Ripening-related changes in Serra da Estrela cheese: A stereological study

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ABSTRACT

Despite its relevance to sensory features and to fundamental explanation of the changes observed throughout cheese ripening, microstructural studies of specialty cheeses have lagged far behind those of industrialized cheeses. Hence, the purpose of this study was to pinpoint microstructural differences in the gel network of traditional Serra da Estrela cheese throughout ripening, using 2-dimensional image analysis, and to unfold correlations of such microstructural indicators with classical bulk chemical and textural parameters. Hence, samples were taken throughout the ripening period, following a nested design; uniform thin sections were systematically observed via light microscopy (LM, 200 ×) and transmission electron microscopy (TEM, 4,400 ×), and computer-assisted quantitative analysis of digital images was comprehensively performed following standard stereological methodology. Fresh cheeses exhibited the highest porosity and ratio of surface area to volume. Significant negative correlations were found between microstructural parameters and proteolysis indicators. Light microscopy images suggested that rearrangements exist, up to 21 d, of the cheese matrix that leave porosity and pores unchanged, whereas TEM images indicated a significant decrease in number of pores within the same time frame, especially those above $1 \times 10^{-2} \mu\text{m}^2$ in area. The larger pores, chiefly with cross-sectional areas above $40 \mu\text{m}^2$, were less represented by the end of ripening—and likely explain the observed significant decrease of cheese porosity without a change in number of pores. Field viewing significantly affected the microstructural parameter values, whereas section viewing affected significantly only LM-based ones. Categorical principal component analysis between the 2 types of microstructural data sets was performed, and permitted discrimination of each stage of ripening. Multiple linear regression analysis indicated that

the variables associated with the nitrogen fraction were well predicted by stereological-based parameters ($R^2 \geq 0.96$). Therefore, our findings demonstrate the potential of image analysis to monitor microstructure throughout ripening, and that the microstructure revealed by LM reflects more clearly cheese aging than that revealed by TEM.

Key words: light microscopy, transmission electron microscopy, microstructure, pore size distribution

INTRODUCTION

Over the last 2 decades, Serra da Estrela, the most famous and unique Portuguese cheese, has been investigated, aiming not only at its technological optimization, but also at a better fundamental understanding of its microbiological, physicochemical and rheological evolution during ripening (Macedo et al., 1993, 1996; Tavaría and Malcata, 1998; Tavaría et al., 2003); however, a knowledge gap relating to its microstructure remains. Parker et al. (1998) originally used light and electron microscopies to ascertain the distribution of microorganisms within mature Serra da Estrela cheese, whereas more recently Tavaría et al. (2006) attempted to correlate microbiological data with scanning electron micrographs of cheeses manufactured in distinct dairy farms (including cheesemaking milk subjected to previous refrigeration). Despite these efforts, there is still a lack of information on how microstructure evolves throughout ripening of this cheese—especially on the relationships between the porous nature of the curd network and the classical indices of proteolysis.

Microimaging of cheese has been described by several authors, using transmission electron microscopy (TEM; Tamime et al., 1999; Kheader et al., 2002), scanning electron microscopy (Rodríguez et al., 1999; Sipahioglu et al., 1999; Özer et al., 2003), and confocal light microscopy (LM; Pavia et al., 1999; Guinee et al., 2000; Buffa et al., 2001; O'Reilly et al., 2002). It is indeed generally accepted that food texture relates directly to microstructure, which, in turn, is determined by chemical composition. During ripening of Serra da

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Estrela cheese in particular, extensive changes occur in terms of proteolysis (Macedo and Malcata, 1997; Tavaría et al., 2003) that eventually determine texture, which evolves toward a rather soft and buttery one. Primary proteolysis brought about by plant rennet enzymes is believed to play a fundamental role in changes of cheese texture early in ripening, by disrupting the relatively extensive network of α_{s1} -casein in the fresh curd (Creamer and Olson, 1982; Fox et al., 1993; Johnston et al., 1994; Watkinson et al., 2001). However, details on the relationship between changes in cheese structure during ripening and texture are scant, so efforts to help improve the quality of such a complex and heterogeneous dairy product still rely on essentially empirical approaches.

Microscopy provides rich qualitative information about the microstructure of cheese and changes thereof during ripening, but this type of information is often hard to quantify; comparisons between samples, as well as attempts to rationalize textural development based on morphology of the protein network, are, thus, difficult. On the other hand, design-based techniques have been made available in recent times, which enable estimates of germane parameters (e.g., number, length, area, and volume of pores) to be determined, while allowing quantification of arbitrary-shaped features in sections. These stereological methods include unbiased sampling schemes in combination with geometric probes (e.g., points, lines, and planes) to quantify first- and second-order stereological parameters, and do not require simplistic assumptions relating to the shape of the pore network. Three-dimensional interpretation of the 2-dimensional (2D) images obtained by microscopy is then possible, based on the stereological concept (Gundersen et al., 1988a). Choice of an appropriate grid is primarily dictated by the need for isotropic, uniform, and random sampling of the microstructure. If isotropy occurs, then any array of lines provides an unbiased sample; however, if the matrix is anisotropic, then the grid should guarantee uniform sampling in all directions. For vertical sections, either cycloid grids or lines that are sine-weighted, rather than randomly orientated, are required (Baddeley et al., 1986; Mouton, 2002; Russ, 2005). If the sample is assumed to be random, isotropic, and uniform, then any sample, section, and image can be used, and it is sufficient to examine enough fields of view to obtain adequate statistical precision.

To date, approaches using stereological concepts have been applied with success to a few cheeses (Guinee et al., 1995; Wium et al., 2003; O'Mahony et al., 2005), as well as particulate whey protein gels (Langton and Hermansson, 1996), stirred yogurt (Skriver et al., 1997), mayonnaises (Langton et al., 1999), pasta dough

(Thorvaldsson et al., 1999), whipped cream (Smith et al., 1999), apple cubes (Funebo et al., 2000), gelatin/maltodextrin systems (Lorén et al., 1999; Hermansson et al., 2000), β -LG/amylopectin gels (Olsson et al., 2002), micellar casein gels (Auty et al., 2005), and baked bread (Datta et al., 2007) to obtain representative measures of 3-dimensional structures from these samples. To our knowledge, the only study that focused on the relationship between textural properties and microstructural data generated via image analysis during cheese maturation was carried out by O'Mahony et al. (2005); and none has ever investigated such a unique (and traditional) cheese as Serra da Estrela in this regard.

Hence, the major objective of this research effort was to find relationships between microstructural parameters and age-related physicochemical changes in ovine Serra da Estrela cheese. To this goal, stereological estimates were produced, and used to generate quantitative information on its 3-dimensional network.

MATERIALS AND METHODS

Cheesemaking and Sampling

Two independent batches of Serra da Estrela cheese were manufactured from raw ewe's milk, following the conventional protocol approved by the regulatory board of its Appellation d'Origine Protégée status (Macedo et al., 1993). Milk was warmed to 32°C, and then coagulated for 45 min using a mixture of macerated thistle flower (*Cynara cardunculus* L., added at a rate of 0.2 g per L of milk) and salt (added at a rate of 20 g per L of milk). Afterward, the curd was cut into small particles, using knives in a circular movement. Ten minutes later, the curds were placed in a cheesecloth, and pressed manually to aid in whey expression. Drainage of whey was completed via pressing the fresh cheese in a pneumatic press, at 250 kPa for 4 h. The cheeses were ripened for 60 d: they were first kept at 8°C and a relative humidity of 99% for 3 wk, and then at 11°C and a relative humidity of 85% for the remaining time. Two independent cheeses were selected at random at each of 3 ripening times (1, 21, and 60 d), so 4 true replicates were subjected to chemical, rheological, and microstructural analyses.

Chemical Analyses

The determination of moisture, fat, salt, titratable acidity, pH, and protein contents, as well as total nitrogen, water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCASN) and phosphotungstic

Figure 1. Sequence of sampling procedures encompassing cheese sections. (A) Parallelepipedal blocks are taken at random from cheese ($a \times a \times h$); (B) sections (ca. 1-mm thick, t) are cut parallel to the vertical axis; (C) small sections are taken at random and embedded in fixed resin; and (D) semi-thin ($0.4 \mu\text{m}$) sections are placed on slides for observation by light microscopy (a), and ultra-thin (70 nm) sections are placed on copper grids for observation by transmission electron microscopy (b).

acid-soluble nitrogen (**PTASN**) were described in detail elsewhere (Reis et al., 2000). All analyses were performed in duplicate.

Rheological Analyses

Each cheese sample was taken at random from each of the true cheese replicates, and kept at 20°C for 1 h before analysis. Texture profile analysis was performed on the cheese surface, using a texture analyzer TA-XT Plus (Stable Micro Systems, Surrey, UK) connected to a 2-mm probe. Each test was conducted as 2 sequential penetration events, of 20-mm penetration at a rate of 0.83 mm/s , separated by a rest period of 10 s. The tests generated a force-time curve, from which fracturability, firmness, cohesiveness, adhesiveness, springiness, gumminess, chewiness, and Young's modulus were automatically calculated. All analyses were performed in quintuplicate.

Sample Preparation for Microscopic Analyses

Cheese samples were examined for their microstructure using LM and TEM. Blocks of cheese ($10 \times 10 \times 20 \text{ mm}$) were taken with a trier inserted vertically through the cheeses from their top surface (see Figure 1); those samples were completely submerged in 4% (vol/vol) formalin, and fixed for at least 1 mo before

cutting (Parker et al., 1998). These blocks were then cut into vertical sections, of approximately $2 \times 2 \times 1 \text{ mm}$, postfixed in 1% (wt/vol) osmium tetroxide, dehydrated using a graded ethanol series (10 to 100%), and placed in a transitional solvent (propylene oxide). Infiltration with resin was sequentially performed using a mixture of Epon 812 (TAAB Laboratories, Berkshire, UK):propylene oxide at 1:50 for 6 h, 75:25 for 6 h, and finally 100:0 overnight in a refrigerator. On the next day, the Epon was replaced by fresh Epon, and left for an extra 8 h; finally, the samples were placed in Epon gelatin capsules, which were polymerized for ca. 48 h at 55°C . Semi-thin sections (approximately $0.25 \mu\text{m}$ in thickness) for LM and ultra-thin sections (approximately $0.06 \mu\text{m}$) for TEM were cut using an LKB 8800 ultramicrotome (LKB, Bromma, Sweden), equipped with glass and diamond knives, respectively.

The aforementioned semi-thin sections were collected in (filtered) pure water, stained using a 1:1 mixture of 1% toluidine blue and 1% sodium borax to visualize the protein phase, and mounted on glass microscope slides (see Figure 1). Such sections were then covered with a cover glass and examined with an Olympus BH-2 microscope (Olympus, London, UK), fitted to a Sony XC 77 camera (Sony, Tokyo, Japan). The ultra-thin sections were, in turn, collected on copper grids (see Figure 1), and contrasted with 2% of uranyl acetate for 7 min, followed by lead citrate for another 5 min

(Reynolds, 1963); examination and image acquisition proceeded afterward with a Zeiss AxioCam (Carl Zeiss, Oberkochen, Germany), fitted to a Zeiss EM 902A TEM (Carl Zeiss) at 50 kV.

For image analysis, independent nonoverlapping LM and TEM micrographs (characterized by $1,280 \times 1,024$ and $1,294 \times 1,020$ pixels, respectively) were acquired at $200 \times$ and $4,400 \times$ magnifications, respectively, thus yielding a resolution of $0.333 \mu\text{m}/\text{pixel}$ and $7.54 \text{ nm}/\text{pixel}$, respectively (see Figure 2). The relevant image parameters (described below) were then estimated for each micrograph, and averaged among micrographs pertaining to each cheese age.

Image Analysis and Stereological Procedures

Quantitative measurements were made along vertical sections, via a stereological method and then subjected to image analysis using Fovea Pro v. 3.0 software (Reindeer Graphics, Ashville, NC) with Adobe Photoshop v. 7.0 (Adobe Systems, San Jose, CA) as the host program. Said measurements were performed on LM and TEM binary images created by simple thresholding, using a gray-level established after examination of the histogram of gray-level values of the image at stake. From each semi-thin (and ultra-thin) section, every 8th (or every 6th) field was systematically sampled, and 3 stereological measures were taken on each image (relating to a field): $\mathbf{A}_A$, the area fraction of the nonprotein phase, which is related to the void fraction; $\mathbf{P}_L$, half of the surface area/volume ratio; and $\mathbf{L}_3$, the mean intercept length. The binary image was thereafter skeletonized and pruned; on this latter representation, 3 structural parameters were further measured: ratio of protein skeleton length to area (hereafter, length), ratio of node count to area (hereafter, nodes), and ratio of ends count to area (hereafter, ends; see Figure 3).

All stereological measurements were taken in between 6 and 8 systematic random microscopic fields, within 3 random sections taken from 2 or 3 cheese blocks of each cheese (thus leading to either 9 or 6 analytical replicates, respectively). Arithmetic means and standard errors were determined as appropriate.

Statistical Analyses

Nonprotein phase densities, obtained via the profile counting method, and skeleton data and estimated volume-weighted mean volume, obtained via the stereological point-sampled intercepts method, were all analyzed using 3-level, nested ANOVA (with time, block nested within time, and section nested within block as parameters), in an attempt to ascertain the significance of differences, as well as the intensity of variation within

and between microscopic fields, sections, and blocks. A mixed model was accordingly applied, with block and section treated as random factors, and time considered as fixed factor. Blocks and sections were randomly sampled, so their interactions with treatments were also considered as random effects. The variability among sections, the variability among blocks and the variability among ripening times were consequently estimated. Finally, principal component analysis (**PCA**) was used to reduce the information conveyed by the too large number of microstructural variables considered, to the level of only essential information, and to describe their interrelation pattern, as well as their putative relationships with cheese aging.

The hypothesized correlations between cheese proteolytic indices and microstructural parameters were assessed by regression analysis. For multiple regressions, and to optimize the number of variables included in the model, the backward selection method was pursued. Multicollinearity may be present when the independent variables are highly correlated with each other; in such a case, even though the coefficient of determination (R^2) values look good, the parameter estimates may change erratically in response to small alterations of the model or disturbances of the data; hence, a principal component regression analysis was performed (Liu et al., 2003) in attempts to anticipate (and overcome) this problem, as the regression equation will be built upon a set of essentially uncorrelated regressors. The principal component scores of the variables included in the model were thus used as regressors, and the proteolytic variables as functions; and nonsignificant score values were excluded from the model by a stepwise methodology. Student's tests were used to assess the regression coefficients. Finally, the coefficient of determination was used as criterion for success of the prediction model.

All statistical analyses were conducted with SPSS v. 16.0 (SPSS, Chicago, IL), and the level of significance was arbitrarily set at 0.05 (or 0.01 or even lower, as appropriate).

RESULTS AND DISCUSSION

Microstructural Profile

The microstructure of Serra da Estrela cheese was assessed at different levels by LM and TEM (see Figure 2). Irrespective of magnification, these micrographs show a continuous, yet irregular protein phase as a black background; and a nonprotein phase, represented in white, which includes void spaces, namely those resulting from spaces previously occupied by fat globules or gas bubbles. From these typical (and many other) micrographs, the microstructural properties of

the cheese matrices were analyzed at various ripening times, namely in terms of number of pores per unit area, fraction of nonprotein phase, and contact area between nonprotein and protein phases. It should be noted that void space appears as patches, which can represent single pores or a network of pores connected to each other. The corresponding stereological parameters, obtained from LM and TEM images of cheese samples, are tabulated in Table 1; note that the 6 (or 9) analytical replicates referred to are actually averages of 36 (or 72) fields of view each, as required for a representative and meaningful statistical analysis.

The average size of the pores was evaluated via L_3 ; this is a unique assumption-free value, which is valid for particles/channels of any size and configuration. As shown in Table 1, L_3 values based on LM images were, on average, 2.70 ± 0.04 , 2.90 ± 0.06 , and 2.74 ± 0.05 μm , for samples taken at 1, 21, and 60 d, respectively. Although L_3 appeared higher by 21 d, such a difference was not significant ($P > 0.05$). Likewise, the ripening time played no significant effect on the number of pores if based on LM images; this is likely a consequence of the great variability among consecutive images, which accounted for 74.5 and 65.2% of L_3 and number of pores, respectively.

In our study, A_A relates to the space occupied by the nonprotein phase in the cheese matrix; it is given by the volume fraction, as cheese samples were analyzed by stereological design-based methods (Gundersen et al., 1988b). Inspection of Table 1 indicates that the area fraction underwent a decreasing trend along ripening time with statistically significant differences ($P < 0.05$) between 21 and 60 d in the case of LM data, and between 1 d and later times (21–60 d) in the case of TEM data. These findings indicate that porosity was lowest in cheeses by 60 d of ripening, so an increase in protein phase density apparently took place as time elapsed. This microstructural feature is consistent with the evolution of moisture content of cheeses ($r = 0.738$, $P < 0.01$): once again, no significant changes were observed in moisture content during the first 21 d of ripening (within 55–54%), but the moisture decreased significantly afterward ($P < 0.05$), from 54.0 % by 21 d to 47.0% by 60 d of ripening.

The frequency of pores in cheese, sorted by increasing area, is plotted in Figure 4A for 3 times of ripening (note that the pores with areas above $40 \mu\text{m}^2$ were not considered in Figure 4A, so the cumulative values do not add up to 100%). From the 2D LM images, one realizes the importance of the pores ranging between 1 and $5 \mu\text{m}^2$ (which account for ca. 45% of the total pores), even though they represent only 3 to 4% of the cheese porosity; this happens because few large pores

account for most void volume. In terms of number of pores, ripening time played a significant ($P < 0.05$) role in almost all pore classes; significant statistical differences were indeed found when the percents of each pore class by 1 d were compared with their 21-d counterparts. As apparent in Figure 4A, pores with areas smaller than $5 \mu\text{m}^2$ decreased in number from 1 to 21 d, whereas those with areas between 5 and $40 \mu\text{m}^2$ (i.e., ca. 40% of the pores) increased in number. This observation is consistent with their contribution to cheese porosity: pores with areas greater than $40 \mu\text{m}^2$ increased significantly in number from 1 to 21 d ($P < 0.05$), despite representing only 15% of the pores, so their contribution to porosity decreased (data not shown). These trends suggest a rearrangement of the cheese matrix from 1 to 21 d, without changes in terms of pores or porosity; however, P_L decreased significantly ($P < 0.05$) throughout ripening time.

From 21 to 60 d, the pores seemed to become smaller, on average. Despite the behavior of pores with areas below $1 \mu\text{m}^2$, the contribution of all pore classes to porosity was statistically different by 60 d relative to that by 21 d: an increase in the number of pores smaller than $20 \mu\text{m}^2$ was indeed observed, with a concomitant decrease in the numbers belonging to the other classes. The significant decrease of the fraction of larger pores, especially those above $40 \mu\text{m}^2$ (which account for ca. 75% of porosity), might explain the significant decrease in cheese porosity without a relevant change in number of pores.

Based on TEM images, one concluded that ripening time had essentially no effect ($P > 0.05$) upon cheese microstructure, except with regard to pores ($P < 0.05$; see Table 1). No statistically significant differences were found in terms of porosity between 1 and 21 d, but the number of pores was lower by 21 d than by 1 d; hence, some pores grew along that period. The results depicted in Figure 4B indicate clearly a larger increase of pores in the classes below $1 \times 10^{-2} \mu\text{m}^2$, which was particularly significant ($P < 0.05$) in the case of pores with areas ranging from 0.10×10^{-2} to $0.55 \times 10^{-2} \mu\text{m}^2$. Conversely, the decrease in number of pores in the classes above $1 \times 10^{-2} \mu\text{m}^2$ was more pronounced than the increase in number of those below such a threshold. Consequently, the period from 1 to 21 d was characterized by a statistically significant increase of the contribution of the pores with areas ranging from 0.10×10^{-2} to $0.55 \times 10^{-2} \mu\text{m}^2$ to porosity, with a simultaneous decrease in the number of pores with areas ranging from 5.5×10^{-2} to $10 \times 10^{-2} \mu\text{m}^2$. This realization explains why porosity did not essentially change from 1 to 21 d. Later during ripening, no significant changes ($P < 0.05$) were observed

in the frequency of pores by area, except that pores with areas below $0.1 \times 10^{-2} \mu\text{m}^2$ increased in number, but without any relevant effect upon cheese porosity. Note that in Figure 4B, unlike Figure 4A, the fraction of pores in each class was normalized by the amplitude of said class.

Intrinsic Validity and Variability of Measurements

The relative contributions of ripening time on the one hand, and of block and section on the other, to the stereological parameters calculated from LM and TEM micrographs are depicted in Figure 5. Analysis

Figure 2. Typical micrographs of cheese samples obtained by light microscopy (A) and transmission electron microscopy (B), by (a) 1 d, (b) 21 d, and (c) 60 d. The bar represents 50 μm (A) and 1 μm (B).

B

Figure 2 (Continued). Typical micrographs of cheese samples obtained by light microscopy (A) and transmission electron microscopy (B), by (a) 1 d, (b) 21 d, and (c) 60 d. The bar represents 50 μm (A) and 1 μm (B).

Figure 3. Sequence of imaging procedures encompassing cheese sections: (A) binary image obtained directly, with protein network shown in white and nonprotein phase in black; (B) inverted binary image of A; (C) skeletonized binary image of the protein network of B; and (D) schematic detail of a skeleton in C, with indication of nodes and ends.

of variance revealed that ripening time had a significant effect upon such microstructural parameters as A_A , P_L , length, nodes, and ends, based on LM images. In the case of L_3 and number of pores, ripening time played no significant role; for these parameters, the predominant factors were indeed the fields of view, which accounted for 74.5 and 65.2%, respectively, of the overall variability. A similar trend was observed for the microstructural parameters measured from TEM images, especially A_A and L_3 ; here, the fields of view accounted for an even higher proportion, namely 94.1 and 98.8% of the observed variability, respectively. This high relative variability between viewing fields is often found, and tends to increase with the level of magnification; cheese section had a significant ($P < 0.05$) effect toward microstructural parameters measured by LM, but not toward those measured by TEM, and typically

followed a Poisson distribution. Although ripening time could explain, to a higher degree, the variation of such TEM-based parameters as P_L , length, ends, and pores, the effect of ripening time was significant ($P < 0.05$) only for the latter (Figure 5). The high heterogeneity detected in Serra da Estrela cheese should, therefore, be emphasized, as it does not occur to such a high level in industrially processed cheeses at large.

With regard to the frequency of pores by area based on TEM images, ANOVA suggests that the viewing fields contributed with most variability, either in terms of number of pores or porosity, whereas the variability between sections explained a large proportion thereof only for those pores in the area range from 0.10×10^{-2} to $0.55 \times 10^{-2} \mu\text{m}^2$ (data not shown). On the other hand, the variability accounted for by ripening time was statistically significant only for pores with areas

Table 1. Changes in stereological parameters of cheese throughout ripening time

Stereological parameter ¹	Ripening time ²			Time effect (<i>P</i> -value)
	1 d	21 d	60 d	
LM images ³				
A _A (%)	36.2 ± 0.5 ^a	35.3 ± 0.9 ^a	30.0 ± 1.1 ^b	0.001
P _L (μm ⁻¹)	0.406 ± 0.007 ^a	0.370 ± 0.010 ^b	0.329 ± 0.009 ^c	0.007
L ₃ (μm)	2.70 ± 0.04 ^a	2.90 ± 0.06 ^a	2.74 ± 0.05 ^a	0.164
Length (μm ⁻¹)	0.103 ± 0.002 ^a	0.076 ± 0.003 ^b	0.066 ± 0.004 ^b	0.000
Nodes (× 10 ⁻³ μm ⁻²)	9.6 ± 0.4 ^a	6.4 ± 0.4 ^b	5.9 ± 0.5 ^b	0.000
Ends (× 10 ⁻³ μm ⁻²)	27.8 ± 0.3 ^a	24.3 ± 0.9 ^b	23.3 ± 0.5 ^b	0.006
Pores (× 10 ⁻³ μm ⁻²)	10.7 ± 0.1 ^a	10.6 ± 0.5 ^a	10.7 ± 0.2 ^a	0.930
TEM images ⁴				
A _A (%)	6.5 ± 0.9 ^a	3.4 ± 1.3 ^a	4.7 ± 0.4 ^a	0.331
P _L (μm ⁻¹)	79.0 ± 10.5 ^a	26.2 ± 3.5 ^b	32.2 ± 4.8 ^b	0.055
L ₃ (μm)	0.096 ± 0.006 ^a	0.118 ± 0.025 ^{ab}	0.169 ± 0.019 ^b	0.220
Length (μm ⁻¹)	0.42 ± 0.06 ^a	0.14 ± 0.03 ^b	0.21 ± 0.02 ^b	0.080
Nodes (× 10 ⁻³ μm ⁻²)	792 ± 119 ^a	271 ± 47 ^b	463 ± 102 ^b	0.170
Ends (× 10 ⁻³ μm ⁻²)	2,698 ± 299 ^a	1,081 ± 148 ^b	1,404 ± 267 ^b	0.068
Pores (× 10 ⁻³ μm ⁻²)	972 ± 99 ^a	415 ± 52 ^b	483 ± 88 ^b	0.040

^{a-c}Means within a row, not followed by the same superscript, are statistically different ($P < 0.05$).

¹LM = light microscopy; TEM = transmission electron microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L_3 = mean intercept length.

²Values are expressed as mean $\pm$ SEM.

³Four true replicates $\times$ 9 analytical replicates.

⁴Four true replicates $\times$ 6 analytical replicates.

Figure 4. Fractional distribution of pores per area classes, as obtained by light microscopy (A) and transmission electron microscopy (B), throughout ripening time, in absolute (symbols with dashed line, left axis) and cumulative terms (symbols with solid line, right axis).

from 0.10×10^{-2} to 0.55×10^{-2} , and from 55×10^{-2} to $100 \times 10^{-2} \mu\text{m}^2$ (33.1 and 11.0%, respectively). When porosity was considered, the variability accounted for by ripening time was significant ($P < 0.05$) only for pores with individual areas below $0.1 \times 10^{-2} \mu\text{m}^2$. If LM images were considered instead, ripening time and cheese section had a significant effect upon the pore distribution for pores in the ranges from 25 to 30 and from 35 to $40 \mu\text{m}^2$, and likewise in terms of porosity, ex-

cept for pores in the range of 5 to $10 \mu\text{m}^2$. As expected, cheese block had no significant effect ($P > 0.05$) upon any of the structural parameters studied, so random sampling appeared to be effective.

When performing the textural assays, it might be argued that the 2-mm probe used was too thin to cause significant compression of the sample; however, Breuil and Meullenet (2001) found a significant correlation between measurements using a similar probe and textural

Figure 5. Contributions of ripening time (black), block (white), and section factors (dark gray), and of intrinsic replicate variabilities (light gray) to the total variance of stereological data. TEM = transmission electron microscopy; LM = light microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L₃ = mean intercept length.

characteristics of a wide range of commercial cheeses (also validated by a sensory panel), so this approach was elected here.

Relating to microscopic observations, formalin has the advantage of penetrating quickly (within a matter of minutes) the cheese sample, owing to the porosity of the latter and the small molecular size of the former, so ripening vectors were readily inactivated; however, complete structural stabilization of the samples took longer, because crosslinking with molecules is a relatively slow process. In any case, the aforementioned fixative disables enzymes (e.g., proteases) fast that would otherwise change the sample, and its toxicity to most microorganisms precludes further metabolic changes in the sample as well. On the other hand, LM should preferably be used on native cheese samples; however, the unique composition of this cheese led to poor micrographs, and confocal microscopy produced observations excessively blurred to be useful; therefore, only fixed samples were exposed to LM (which would anyway be strictly required, to obtain valid quantitative information by stereological techniques).

Finally, each stereological parameter was estimated from 72 and 36 fields of view per cheese, for LM and TEM, respectively. Despite the large percent of variability

among fields of view, their number could not grow without limit, so a balance between limited analytical resources and intrinsic heterogeneity of the samples was eventually attained.

Relationships Between Microstructural Parameters

In attempts to unfold interrelationships among stereological parameters, while simultaneously detecting the most important causes of variability, PCA was applied. First, the Kaiser-Meyer-Olkin (KMO) and the Bartlett test of sphericity indicated that the environment data were suitable for that analysis (KMO = 0.682 and $\chi^2 = 3938.885$, respectively, with $P < 0.01$). Four principal components could, thus, be extracted, which accounted for 90.8% of the whole variability within the original data set (see Table 2); commonalities involving the variables selected were high, so they also shared a considerable variance.

Based on the rotation sum of squares loadings, the first principal component accounted for 45.1% of the total variance; variables that loaded higher onto this component reflected mainly the microstructural characteristics assessed by TEM, except A_A and L₃. The second principal component accounted for 22.5% of the

Table 2. Significant ($P < 0.01$) correlations between stereological parameters, expressed as principal components¹

	Principal component				
Item ²	1	2	3	4	Commonalities
LM images					
A _A (%)		0.872	−0.328		0.887
P _L (μm ^{−1})		0.804	0.440		0.887
L ₃ (μm)			−0.833		0.827
Length (μm ^{−1})		0.910			0.909
Nodes (× 10 ^{−3} μm ^{−2})	0.320	0.815			0.775
Ends ³ (× 10 ^{−3} μm ^{−2})		0.596	0.731		0.945
Pores (× 10 ^{−3} μm ^{−2})			0.914		0.848
TEM images					
A _A (%)	0.470			0.852	0.948
P _L (μm ^{−1})	0.909				0.959
L ₃ (μm)				0.936	0.934
Length (μm ^{−1})	0.800			0.530	0.966
Nodes (× 10 ^{−3} μm ^{−2})	0.917				0.928
Ends (× 10 ^{−3} μm ^{−2})	0.967				0.975
Pores (× 10 ^{−3} μm ^{−2})	0.939				0.930
Variability					
Initial eigenvalues	6.3130	3.1440	2.036	1.225	
Variance explained (%)	45.093	22.457	14.544	8.751	
Cumulative variance explained (%)	45.093	67.550	82.094	90.845	

¹Twelve true replicates × 36 analytical replicates.²LM = light microscopy; TEM = transmission electron microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L₃ = mean intercept length.³Parameter dropped from final solution because of high loadings on more than one factor.

total variance and was defined by almost all LM variables, except L₃, ends, and number of pores. The third principal component accounted for 14.5% of the total variance; it was related to such LM characteristics as L₃, ends, and number of pores. Finally, the fourth principal component was dominated by A_A and L₃, both determined by TEM, and accounted for 8.8% of the total variance. A deeper analysis of the first principal component indicates that almost all variables measured by TEM are highly correlated with each other ($r > 0.8$, $P < 0.01$), whereas the variables with high factor loadings on the second principal component are more related to cheese microstructure as dictated by LM observations.

In order to more easily ascertain the influence of ripening time, the scores of cheese samples by 1, 21, and 60 d, as well as the relevant processing variables were displayed on the same 2D plot (Figure 6); each variable is represented by a line starting at the origin of the plot, and ending at a point with coordinates given by the loadings on the 2 dimensions. Note that the TEM variables appear close to each other (at the bottom right corner of Figure 6), thus indicating a strong relationship between them (i.e., the smaller the angle between the associated vectors, the higher the correlation between the corresponding variables). A similar rationale applied to the 4 lines depicting variables A_A, P_L, length, and nodes (at the upper right corner): al-

though all retained variables exhibited high (positive) loadings along dimension 1, dimension 2 could discriminate the 4 variables based on LM from the 5 variables based on TEM: variables associated with LM loaded positively along dimension 2, whereas those associated with TEM did so negatively. This indicates that cheese samples with high values on dimension 1 will report relatively high values on both LM and TEM vectors, and vice-versa; cheese samples with high positive, or negative values on dimension 2 will be more, or less, oriented toward LM than TEM vectors, respectively. Because categorical PCA was able to explain 85.6% of the total variance via its first 2 factors, the associated dimensionality of the results could be reduced to 2 components at the expense of only 14.4% loss in variance, which is an interesting and useful practical result. Furthermore, Figure 6 suggests that microstructural parameters are closely related with ripening time; on average, 21- and 60-d cheeses scored similarly on dimension 1, but yielded opposite values on dimension 2, although they partially overlapped on this dimension; their values on dimension 1 differed significantly from those by 1 d.

One observes that 1-d cheeses were spread over 2 quadrants that are formed by the positive part of the LM lines and the positive part of the TEM lines; 21-d cheese samples tended to lie on the negative side of TEM vectors, thus implying that nanostructure plays

a less important role; and 60-d cheeses lay chiefly on the quadrant formed by the negative side of the lines representing LM, so microstructural differences were also important at later stages of ripening. In other words, cheeses by 1 d are characterized by relatively high values on the scales that represent both dimensions, whereas 21 d-old cheeses lead to low values on the scales representing TEM and LM (except A_A), and 60 d-old cheeses are characterized by low values on the scales representative of both dimensions. The plot of component loadings (Figure 6) also outlined the fact that A_A can be considered as being independent from any TEM-associated parameters because the latter lines are more or less perpendicular to the A_A line.

Relationships Between Microstructural, Chemical, and Rheological Parameters

No significant changes were observed in fat, NaCl, and protein contents (which yielded 49.8%, 3.4%, and 41.6% on average, respectively, on a dry matter basis) along ripening. The initial pH of cheese was ca. 5.8, but decreased significantly thereafter until 21 d, remaining at ca. 4.9 throughout the remainder of ripening. The titratable acidity also decreased significantly throughout the 60-d period. No significant changes were perceived in moisture content during the first 21 d of ripening, unlike between 21 and 60 d (with significant decreases from 54.0 to 46.9%, between 21 and 60 d of ripening, as mentioned before). The concentrations of WSN, TCASN, and PTASN, expressed as percentage of total nitrogen, increased significantly during ripening: from 9.62 to 23.33%, from 1.48 to 3.71%, and from 0.56 to 2.60%, respectively, between 1 and 60 d.

Pearson correlation coefficients were calculated to back up any suspected linear relationships between microstructural parameters (based on LM and TEM images) and nitrogen fractions (i.e., WSN, TCASN, and PTASN) throughout ripening time. The corresponding results, listed in Table 3, show that all microstructural parameters bore a negative correlation with nitrogen fractions, except L_3 , which held a positive correlation; however, it was significant ($P < 0.05$) only for those pores measured by TEM. Hence, it seems that L_3 increases as proteolysis takes place. Apart from L_3 and number of pores, all LM-based parameters entertained a significant correlation ($P < 0.01$) with the aforementioned nitrogen fractions. In general, said parameters were better correlated with the nitrogen fractions (with absolute correlation coefficients ranging between 0.6 and 0.9) than were TEM-based ones (with absolute correlation coefficients ranging merely from 0.4 to 0.7), hence suggesting that structure revealed by LM (i.e.,

Figure 6. Loading score plot in terms of the first 2 principal components ($n = 108$), pertaining to light microscopy ($_L$) and transmission electron microscopy ($_T$), throughout ripening time (1, 21, and 60 d), and encompassing such stereological parameters as area fraction of the nonprotein phase (A_A), half of the surface/volume ratio (PI), length, nodes, ends, and pores.

microstructure) could more clearly reflect cheese aging than that revealed by TEM (i.e., nanostructure).

The most important biochemical reactions occurring in most varieties of cheese during ripening are related to proteolysis (Fox and McSweeney, 1996); primary proteolysis (measured chiefly by WSN) causes softening of the cheese matrix early during ripening, via disruption of its 3-dimensional protein network (Fox et al., 1993), whereas secondary proteolysis, brought about by the microflora rather than the residual rennet (and measured chiefly by PTASN), causes a more localized breakdown. This is somewhat consistent with the good correlations found between A_A (measured by LM) and PTASN ($r = -0.798$, $P < 0.01$), and to a slightly lesser extent with TCASN ($r = -0.717$, $P < 0.01$) and WSN ($r = -0.670$, $P < 0.01$); hence, it appears that ripening depth reflected changes in A_A . The highest correlation coefficients were found between length (measured by LM) and WSN ($r = -0.890$, $P < 0.01$); length was also well correlated with PTASN ($r = -0.777$, $P < 0.01$) and TCASN ($r = -0.772$, $P < 0.01$), but to lesser degrees. Likewise, P_L (as measured by LM) did correlate well with WSN ($r = -0.787$, $P < 0.01$), PTASN ($r = -0.788$, $P < 0.01$), and TCASN ($r = -0.755$, $P < 0.01$). There-

Table 3. Significant correlations between stereological parameters and cheese ripening parameters

Stereological parameter ¹	Ripening parameter ²		
	WSN (%, wt/wt)	TCASN (%, wt/wt)	PTASN (%, wt/wt)
LM images ³			
A _A (%)	-0.670**	-0.717**	-0.798**
P _L (μm ⁻¹)	-0.787**	-0.755**	-0.788**
L ₃ (μm)			
Length (μm ⁻¹)	-0.890**	-0.772**	-0.777**
Nodes (× 10 ⁻³ μm ⁻²)	-0.780**	-0.655**	-0.645**
Ends (× 10 ⁻³ μm ⁻²)	-0.755**	-0.623**	-0.625**
Pores (× 10 ⁻³ μm ⁻²)			
TEM images ⁴			
A _A (%)			
P _L (μm ⁻¹)	-0.716**	-0.562**	-0.452*
L ₃ (μm)	0.536*	0.596**	0.570**
Length (μm ⁻¹)	-0.630**	-0.437*	
Nodes (× 10 ⁻³ μm ⁻²)	-0.488*		
Ends (× 10 ⁻³ μm ⁻²)	-0.628**	-0.483*	
Pores (× 10 ⁻³ μm ⁻²)	-0.675**	-0.548**	-0.413*

¹LM = light microscopy; TEM = transmission electron microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L₃ = mean intercept length.

²WSN = water-soluble nitrogen; TCASN = trichloroacetic acid-soluble nitrogen; PTASN = phosphotungstic acid-soluble nitrogen.

³Twelve true replicates × 9 analytical replicates.

⁴Twelve true replicates × 6 analytical replicates.

* $P < 0.05$; ** $P < 0.01$: significant correlation (single-tailed).

fore, as proteolysis occurs, length, P_L and A_A decrease, and thus reflect a coarser protein network.

Considering TEM-based parameters, the highest correlations were found between P_L and WSN ($r = -0.716$, $P < 0.01$) and TCASN ($r = -0.562$, $P < 0.01$). The area occupied by pores exhibited no significant correlations with the aforementioned nitrogen fractions; this realization is consistent with the data tabulated in Table 1. The L₃ was poorly correlated with WSN ($r = 0.536$, $P < 0.05$), TCASN ($r = 0.596$, $P < 0.01$), and PTASN ($r = 0.570$, $P < 0.01$). From TEM images, it seemed that the volume of individual pores increased as ripening time elapsed; however, the variability among fields of view accounted for more than 80% (Figure 5).

Correlation coefficients were likewise calculated as a means to ascertain putative relationships between microstructural and rheological parameters (Table 4). As expected, A_A was negatively correlated ($P < 0.01$) with fracturability, firmness, gumminess, chewiness, and Young's modulus (with absolute correlation coefficients above 0.7, $P < 0.01$). It is well known that increasing the moisture content or the ratio of moisture to protein weakens the cheese continuum, because the volumetric fraction of protein (i.e., the backbone of the curd) decreases. On the other hand, the adhesiveness yielded a positive correlation with A_A ($r = 0.757$, $P < 0.01$); recall that adhesiveness in Serra da Estrela cheese remained essentially constant up to 21 d, but

decreased significantly by 60 d. Similar correlations were observed between P_L and the foregoing rheological parameters. In particular, it is interesting to note that L₃ (measured by LM) entertained a significantly better correlation with springiness ($r = -0.498$, $P < 0.01$); it seems that L₃ may be directly related to weakening of the proteinaceous network.

As happened with the nitrogen fractions, LM parameters, in general, correlated better with rheological parameters than did TEM-based ones. Surprisingly, springiness exhibited a stronger correlation with TEM- than LM-based parameters, in particular with P_L ($r = 0.769$, $P < 0.01$) and length ($r = 0.767$, $P < 0.01$). Finally, length correlated better with rheological parameters, mainly fracturability.

In view of the above, changes in the extent of proteolysis can apparently be predicted via microstructural parameters (and vice-versa), especially in the case of TCASN and PTASN. Furthermore, several rheological features of cheese, in particular fracturability, adhesiveness, and Young's modulus, can also be accurately predicted by microstructural parameters (and vice versa).

The underlying hypothesis of this study was that changes in cheese microstructure are associated with changes in chemical parameters throughout ripening. Multiple regression analysis was, thus, carried out, encompassing microstructural parameters and proteolytic indices. The results are listed in Table 5, and indicate

Table 4. Significant correlations between stereological parameters and texture parameters

Stereological parameter ¹	Texture parameter						
	Fracturability (N)	Firmness (N)	Adhesiveness (N·s ⁻¹)	Springiness (–)	Gumminess (N)	Chewiness (N)	Young's modulus
LM images ²							
A _A (%)	–0.664**	–0.647**	0.757**		–0.668**	–0.664**	–0.794**
P _L (μm ⁻¹)	–0.729**	–0.528**	0.683**	0.332*	–0.547**	–0.543**	–0.725**
L ₃ (μm)				–0.498**			
Length (μm ⁻¹)	–0.753**	–0.368*	0.561**	0.533**	–0.361*	–0.355*	–0.672**
Nodes (× 10 ⁻³ μm ⁻²)	–0.668**		0.499**	0.518**			–0.572**
Ends (× 10 ⁻³ μm ⁻²)	–0.614**		0.452**	0.534**			–0.518**
Pores (× 10 ⁻³ μm ⁻²)							
TEM images ³							
A _A (%)				0.528*			
P _L (μm ⁻¹)	–0.604**			0.769**			
L ₃ (μm)	0.585**	0.478*			0.410*	0.411*	0.571**
Length (μm ⁻¹)	–0.487*			0.767**			
Nodes (× 10 ⁻³ μm ⁻²)				0.686**			
Ends (× 10 ⁻³ μm ⁻²)	–0.515*			0.716**			
Pores (× 10 ⁻³ μm ⁻²)	–0.563**			0.706**			

¹LM = light microscopy; TEM = transmission electron microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L₃ = mean intercept length.

²Twelve true replicates × 9 analytical replicates.

³Twelve true replicates × 6 analytical replicates.

* $P < 0.05$; ** $P < 0.01$: significant correlation (single-tailed).

that the proportion of variance explained by LM- and TEM-based parameters considered together was similar to that explained by LM- or TEM-based parameters considered independently. However, by leaving LM- or TEM-based parameters out of the regression analysis, the adjusted coefficient of determination was not greatly reduced, so LM- and TEM-based parameters entertain similar importance with regard to nitrogen fractions (WSN, TCASN, and PTASN). Surprisingly, when the parameters estimated from TEM images were considered, a slight improvement resulted relative to using only LM-based parameters in attempts to predict the aforementioned proteolytic indices.

All models used to predict WSN, TCASN, and PTASN were highly significant ($P < 0.0001$; Table 6). The regression coefficients associated with the standardized variables implied that P_L and number of pores, estimated from LM images, as well as A_A, L₃, and nodes, estimated from TEM images, were significant in the models encompassing WSN, TCASN, and PTASN. This result was not at all unexpected, because P_L measured by LM correlated well with the nitrogen fractions; however, L₃ and nodes from TEM, which are poorly related to each other ($|r| \leq 0.6$), and pores from LM images and A_A from TEM images, which bear no significant correlations with any nitrogen fraction, were considered significant in terms of the regression models (Table 6). As happened with the correlation coefficients, length from TEM was significant in efforts to predict WSN ($P < 0.01$) and TCASN ($P < 0.05$); when

said length increased, WSN and TCASN decreased, whereas when P_L from TEM increased, all of those proteolytic indices decreased. Therefore, such LM-based parameters as A_A, P_L, length, nodes, and ends appear to be good candidates to relate the extent of proteolysis and the rheological parameters to the microstructural properties of Serra da Estrela cheese.

CONCLUSIONS

Stereological image analysis was found useful in discriminating among cheeses ripened for different periods. By combining microstructural data with chemical and textural ones, LM-based parameters appeared, in

Table 5. Adjusted coefficient of determination (± SE of the estimate) for linear regression equations between stereological parameters and ripening parameters

Stereological parameter ¹	Ripening parameter ²		
	WSN ³ (% wt/wt)	TCASN ³ (% wt/wt)	PTASN ⁴ (% wt/wt)
LM	0.815 ± 0.436	0.621 ± 0.611	0.651 ± 0.604
TEM	0.838 ± 0.420	0.858 ± 0.370	0.761 ± 0.523
LM + TEM	0.981 ± 0.143	0.960 ± 0.197	0.973 ± 0.177

¹LM = light microscopy; TEM = transmission electron microscopy.

²WSN = water-soluble nitrogen; TCASN = trichloroacetic acid-soluble nitrogen; PTASN = phosphotungstic acid-soluble nitrogen.

³Twelve true replicates × 6 analytical replicates.

⁴Twelve true replicates × 9 analytical replicates.

Table 6. Linear regression equations (expressed as partial regression coefficients) between stereological parameters and ripening parameters

Item ¹	Ripening parameter ²		
	WSN ³ (%, wt/wt)	TCASN ³ (%, wt/wt)	PTASN ⁴ (%, wt/wt)
Intercept	27.099***	4.469***	43.319*
LM images			
A _A (%)	0.858**		
P _L (μm ⁻¹)	-121.679***	-17.250***	-133.571*
L ₃ (μm)			-13.918*
Length (μm ⁻¹)	-391.102***	38.991*	
Nodes (× 10 ⁻³ μm ⁻²)	1,740.875***		429.626**
Ends (× 10 ⁻³ μm ⁻²)			-294.842**
Pores (× 10 ⁻³ μm ⁻²)	1,714.354***	642.981**	960.611**
TEM images			
A _A (%)	-2.089***	-0.592***	-0.464***
P _L (μm ⁻¹)			
L ₃ (μm)	83.037***	20.217***	14.750**
Length (μm ⁻¹)			
Nodes (× 10 ⁻³ μm ⁻²)	12.380***	6.016***	3.244***
Ends (× 10 ⁻³ μm ⁻²)			
Pores (× 10 ⁻³ μm ⁻²)		-2.662**	
Adjusted R ²	0.981	0.960	0.973
SE of the estimate	0.143	0.197	0.177

¹LM = light microscopy; TEM = transmission electron microscopy; A_A = area fraction of the nonprotein phase; P_L = half of surface/volume ratio; L₃ = mean intercept length.

²WSN = water-soluble nitrogen; TCASN = trichloroacetic acid-soluble nitrogen; PTASN = phosphotungstic acid-soluble nitrogen.

³Twelve true replicates × 6 analytical replicates.

⁴Twelve true replicates × 9 analytical replicates.

P* < 0.05; *P* < 0.01; ****P* < 0.001: significant correlation.

general, to be good candidates for attempts to relate the extent of proteolysis and the texture to the cheese microstructure. It can be stated that a decrease in A_A, P_L, length, nodes, and ends is closely associated with an increase in firmness, fracturability, Young's modulus, and proteolysis, but with a decrease in springiness and adhesiveness. Moreover, the viewing fields and sections (only accessible by LM) affected significantly the microstructural parameters analyzed, so sampling at the field of view level is preferable to sampling at the cheese block level, or even at the cheese section level.

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