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IMAGE ANALYSIS OF THE FAT DISPERSION IN A COMMINUTED MEAT SYSTEM

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Abstract

In a series of experiments on a comminuted meat system, image analyses were conducted to quantify changes in structure as affected by polyphosphate, fat level, and chopping time and temperature. Four batches of comminuted meat were investigated which contained neck beef, pig head meat, rind, ice, salt (2%), fat at a level of either 27 or 40%, and polyphosphate at a level of either 0 or 0.5%. Structural changes in different stages of comminution coincided with a shift in size distribution, shape factor, density and area percentage of fat particles. The density of the protein matrix (matrix defined as all disintegrated tissues, without intact pieces of muscle fibres, connective tissue and fat cell clusters) was markedly affected by polyphosphate. Polyphosphate led to a fine dispersion of the fat, as reflected by the formation of a larger proportion of fat droplets smaller than 3.5 μm^2. Results indicate that in meat batters physical entrapment of larger fat particles as well as emulsification of smaller droplets occur simultaneously in the course of fat stabilization.

Introduction

The mechanisms of fat and water binding have been the subject of many studies which were recently reviewed by Hermansson (1986). In addition to physico-chemical studies, microscopy is increasingly applied to investigate the relationship between microstructure and functional properties of comminuted meat products (Lee, 1985; Schmidt et al., 1985; Cassens et al., 1987; Hermansson, 1987; Oelker and Dehmlow, 1988). Based on microscopical observations, two different theories have been postulated about fat stabilization in comminuted meats, namely, fat emulsification (Hansen, 1960) and physical entrapment of fat in the protein matrix (Van den Oord and Visser, 1973). Additional research supported both these theories (Jones and Mandigo, 1982; Lee, 1985). However, more and more it became clear that the microstructure of a comminuted meat system is very complicated and cannot be explained by one phenomenon only (Swasdee et al., 1982; Schmidt, 1984). Moreover, the importance of fat emulsification versus physical restriction of fat coalescence may vary with different processing conditions (Smith, 1988). It is the current view that a comminuted meat product represents a complex multiphase system consisting of a solution, a suspension, a gel and an emulsion (Wirth, 1985; Hermansson, 1988).

Until recently image analysis was only applied to meat quality control, i.e., the quantification of the tissue composition of comminuted meat products (Hildebrandt and Hirst, 1985). The application of image analysis for meat technology research remained limited (Kempton and Trupp, 1983; Lee, 1985) in spite of its broad application possibilities. Not only in microstructural studies, but also in the examination of the macrostructure of meat and meat products, video image analysis has an increasingly important role (Newman, 1987). The additional information about microstructural and thus functional properties provided by image analysis can be useful in process control as well as product development.

In our study image analysis was used in an attempt to relate microstructural changes with morphological variables. Particular attention was paid to the dispersion of fat particles in the

protein matrix of raw meat batters with a different formulation.

Materials and Methods

Experiments were conducted on four different batches of comminuted meat. The batches were prepared according to the recipe given in Table 1. Beef neck meat, ice, salt (containing 0.6% $NaNO_2$) and polyphosphate (Latuw, commercial mixture of tetrasodium diphosphate and potassium polyphosphate at a 1/2 ratio, containing 60% P_2O_5, Degens, Vlaardingen, The Netherlands) were chopped in a bowl chopper (Laska, model KT 60-3 MV, Linz, Austria) until a temperature of 9°C was reached. Subsequently, the other ingredients were added and further comminuted until a final temperature of approximately 30°C was reached. During the comminution process, 200 g cans were filled with the meat batters. Subsequently, these were heated for 75 min at 115°C simulating a retort process at a F_0 value of 2.0. After cooling to 23°C, fat and water loss was determined as described by Tinbergen and Olsman (1979). The chemical composition of the unheated meat batters was determined by International Organization for Standardization (ISO/R937 (1969); ISO 1442 (1973); ISO 1444 (1973)) procedures and is shown in Table 1.

Sampling for microscopical examination was done in the course of the comminution process at 2 or 3 min intervals depending on the temperature rise. This means that four samples were taken, each measuring 1.5 x 1.5 x 0.5 cm, which were frozen in isopentane cooled with liquid nitrogen. The samples were stored at -80°C. Simultaneously, eight similar samples were collected and fixed in a buffered neutral formalin solution for 24 h.

To investigate structural changes at different stages of the comminution process, microscopical examination was conducted at the following time intervals: batch 1 at 9, 11 and 14 min; batch 2 at 8 and 16 min; batch 3 at 8, 10 and 13 min and batch 4 at 4 and 15 min. Samples at these time intervals represented stability or instability of the raw batter microstructure. The frozen samples were cryosectioned (cryostat, type HR, Slee, London, UK) whereafter 8 µm thick sections were stained for fat with Oil red O (Cassens et al., 1977). The formalin-fixed samples were embedded in paraffin and sectioned at 5 µm thickness using an American Optical 820 microtome. Subsequently, the sections were stained with toluidine blue (Swasdee et al., 1982).

All sections were examined by light microscopy at different magnifications to observe structural changes in the batters. To quantify these changes the following morphological variables were determined by image analysis: total area (%) and density of fat particles below and above shape factor 0.45 and the total area (%) of the protein matrix. Shape factor, indicating the degree of roundness of particles (e.g. shape factor = 1.0 describes a circle), was calculated by the formula:

$$* \text{ shape factor} = \frac{4 \pi \times A}{U^2} \qquad (1)$$

* where π = pi, A = area, U = perimeter.

The level of 0.45 was chosen after random-measuring of 200 differently shaped fat particles. Intact fat cells and most protein-covered particles had a shape factor well above the 0.45 level. Shape factors below 0.45 were found for coalesced fat and conglomerates which exhibited the random shape the porosity of the surrounding protein matrix provided. Particle density refers to the number of particles per given area. The protein matrix measured included disintegrated muscle- and collagenous tissue, individual intact fat cells and all smaller fat particles that were covered or emulsified by this protein. Larger intact muscle cells, connective tissue and intact fat cell clusters were excluded. The value for protein matrix thus determined is a fair indication of the amount of fat stabilized by released muscle protein.

In a pilot experiment, all morphological variables were measured in a number of fields of view in different sections. Data were subjected to analysis of variance to determine the optimum number of fields of view and sections, required to obtain reliable results.

Image analysis was done with an IBAS system (Kontron Bildanalyse GmbH, Eching b. München, FRG) and a television camera (Plumbicon Video 50, type LDH 0500/01, Philips) connected directly to the microscope. The measurements were conducted at two magnifications, viz. 40x and 250x. At the 250x magnification, all fat particles ranging from 3.5 to 200 µm² were measured. Image analysis at 40x magnification included all particles larger than 200 µm². In addition, size distributions of all the fat particles from each sample with a shape factor larger than 0.45 were plotted in histograms of 25 class ranges. The average area of 325 intact fat cells from adipose tissue was determined in both paraffin and frozen sections to evaluate the contribution of intact fat cells in terms of size range in the total size distribution of fat particles.

Data from the different morphological variables were subjected to Student t-tests.

Results and Discussion

The effect of fat content and the addition of polyphosphate on water and fat holding as affected by chopping time and temperature is shown in Figure 1. Instability in batches 1 and 3 increased rapidly after 9 min of chopping, whereas in batches 2 and 4 water loss remained low and fat loss negligible. Examination by light microscopy revealed structural changes at the different stages of the comminution process. In batch 1, the microstructure of the meat batter after 9 min of comminution was characterized by a continuous protein matrix of disintegrated muscle- and collagenous tissue in which intact adipose tissue, individual fat cells and many smaller fat particles were distributed (Figure 2). At 11 min chopping time, fat was partly coalesced into larger fat particles (Figure 3). At 14 min chopping time, fat had run together into large channels which disturbed the integrity of the protein matrix (Figure 4). Similar features were observed in batch 3 in which the formation of large fat

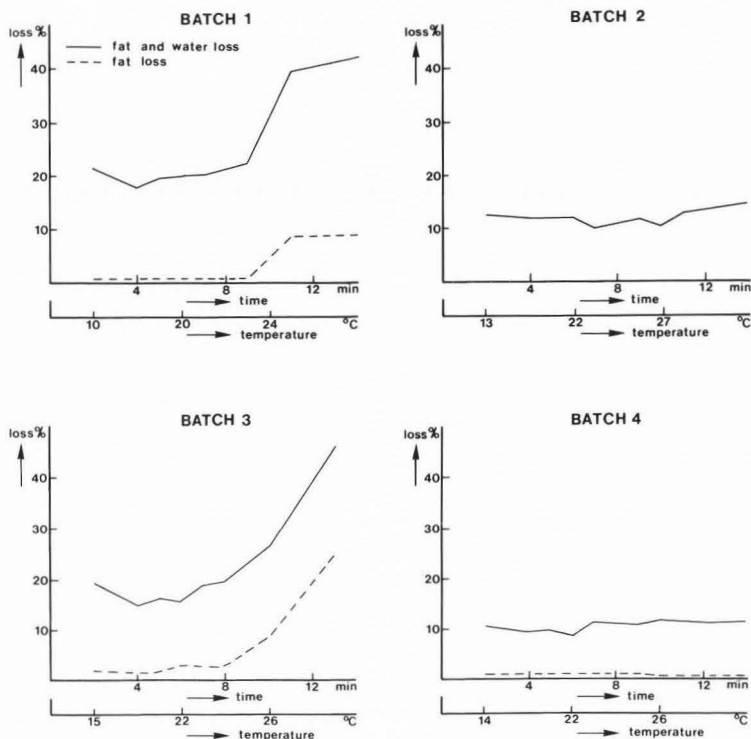**BATCH 1**

BATCH 2

BATCH 3

BATCH 4

Figure 1. Fat and water loss percentages as a function of chopping time and temperature. There was no fat loss in batch 2.

Table 1. Recipe and chemical composition of the meat batters in percentages.

Batches	1	2	3	4
Beef neck meat	26	26	26	26
Pork rind	5.5	5.5	5.5	5.5
Added ice/water	15	15	15	15
Salt	2	2	2	2
Pig head meat	31	31	11	11
Pork fat	20.5	20	40.5	40
Polyphosphates		0.5		0.5
Fat	27.2	27.0	40.6	39.8
Moisture	58.3	58.3	48.6	48.4
Total protein	12.8	12.8	10.4	10.4

channels and the discontinuity of the protein matrix were even more pronounced due to the higher fat content of the meat batter. Scattered between the discontinuous protein matrix intact fat cell membranes were observed. Batches 2 (Figures 5 and 6) and 4 (Figure 7) revealed a different microstructure. The protein matrix was more dense and the large number of finely distributed small fat particles, especially in batch 4, was striking. Extended chopping times did not affect the integrity of the protein matrix and all fat particles remained surrounded by protein.

By image analysis the area percentage, density and shape factor of all fat particles were measured simultaneously. Through analysis of
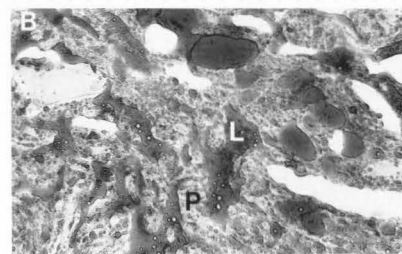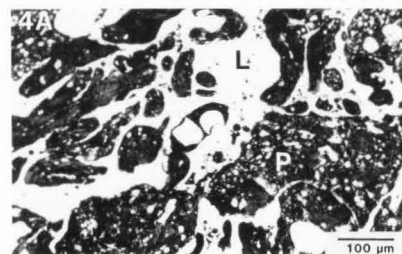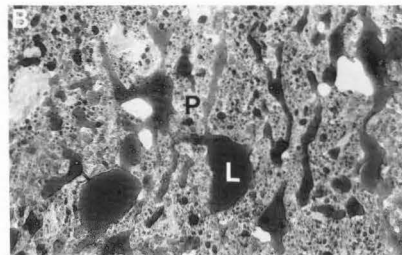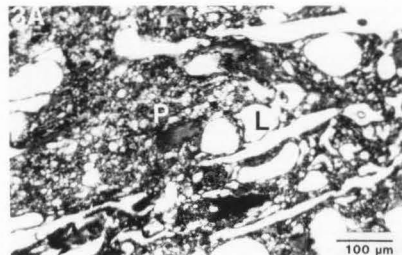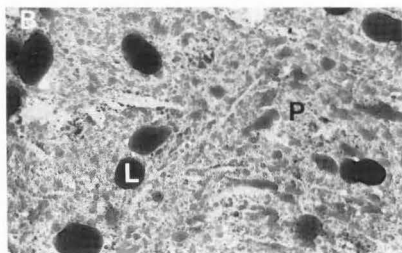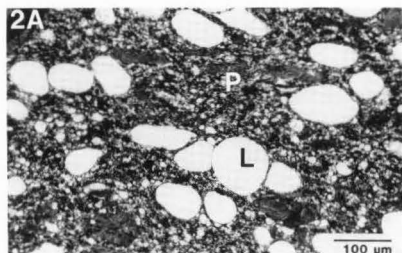
Figure 2. Micrograph of the meat batter microstructure (batch 1) after 9 min of chopping showing a continuous protein matrix. (A) Toluidine blue staining, (B) Oil Red O staining, P = protein, L = lipid.

Figure 3. Micrograph of the meat batter microstructure (batch 1) after 11 min of chopping showing the formation of fat channels.

Figure 4. Micrograph of the meat batter microstructure (batch 1) after 14 min of comminution showing the discontinuity of the protein matrix and coalescence of fat.

variance, it was verified in a pilot experiment that the examination of four fields of view per section in four different sections, i.e., a total of 16 fields of view for each sample, yielded reliable results at both magnifications for all morphological variables measured. The results of the image analysis are shown in Figure 8. In batch 1, the optimum fat stability was reached after a chopping time of 9 min. This stability was reflected by an area percentage of 15 for fat, mainly covered by spherical particles at both magnifications. The protein matrix covered an area of approximately 80%. At 11 min chopping time, instability started to occur. This coincided with a shift towards more large fat particles with a shape factor lower than 0.45 (p<0.001).
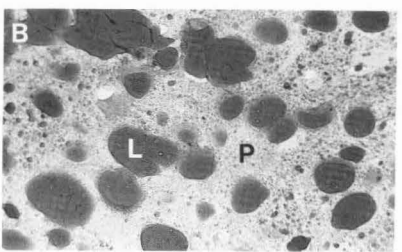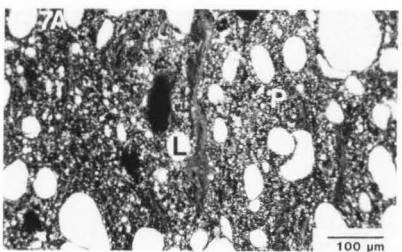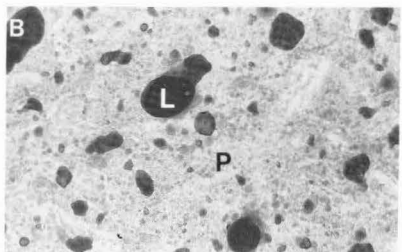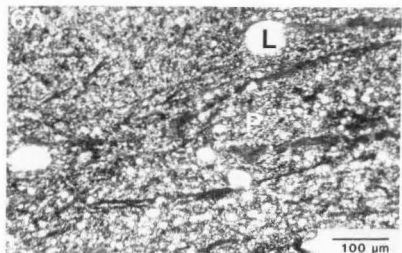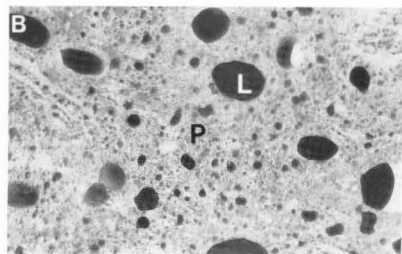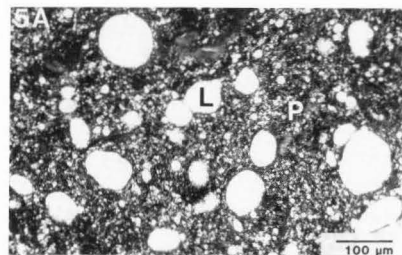
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Figure 5. Micrograph of the meat batter microstructure (batch 2) after 8 min of chopping showing a continuous protein matrix and a uniform dispersion of fat particles. (A) Toluidine blue staining, (B) Oil Red O staining, P = protein, L = lipid.

Figure 6. Micrograph of the meat batter microstructure (batch 2) after 16 min of comminution. A more dense protein matrix with finely dispersed fat particles can be observed.

Figure 7. Micrograph of the meat batter microstructure (batch 4) after 15 min of comminution. Besides intact fat cells, finely dispersed fat particles were present in the dense protein matrix.

After 14 min of chopping, the increased instability was marked by a distinct increase in the area for fat, a decrease in the particle density for both magnifications and a decrease in the area for protein matrix (p<0.001).

Batch 3 revealed a similar pattern as observed in batch 1. Stability was associated with a large area of protein matrix, a high fat particle density and a large area of fat that mainly consisted of particles with a shape factor larger than 0.45. Here also instability was associated with a decreased density of small fat particles and an increase in the density of larger fat particles with a lower shape factor. "Overchopping" of this batch with a high fat content, which was observed at 13 min chopping time, caused phase separation and large fat channels occupied 50% of
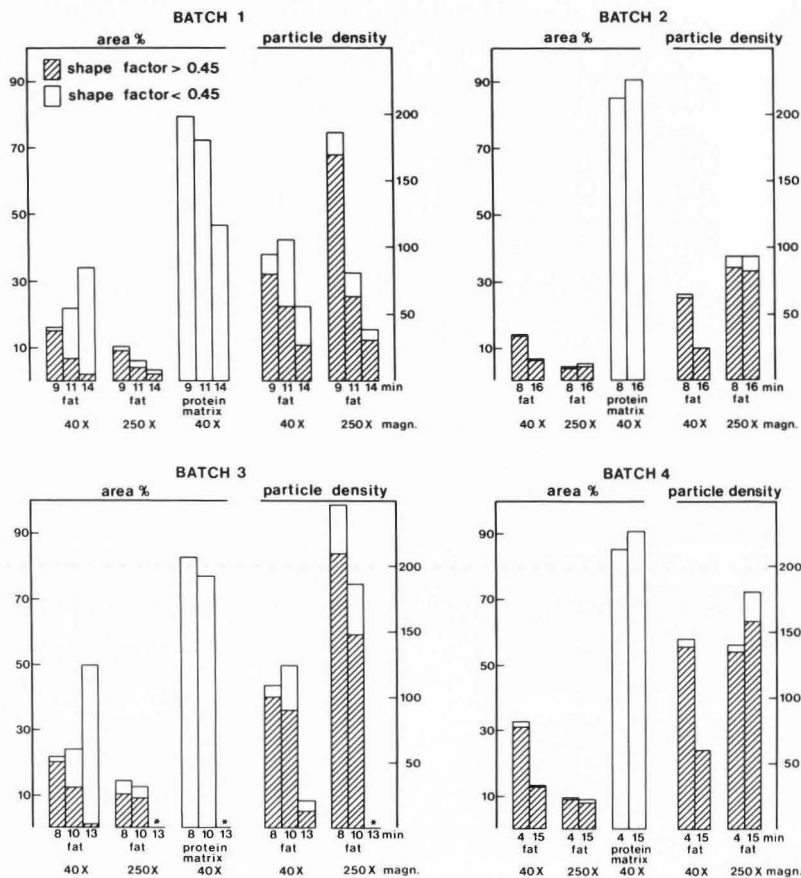
Figure 8. Effect of chopping time on the total area and density of fat particles and the total area of the protein matrix; density expressed as number per $1.102\ mm^2$ (magnification 40x) and number per $0.0268\ mm^2$ (magnification 250x). * could not be determined.

the area. Due to the discontinuous microstructure, it was impossible to properly measure the protein matrix area and the particle density at the 250x magnification.

The microstructure of batches 2 and 4 differed considerably from that of batches 1 and 3. The addition of polyphosphate resulted in a higher density of the protein matrix which occupied 85-90% of the area. As compared with batches 1 and 3, the fat particle density and area of fat in batch 2 at the 40x magnification were lower ($p<0.05$). The area and density of small fat particles, measured at the 250x magnification, remained at the same level even after extended chopping. Most fat particles did not coalesce and held a shape factor larger than 0.45. The same trend was visible in batch 4. In contrast with batch 3, there was a clear shift towards smaller

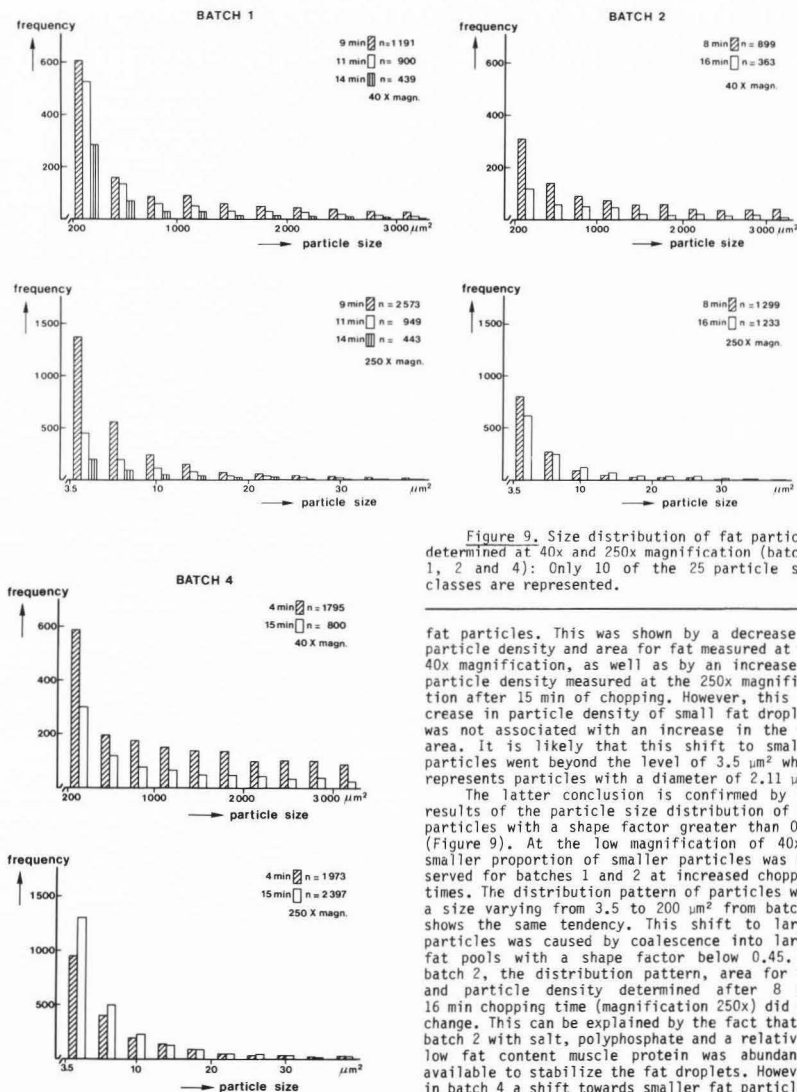Figure 9. Size distribution of fat particles determined at 40x and 250x magnification (batches 1, 2 and 4): Only 10 of the 25 particle size classes are represented.

fat particles. This was shown by a decrease in particle density and area for fat measured at the 40x magnification, as well as by an increase in particle density measured at the 250x magnification after 15 min of chopping. However, this increase in particle density of small fat droplets was not associated with an increase in the fat area. It is likely that this shift to smaller particles went beyond the level of 3.5 μm² which represents particles with a diameter of 2.11 μm.

The latter conclusion is confirmed by the results of the particle size distribution of fat particles with a shape factor greater than 0.45 (Figure 9). At the low magnification of 40x a smaller proportion of smaller particles was observed for batches 1 and 2 at increased chopping times. The distribution pattern of particles with a size varying from 3.5 to 200 μm² from batch 1 shows the same tendency. This shift to larger particles was caused by coalescence into larger fat pools with a shape factor below 0.45. In batch 2, the distribution pattern, area for fat and particle density determined after 8 and 16 min chopping time (magnification 250x) did not change. This can be explained by the fact that in batch 2 with salt, polyphosphate and a relatively low fat content muscle protein was abundantly available to stabilize the fat droplets. However, in batch 4 a shift towards smaller fat particles, determined at the 250x magnification occurred, at

extended chopping time, while the particle density increased and the area for fat remained unchanged. In this meat batter with a higher fat content, an extended chopping resulted in the formation of numerous small fat droplets that required a large proportion of the available muscle protein to be covered. Figure 1 clearly shows that enough protein was available to stabilize these droplets. Both the increased particle density and the shift to smaller particles indicate that fat was partly comminuted into droplets smaller than 3.5 μm². This was confirmed by the fact that measuring particles from 3.5 μm² onwards did not increase the total area % for fat which we expected after examining the same sections by light microscopy.

The formation of numerous fat droplets smaller than 3.5 μm² which were stabilized by the polyphosphate-induced improved protein functionality suggests that emulsification of smaller droplets occurred in addition to fat dispersion of larger particles. Our study afforded limited information on size distribution due to section thickness and magnifications used. Amongst others, Jones and Mandigo (1982) described an emulsification mechanism of fat droplets by protein membranes. All of these studies involved scanning or transmission electron microscopy to qualitatively evaluate the microstructure. More research on quantitative microscopy at this magnification level is essential to elucidate the size distribution and binding of fat particles smaller than 3.5 μm².

The average fat-cell shape factor determined from 325 individual cells was 0.75 with a standard deviation of 0.07. The average size of these fat cells was 3400 μm² with a standard deviation of 1100 μm². This area coincided with largest diameters of 66 to 105 μm depending on where the slicing plane intersected the fat cells. However, these diameters are quite arbitrary. In order to refine on measurements of particle size, we preferred to express particle size not only in μm² (Lee, 1985), but both in μm² and shape factor. Particle size of all fat particles with a shape factor greater than 0.45 ranged from 3.5 to 8200 μm². In all four meat batters, we observed fat particles in the 2300-4500 μm² range with shape factors greater than 0.65. Our data strongly indicate that particles within this size range mainly consisted of intact fat cells. This agrees with observations of Lee (1985) who also stressed the importance of particles in the range from 20-80 μm diameter in the fat holding mechanism. The observed size distribution and the intact fat cell membranes in the disrupted protein matrix of batch 3 indicate that integrity of the fat cell membranes represents an important factor in the mechanism of fat stabilization. Schut (1978) reported likewise and Tinbergen and Olsman (1979) came to the same conclusion by their fat extraction technique.

Adipose tissue fragments, intact fat cells and larger droplets of membrane free fat were physically entrapped in the pores and between other components of the protein matrix. This could clearly be observed in the 9 and 8 min samples of batches 1 and 3. The dispersion of

larger fat particles throughout the continuous phase to a large extent contributed to fat-holding. Fat stabilization by emulsification is likely to occur in finely comminuted batters with very small particles and at ample protein availability (Hermansson, 1986; Smith, 1988). Our experiments indicate that emulsification took place to a greater extent in batches 2 and 4 with polyphosphate addition, than in batches 1 and 3.

In addition, the results show that shape factor as a morphological variable can be used to classify fat particles in terms of stability. Shape factor in combination with particle density and area percentage can be effectively employed to distinguish between stabilized and non-stabilized fat particles.

Conclusions

Differences in chopping time, phosphate addition and fat content resulted in microstructural changes of the meat batters. These changes were quantified in morphological data determined by image analysis. With the aid of polyphosphate a dense protein matrix was formed in which numerous small fat droplets were stabilized, even after extended chopping time and increased fat content. Without polyphosphate, meat batters became less stable to increased chopping time and fat content. The predominant way of fat stabilization in the absence of polyphosphate was effected through physical entrapment of the larger fat particles in a coarser matrix. Both physical entrapment and emulsification contributed to fat-binding in all meat batters investigated, but varied with different formulations. The shape factor proved to be a useful morphological tool in discriminating between stabilized and coalesced fat particles.

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Discussion with Reviewers

C.M. Lee: On what basis can you conclude that emulsification occurred in batches 2 and 4 with polyphosphate? According to your micrographs, fat was simply finely dispersed. Without a fine micrograph such as TEM, it would be presumptuous to state such.

Authors: We agree that without micrographs at higher magnifications, it is impossible to demonstrate emulsification of small fat particles. Our results show that in batches 2 and 4, in contrast with batches 1 and 3, a distinct shift to numerous smaller fat particles occurred. However, this increased particle density in batches 2 and 4, as measured at 250x magnification, was not associated with an increase of the fat area. Chopping fat cells into many smaller fat particles involves a tremendous surface area increase (Barbut, 1988). Since no increase in fat area in batches 2 and 4 was found for particles of $3.5 \ \mu m^2$ or larger, the area increase must be attributed to many particles smaller than $3.5 \ \mu m^2$. As a result of phosphate addition, enough muscle protein was available to stabilize these droplets. It is likely, that under these circumstances emulsification occurred.

A.-M. Hermannson: Fat staining was made with unfixed sections, probably at room temperature. From the micrographs it seems as if the fat phase has been affected by the staining solution and not only by, e.g., overchopping in the bowl chopper. Have the authors any comments on the fat staining procedure?

Authors: Fixation of the sections was done in a formal-calcium solution as described by Cassens et al. (1977). Fat staining by Oil red O was performed at room temperature. It is true that the staining procedure somewhat affected the morphology and location of the fat phase. Any fat-staining solution, as well as the mounting procedure, will cause some dislocation of the non protein-covered small fat droplets at the surface of the sections. Further it was noticed that during sectioning of the batters with phase separation, some disruption occurred, which was not the case in sections from batters without phase separation. However, this limitation did not detract from the fact that an accurate comparison between the batters could be made. This was verified by polariscopy of unstained cryosections, through which it became clear that the staining procedure had affected the overall picture only to a limited extent.

A.-M. Hermannson: There is no discussion of the presence of pores in the meat batters, e.g. the correlation between the fat-stained and the protein-stained sections with regard to the discontinuous phase. Can the authors please make some comments?

Authors: In order to evaluate the presence of air pockets, pores, cracks, etc. in the sections, we applied dark-ground microscopy and polariscopy to unstained cryosections. In this way it was possible to distinguish between fat, protein and empty areas in the sections. Empty areas were only observed in the batters with phase separation. This was caused by some disruption during sectioning, resulting in slippage of fat globules. From this observation we concluded that the contribution of

pores to the total surface area, measured in the protein stained sections, was negligible. Hence, the presence of only a few pores could not have had a significant effect on the fat dispersion pattern determined.

A.-M. Hermansson: Did image analysis at the lower magnification not include any particles less than 200 μm^2, or was there any overlap between the two magnifications?
Authors: There was no overlap between the two magnifications used. The IBAS system offers the possibility of putting lower and upper size limits (μm^2) to particles to be measured. In this way one is able to define size ranges in which particles can be measured. We chose for the lower limit of 200 μm^2 for the 40x magnification. Below this limit particles could be measured more accurately at higher magnifications.

A.-M. Hermansson: How did the measured fat area correspond to the fat content of the batters?
Authors: The measured fat area corresponded well with the fat content of the different batches. This can be seen from figure 8. A higher fat level was associated with a higher fat area and a lower protein area; see batch 1 versus batch 3, and batch 2 versus batch 4.

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