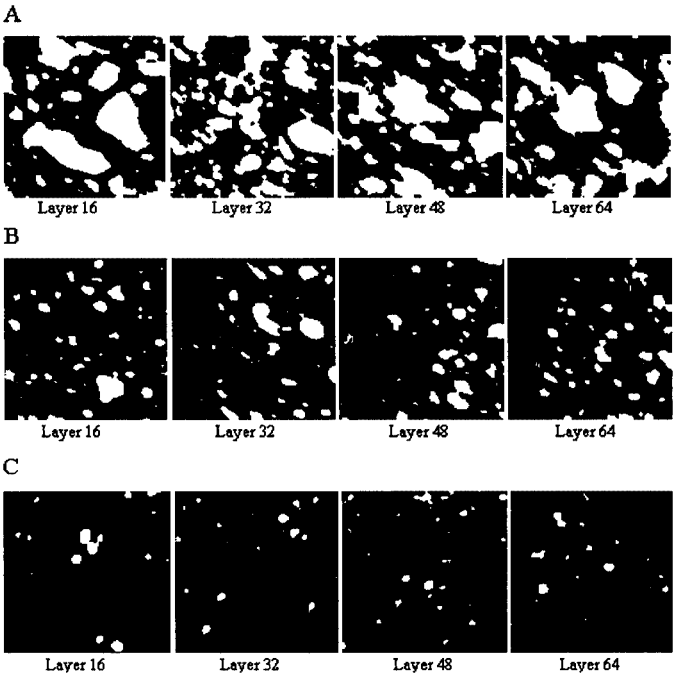

Figure 1. Two-dimensional layered images of 1-mo-old Cheddar cheeses (the width of each microscopic image is 77 μm). A) Top view, layered images of full-fat cheeese; B) top view, layer images of low-fat cheese; C) top view, layered images of very low-fat cheese.

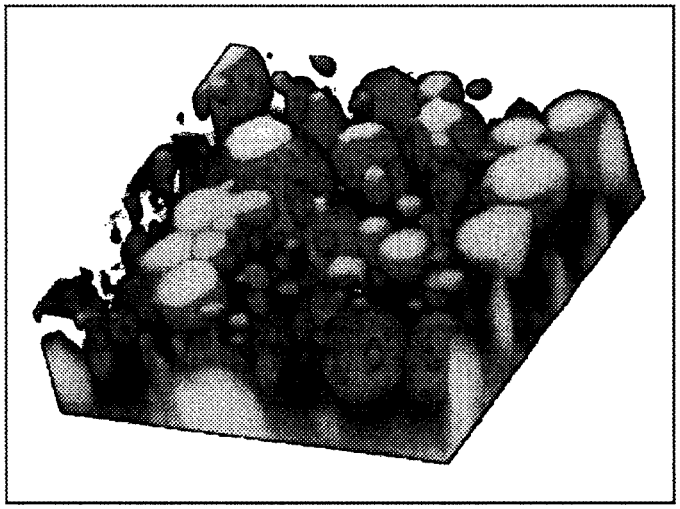

Figure 2. Three-dimensional reconstructed image of fat globules in low-fat Cheddar cheese. Boundary-cropped globules are seen at the image boundaries. Each side of the image is 77 μm.

Image Feature Extraction

Some of the fat globules, especially those at the sample boundary, were not fully viewed because the observation depth was only 40 μm. These fat globules were labeled boundary-cropped globules as distinguished from those totally contained within the sample boundaries, the whole globules. Because boundary-cropped globules were difficult to acocunt for, they were omitted from further analysis.

Size of Fat Globules

The volume of a fat globule was calculated by counting the number of volume elements in a digitized image (**voxels**) and multiplying per voxel volume as follows. The volume of 1 voxel was $0.5 \times 0.5 \times 0.5$ μm^3. The equivalent sphere diameter (D_S) of a fat globule was calculated from globule volume as follows.

$$D_s = (6v/\pi)^{1/3}$$

where D_s is the diameter of a sphere that has the same volume as that of the fat globule under consideration.

Shape of Fat Globules

The shape of a fat globule was represented by a 3-D shape factor, sphericity (S), which describes how close a shape is to a perfect sphere. For a perfect sphere, S = 0. The further a shape is from being a perfect sphere, the greater the value of S. The sphericity of a fat globule was defined in terms of surface voxels (i.e., voxels along the globule boundary).

S = (|number of surface voxels of a fat globule – number of surface voxels of an equivalent sphere|)/number of surface voxels of a fat globule

For 2-D analysis, circularity (C) was the shape factor used. The circularity of a fat globule in a 2-D image was defined in terms of the perimeter as explained below.

C = (|perimeter of a fat globule – perimeter of an equivalent circle|)/perimeter of a fat globule

where equivalent circle perimeter = $\pi \times$ equivalent circle diameter (D_C)

$$D_C = 2(\text{area of a fat globule in 2-D image}/\pi)^{1/2}$$

TABLE 1. Comparison of boundary-cropped and whole fat globules in low-fat Cheddar cheese.

Fat globule type	N_g[1]	%	Volume[2] (μ^3)	%
Boundary whole	335	74	10,348	41
Boundary cropped	117	26	14,902	59

[1]Total number of fat globules.
[2]Volume of all fat globules.

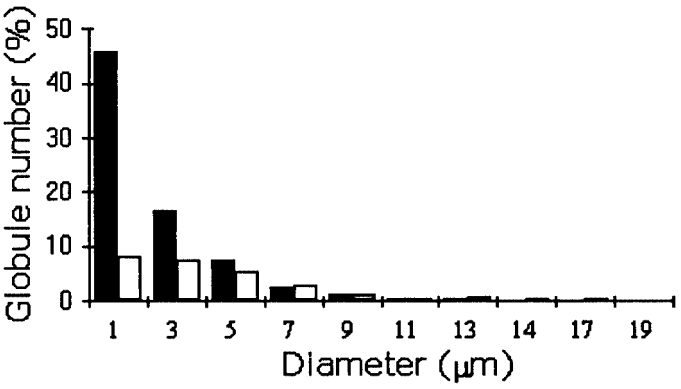

Figure 3. Distribution of globule number with respect to sphere diameter in low-fat Cheddar cheese. Whole fat globule (■); cropped fat globule (□).

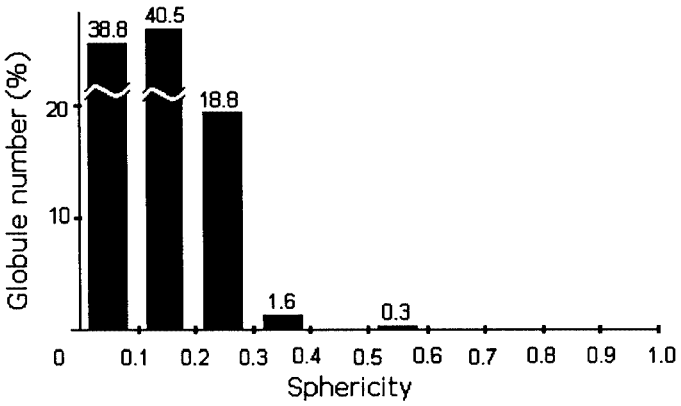

Figure 5. Distribution of globule number with respect to sphericity in low-fat Cheddar cheese.

RESULTS AND DISCUSSION

A typical series of 2-D layers of fat globule images obtained from CLSM is presented in Figure 1. The 3-D, digitally reconstructed view of the low-fat cheese sample is presented in Figure 2. The boundary-cropped globules are clearly seen at the image boundaries of Figure 2.

The total number and volume (cubic micrometers) of cropped and whole fat globules in the low-fat Cheddar cheese specimens examined are presented in Table 1. The cropped globules are obviously larger than the whole globules. Although fewer and incomplete, cropped globules represent a larger percentage of fat volume than do whole globules. This number and size disparity was further verified via globule number and globule volume versus diameter, Figures 3 and 4, respectively. Additionally, Figure 3 shows that most fat globules were very small—more than 40% were smaller than 2 μm in diameter. One reason for this phenomenon is centrifugation of milk used in the experiments. The centrifugation process separates fat from milk during which larger globules are preferentially removed.

The sphericity values of fat globules with respect to the number of globules are presented in Figure 5. Because it is inappropriate to evaluate the shape of boundary-cropped fat globules, they were not included. Hence, shape analysis results must be interpreted with this fact in mind (i.e., it is likely that overall sphericity values of the globules are different from the ones presented here). Note in Figure 5 that most fat globules were spherical ($S < 0.3$). Only a few fat globules (<2% of all whole globules) may be considered nonspherical. The globule size versus sphericity distribution (Figure 6) indicated that the small fat globules ($D_S < 2$ μm) were not distributed randomly. Because small fat globules occupy only a few surface voxels, the sphericity values for these fat globules could not be obtained very accurately. Therefore, results were obtained by considering only the globules larger than 2 μm to avoid this uncertainty.

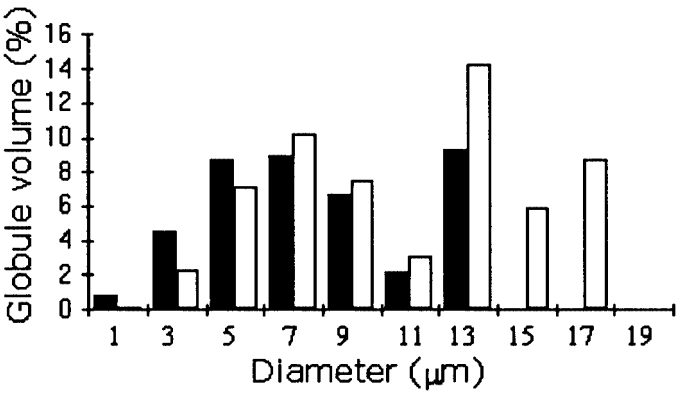

Figure 4. Distribution of globule volume with respect to sphere diameter in low-fat Cheddar cheese. Whole fat globule (■); cropped fat globule (□).

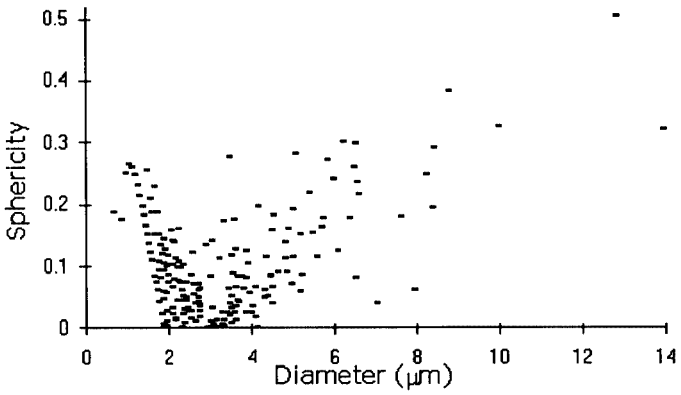

Figure 6. Distribution of fat globule sphericity with respect to spherical diameter in low-fat Cheddar cheese.

TABLE 2. Comparison of three-dimensional parameters of fat globules in Cheddar cheeses of two fat concentrations.

Cheese type	N_g[1]	D_s[2] (μm)		S[3] (all)		S[4] ($D_s > 2\mu$m)	
		$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD
Low-fat cheese	452	2.5	1.9	0.13	0.08	0.08	0.07
Very low-fat cheese	580	1.6	1.2	0.16	0.08	0.09	0.08

[1]Total number of fat globules.
[2]Equivalent sphere diameter.
[3]Sphericity of all fat globules.
[4]Sphericity of fat globules larger than 2 μm equivalent sphere diameter.

The total fat globules, equivalent sphere diameter, and sphericity values of low-fat and very low-fat cheeses are compared in Table 2. Because the laser beam could only penetrate about 40 μm deep from the surface of the sample, many of the large fat globules were cropped by the 3-D image boundaries, and 3-D image analysis could not provide accurate results for the full-fat cheese. As expected, on average, the fat globules in the low-fat cheese were larger than those in the very low-fat cheese. However, the very low-fat cheese had more globules than did the low-fat cheese. The shape of the globules was fairly spherical and about the same in both cheeses. The mean sphericity improved when very small globules ($D_S < 2$ μm) were not included. This result implied that smaller globules were less spherical than the larger ones, or they were more difficult to measure accurately than larger globules.

Analysis of individual 2-D layers are summarized in Table 3. This analysis was performed to compare 2-D and 3-D characteristics. The average size of the fat globules was inversely related to the total fat content. The circularity of the globules seemed to improve with decreasing fat content. Perhaps this improved circularity was due to the larger distance between globules in the lower fat cheeses, which limited the tendency for globules to coalesce. The number of fat globules per layer was higher in the low-fat cheese than in the full-fat cheese. The difference in size of globules between the full-fat and low-fat cheeses probably was caused by centrifugation to separate the fat from milk, during which larger globules were preferentially removed. The shape of fat globules in low-fat cheese was more circular than that in full-fat cheese.

In comparison of the number of fat globules (N_g and n_g) in Tables 2 and 3, very low-fat cheese had less fat globules per layer but more total fat globules than did low-fat cheese, possibly because very low-fat cheese contains numerous small globules, sparsely dispersed. For example, if two globules of 1.0 μm diameter appear in four layers, they will be counted as two globules in 3-D analysis (i.e., $N_g = 2$) but may be counted as one globule in 2-D analysis (i.e., $n_g = 1$). This example shows that a more accurate representation of microstructural characteristics is possible with 3-D rather than 2-D analysis.

Full-fat cheese had larger, less circular or spherical fat globules, which may have been due to the complex, sponge-like structure of its protein matrix (6). The microstructure of full-fat Cheddar cheese has been described as an open, fibrous protein matrix (8). In lower fat cheeses, the protein matrix dominates the structure and may tend to contain fat globules more tightly and prevent them from changing size or shape or both.

Visually (Figure 1), the sizes of fat globules are very different at each fat concentration. However, the mean globule sizes are not different (Tables 2 and 3). To examine these differences, the size distribution of fat globules in 2-D and 3-D views were plotted in Figures 7 and 8, respectively. These plots show that only a few large

TABLE 3. Two-dimensional image analyses of 1-mo-old Cheddar cheese.

Cheese type	n_g[1]	D_c[2] (μm)		C[3] (all)		C[4] ($D_c > 2\mu$m)	
		$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD
Full-fat cheese	40	3.7	0.7	0.22	0.05	0.23	0.13
Low--fat cheese	48	3.0	0.2	0.15	0.02	0.13	0.06
Very low-fat cheese	25	2.1	0.4	0.16	0.06	0.11	0.09

[1]Total number of fat globules per layer.
[2]Equivalent circle diameter.
[3]Circularity of all fat globules.
[4]Circularity of fat globules larger than 2 μm equivalent circle diameter.

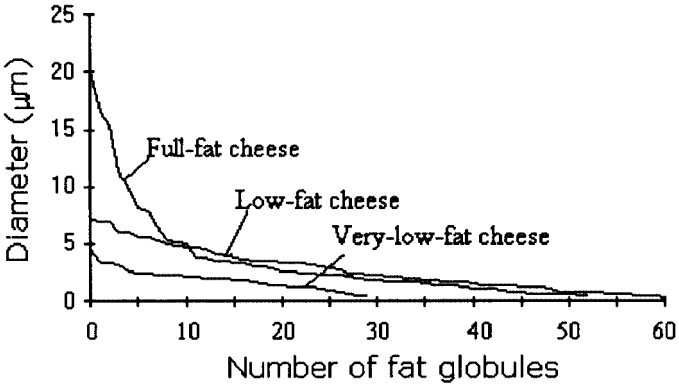

Figure 7. Distribution of fat globule diameters from 2-D images.

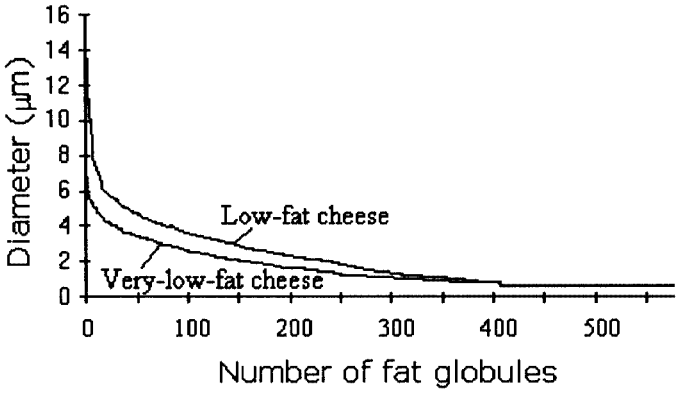

Figure 8. Distribution of fat globule spherical diameters from 3-D images.

globules (D_S or $D_C > 4$ μm) were in each cheese. Therefore, the 10 largest fat globules from each were analyzed separately (Table 4). The 3-D results were obtained from all layers of the 3-D image. The 2-D results were obtained from layer 32 (one of the middle layers, selected arbitrarily) of each sample. In terms of mean globule size, the results presented in Table 4 are very different from those in Tables 2 and 3. Furthermore, visual perception of Figure 1 is similar to that of the data in Table 4, because our visual perception is often obscured by a few large globules, and it is often impossible to view the overall composite of the image.

Table 5 shows results of image analysis of the 2-D layers observed from different viewing directions (obtained electronically) of the 3-D image. The mean sizes of the fat globules in the 2-D view varied from 2.3 to 4.1 μm, depending on the viewing direction. In addition, the circularity of the globules was also different; although essentially the globules are close to being spherical, they are randomly shaped. Because of these random-shaped globules, results of the 2-D analysis of cheese microstructure depend heavily on the location and direction of viewing of a sample. The results should be more representative of the in situ microstructure than those obtained by 2-D image analysis because 3-D image analysis is comprehensive of all layers and all directions. Note that in typical 2-D micrography, different viewing directions of a given sample is impossible.

CONCLUSIONS

The CLSM is a useful tool for evaluating 3-D microstructure characteristics of fat globules in Cheddar cheese. It not only allows visualization of in situ 3-D microstructure but also allows quantification of some important features. Because 3-D analysis provides comprehensive data from a sample much larger than those used for typical 2-D microscopy, 3-D data are more accurate and less dependent on the sample location and viewing direction. However, the limitation of observation depth is a potential problem, especially for evaluating fat globule properties in full-fat cheese in which globules tend to be much larger. Hence, many more of them are cropped at image boundaries compared with lower fat cheeses. To some extent, results of the 3-D

TABLE 4. Properties of ten largest fat globules in 1-mo-old Cheddar cheeses.

Two-dimensional analysis	D_C[1] (μm)		C[2]	
	$\bar{X}$	SD	$\bar{X}$	SD
Full-fat cheese	10.6	5.1	0.33	0.14
Low-fat cheese	5.9	0.8	0.13	0.06
Very low-fat cheese	2.9	0.7	0.13	0.08
Three-dimensional analysis	D_S[3] (μm)		S[4]	
Low-fat cheese	10.2	2.3	0.20	0.09
Very low-fat cheese	5.7	0.7	0.21	0.10

[1]Equivalent circle diamenter.
[2]Circularity.
[3]Equivalent sphere diameter.
[4]Sphericity.

TABLE 5. Comparison of two-dimensional parameters of fat globules from different views.

	Top view		Side view		Front view	
	D_e[1] (μm)	C[2]	D_e (μm)	C	D_e (μm)	C
Minimum	2.4	0.11	2.4	0.21	2.3	0.23
Maximum	4.1	0.28	3.9	0.28	3.4	0.26
Average	3.2	0.17	3.0	0.24	2.8	0.25

[1]Equivalent circle diameter.
[2]Circularity.

image analysis of CLSM images should be interpreted with this fact in mind. Nonetheless, quantification of 3-D image features obtainable with CLSM could serve as an objective criterion for evaluating quality or effect of a number of variables of interest in cheese making.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Wisconsin Milk Marketing Board and The Dairy Manufactures Inc. The comments and suggestions of Norm Olson are also gratefully acknowledged.

REFERENCES

1 Ali, M. Z., and R. K. Robinson. 1985. Size distribution of casein micelles in camel's milk. J. Dairy Res. 52:303–307.
2 Blonk, J.C.G., and H. van Aalst. 1993. Confocal scanning light-microscopy in food research. Food Res. Int. 26:297–311.
3 Brakenhoff, G. J., H.T.M. van der Voort, E. A. van Spronsen, and N. Nanninga. 1988. 3-dimensional imaging of biological structures by high resolution confocal scanning laser microscopy. Scanning Microsc. 2:33–40.
4 Brooker, B. E. 1991. The study of food systems using confocal laser scanning microscopy. Microsc. Anal. 9:13–15.
5 Ding, K., and S. Gunasekaran. 1998. Three-dimensional image reconstruction for food microstructure evaluation using confocal laser scanning microscope. Artif. Intelligence Rev. Kluwer Acad. Publ. 12:245–262.
6 Eino, M. F., D. A. Biggs, D. M. Irvine, and D. W. Stanley. 1979. Microstructural changes during ripening of Cheddar cheese produced with calf rennet, bovine pepsin, and porcine pepsin. Can. Inst. Food Sci. Technol. J. 12:149–153.
7 Emmons, D. B., M. Kalab, E. Larmond, and R. J. Lowrie. 1980. Milk gel structure, X. Texture and microstructure in Cheddar cheese made from whole milk and from homogenized low fat milk. J. Textures Stud. 11:15–34.
8 Green, L. M. 1984. Milk coagulation and the development of cheese texture. Pages 1–34 *in* Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk. Fl. L. Davies and B. A. Law, ed. Elsevier Applied Sci., New York, NY.
9 Hassan, A. N., J. F. Frank, M. A. Farmer, K. A. Schmidt, and S. I. Shalabi. 1995. Formation of yogurt microstructure and three-dimensional visualization as determined by confocal scanning laser microscopy. J. Dairy Sci. 78:2629–2636.
10 Holcomb, D. N. 1991. Structure and rheology of dairy products: a compilation of references with subject and author indexes. Food Struct. 10:45–108.
11 Inoue, S. 1990. Foundations of confocal scanned imaging in light microscopy. Pages 68–97 *in* Handbook of Biological Confocal Microscopy. J. B. Pawley, ed. Plenum, New York, NY.
12 Inoue, S. and K. R. Spring. 1997. Pages 119–157 *in* Video Microscopy—The Fundamentals. Plenum, New York, NY.
13 Jameson, G. W. 1987. Dietary cheeses: low fat, low salt. Food Technol. (Aust.) 39:99–101.
14 Kalab, M. 1979. Microstructure of dairy foods. 1. Milk products based on protein. J. Dairy Sci. 62:1352–1364.
15 Kalab, M. 1981. Electron microscopy of milk products: an overview of techniques. Pages 453–472 *in* Scanning Electron Microscopy. Scanning Electron Microsc., Inc., AMF, O'Hare, IL.
16 Marshall, R. J. 1990. Composition, structure, rheological properties and sensory texture of processed cheese analogous. J. Sci. Food Agric. 50:237–252.
17 Mistry, V. V., and D. L. Anderson. 1993. Composition and microstructure of commercial full-fat and low-fat cheeses. Food Struct. 12:259–266.
18 Ruegg, M., and V. Moor. 1987. The size distribution and shape of curd granules in traditional Swiss hard and semi-hard cheeses. Food Microstruct. 6:35–46.
19 Srilaorkul, S., L. Ozimek, B. Ooraikul, D. Hadziyev, and F. Wolfe, 1991. Effect of ultrafiltration of skim milk on casein micelle size distribution in retentate. J. Dairy Sci. 74:50–57.
20 Stanley, D. W. 1987. Food texture and microstructure. Pages 35–64 *in* Food Texture: Instrumental and Sensory Measurement. Moskowitz, H. R., ed. Marcel Dekker, Inc., New York, NY.
21 Stanley, D. W., and M. A. Tung. 1976. Microstructure of food and its relation to texture. Pages 28–78 *in* Rheology and Texture in Food Quality. J. M. De Man, P. W. Voisey, V. F. Rasper, and D. W. Stanley, ed. AVI, Westport, CT.
22 Tamime, A. Y., M. Kalab, G. Davies, and M. F. Younis. 1990. Microstructure and firmness of processed cheese manufactured from Cheddar cheese and skim milk powder cheese bass. Food Microstruct. 9:23–27.
23 Vodovotz, Y., E. Vittadini, J. Coupland, D. J. McClements, and P. Chinachoti. 1996. Bridging the gap: use of confocal microscopy in food research. Food Technol. 50:74–78.