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QUANTITATIVE ANALYSIS OF CHEESE MICROSTRUCTURE USING SEM IMAGERY

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Abstract.

Structural properties of cheese highly influence its chemical, mechanical and nutritive properties. The analysis and quantification of relevant features in food imagery is the basis of modern food, and in particular cheese, microscopy. Processing Scanning Electron Microscopy (SEM) images is a powerful tool to estimate microstructural cheese features.

In this paper, we present an ad-hoc method to analyse SEM cheese imagery and to quantitatively characterise a number of features of cheese microstructure, using simple and efficient image processing techniques. An experimental analysis is presented on a number of traditional Sicilian cheese varieties.

Keywords: SEM imagery; Image analysis; Food microstructure; Porous materials

1. Introduction and Motivation

Cheese microstructure is the spatial arrangement of the casein micelles that join together into clusters and chains to form a viscoelastic protein network throughout which moisture, fat globules, minerals and bacteria are dispersed.[1,9,11,10,12] Microstructure is one of the major controlling factors of texture (firmness, softness, cohesiveness, rubberiness, elasticity, pastiness, crumbliness) and functional properties of cheese;[8] it also affects the physicochemical, transport and nutritional cheese properties.[1] As texture and functional properties are significant quality requirements for consumers, microstructure analysis play an important role in the quality evaluation of the dairy products; better quality usually brings higher revenues and consumer satisfaction.[9,5,3] In this study we have investigated by Scanning Electron Microscope (SEM), the internal microstructure of the main traditional Sicilian cheeses: *Ragusano P.D.O.*, *Provola dei Nebrodi*, *Palermitano*,

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Vastedda Valle del Belice which are *Pasta Filata* cheeses and *Pecorino Siciliano P.D.O.*, *Piacentinu Ennese*, *Maiorchino*, *Tuma Persa*, *Fiore Sicano* which are *Pressed* cheeses. As a consequence of differences in the making process of each cheese, there are differences in texture between the 9 cheese varieties.[6,7]

Scanning Electron Microscope generates high resolution images, which allow to study cheese microstructure by a qualitative visual evaluation; however, in a scientific study, it is becoming more and more important to describe SEM images in quantitative terms.[9] Computerised image analysis is a powerful system to extract, from a digital image, objective and numerical data[5] which can be handled mathematically or statistically. Indeed, the combination of SEM image acquisition, image processing, and quantification of microstructural features is the basis of the modern food microscopy.[1] Unfortunately, due to the limitations of the acquisition process, the acquired images are noisy and present reflections that prevent the robust extraction of useful information by simple thresholding techniques. Even if some authors use commercial image processing tools to manually threshold SEM images[15] (see Figures **??** and **??**), the binarised images are not accurate enough for quantitative analyses. Therefore, SEM imagery must undergo an enhancing pre-processing step before binarisation.

In the present study we present an ad-hoc method to enhance and threshold SEM images in order to carry out a pore structure characterisation of cheese, and to determine quantitatively the structural differences among the microstructure of the main traditional Sicilian cheeses. Parameters such as porosity, pore number, pore size and shape were measured on 2-dimensional SEM images, and further analysed with statistical methods.

Since image analysis is often run on a large number of samples, for the sake of computational efficiency our method involves only simple and fast image processing techniques. We avoid to compute complex (and time-consuming) shape descriptors, as well. An experimental analysis is presented on a number of traditional Sicilian cheese varieties. We also briefly describe an Open Source software tool that implements the proposed method, probably the main contribution of our work in dairy science.

2. Sample Preparation

Cheeses were chosen and purchased at their best ripening age mostly appreciated by consumers and then prepared using the freeze-fracturing technique according to McManus et al. procedure.[13] Cheese cores were extracted from the central area of each cheese block in vertical and horizontal direction for *pasta filata* cheeses and perpendicularly to the larger surface for *pressed* cheeses.

Strings of cheese (approximately $1 \times 3 \times 8$ mm) were cut from the center of each coin (1 cm diameter) using a blade. Each string was transferred into vials with 2% aqueous glutaraldehyde at room temperature for one hour and after that stored in new solution for 3 days at 4C. Samples were then dehydrated step-wise to 70% ethanol, and then plunged into Freon 22 cooled with liquid nitrogen. In the following steps, samples were transferred to liquid nitrogen and fractured perpendicular to the long axis using precooled insulated forceps. Then, samples were dehydrated with step-wise return to ethanol and defatted using step-wise increasing concentrations of Freon 113. The final step is represented by metallisation done by metal-impregnating in O-F solution (1% OsO_4 and 1.5% $K_4Fe(CN)_6 \cdot 3H_2O$ in 0.1 M sodium cacodylate buffer, pH 7.2), 2% tannic acid in 0.1 M cacodylate buffer pH 7.2, O-F solution again, and finally an aqueous solution of 1%

hydoquinone. Samples were dried by the critical point method in CO_2 and mounted on SEM aluminum stubs using a carbon adhesive and then coated (ca. 4.5 nm thick) with gold-palladium in argon medium. Finally, cheese samples were examined in an scanning electron microscope with a voltage of 15 kV and a working distance of 12 to 14 mm. The internal fractured surface of the samples was observed at $500\times$ and $1,000\times$ magnification. Three representative areas were randomly selected and scanned in vertical direction and a series of sequential images was recorded for each selected area.

3. Image Analysis

Given a SEM scan of a cheese sample, our objective is to compute a number of quantitative measurements about its microstructure. Since SEMs give a set of regularly spaced samples, scans can be regarded as digital greyscale images. Hence, we can benefit from classical image processing algorithms. In particular, we investigate the structure of micropores in the protein matrix in order to quantify useful measures, such as porosity and a number of shape descriptors. By analysing the statistical distributions of such measures, one can investigate the relationships between cheese microstructure and texture.

3.1. *Image Enhancement*

In order to compute statistics about the microstructure of cheese samples, the SEM scans must be binarised to classify which pixels belong to pore or to protein matrix regions. Due to scanning limitations, simple manual or automatic thresholding techniques produce noisy images. Figure 1(a) show the SEM scan of a sample of Ragusano cheese, while Figure 1(d) shows a binary image obtained by manually thresholding the input scan. Clearly, more sophisticated techniques are needed to improve this result, in order to gather affordable statistics about the microstructure of the sample. Hence, cheese SEM scans must be enhanced before binarisation (see Figure 1(f)). Regularisation of input images results in less noisy binary images. The proposed method is composed of four simple phases:

- Denoising and flattening
- Pore shape regularisation
- Binarisation
- Hole filling
- Quantification of relevant features.

SEM images often present speckle noise. Even if sophisticated despeckle methods exist[4] we found that a 3×3 median filter gives sufficiently accurate results for our application. We further apply a Gaussian smoothing filter with a small kernel to remove noise deriving from different sources (see Figure 1(b)).

After removing noise, some images still present reflections causing strong luminance gradients that rule out global thresholding, since the greyscale value of pixels in highly illuminated pore regions can be greater than the value of pixels in lowly illuminated protein matrix areas (Figure 1(c)). In order to remove this effect we flatten the image by removing low frequencies using a bandpass filter. The result of this operation is shown in Figure 1(e). Since automatically finding a global threshold in flattened images may be tricky, care must be taken using this operation in order to avoid unsatisfactory results. For this reason, we require that the process is assisted by the user who enables this

operation only when actually needed. In principle, this operation could be automatically enabled when an intensity gradient is detected. However, since the algorithms for gradient detection are not sufficiently robust, this could lead to disastrous results.

In order to help thresholding, pore borders can be strengthened using simple morphological operations. We employ a closure operator with small support to regularise the shape of pores and to enhance pore borders. We found that better results can be obtained if the dilation and erosion operations are decoupled and thresholding is done before erosion. The well-known Otsu thresholding algorithm[14] is used for automatic binarisation (Figure 1(f)).

Finally, inner holes are filled by flood filling on pores with a closed border.

3.2. *Sample Statistics*

The most relevant measure for the analysis of the microstructure of porous materials (e.g., cheese) is *porosity*, defined as the percentage of pore area with respect to the total sampled area. In the discrete domain of digital images, it can be easily computed as the count of pore pixels over the total number of pixels. Anyway, a number of other important measures are used in food technology.[17] Among all the most common features described

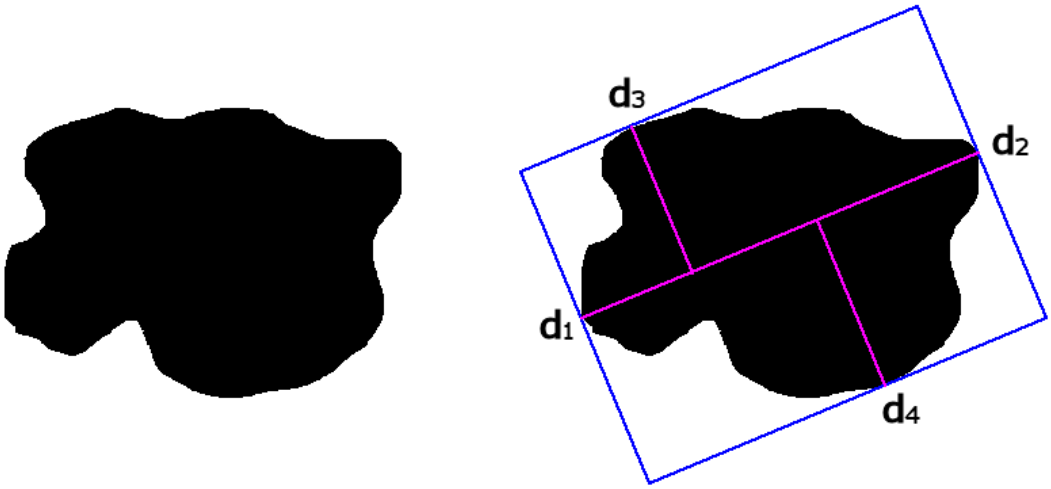
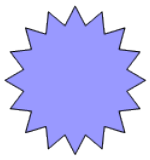
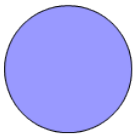
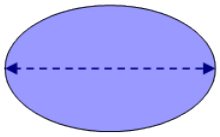
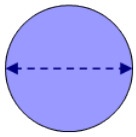
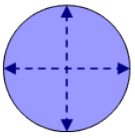
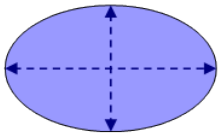


Fig. 1. Directionality. $\overline{d_1 d_2}$ is the *Maximum Diameter*. The *Minimum Diameter* is the sum of the two segments given by d_3 and d_4 and their projections on the Maximum Diameter.

in the literature, we restrict to a little set designed to describe pore shape, orientation, area, and distribution (see Figure 1 and Table 1):

- Area
- Perimeter
- *Maximum Diameter*
- *Orthogonal Diameter*
- *Directionality*
- *Form Factor*
- *Roundness*
- *Aspect Ratio*

Table 1. Shape quantitative descriptors. Meaning of the parameters. A: net area; p: perimeter; D_{max}: length of the maximum diameter; D_{min}: length of the shortest edge of the bounding rectangle whose longest axis is parallel to the maximum diameter.

Parameter name	Formula	Low value	High value
Form Factor	$\frac{4\pi \cdot A}{p^2}$		
Roundness	$\frac{4A}{\pi \cdot D_{max}^2}$		
Aspect Ratio	$\frac{D_{max}}{D_{min}}$		

Maximum Diameter is the distance of the two farthest points in the pore border (d_1 and d_2 in Figure 1). *Orthogonal Diameter* is derived from the Maximum Diameter by taking the farthest point from the Maximum Diameter axis on each side of the axis (d_3 and d_4 in the figure). The Orthogonal Diameter is the sum of the distances of these two points from the axis. *Directionality* is the orientation of the maximum diameter axis of the pores, computed as the angle described by the Maximum Diameter with respect to the x-axis. The descriptors *Form Factor*, *Roundness*, and *Aspect Ratio* quantify the departure of a feature from roundness. The former, which describes the regularity of the pore border, assigns a higher value to features exhibiting uneven edges, while the last two quantify elongations toward more elliptical shapes.

Obviously, these descriptors quantify the aspect of a single structural unit. Statistical parameters such as mean, standard deviation, skewness, and kurtosis can be effectively used to extract useful information on the distribution of the collected data.

4. A Tool for Image Analysis of Cheese SEM Imagery

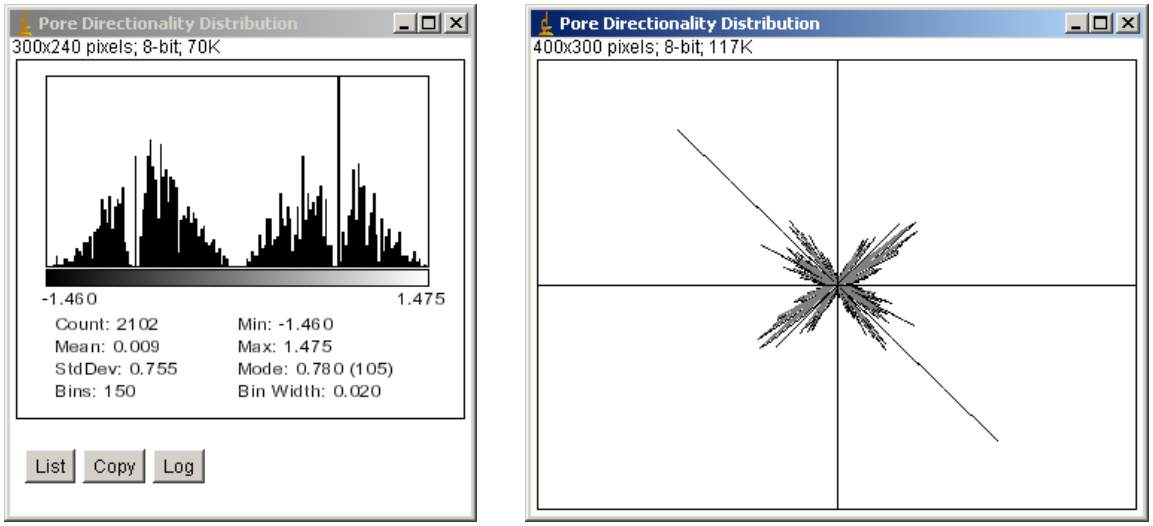
The proposed method has been implemented as a plugin of the *ImageJ* software.[16] We chose this solution for three main reasons:

- *ImageJ* is a well-known and widely-used software for Image Analysis
- It is equipped with a number of tools for image processing and analysis, and
- It is in the public domain.

Hence, implementing our method as a plugin for this software offers a number of advantages over a stand-alone executable, such as reliability, maintenance, ease of implementation, and portability. Moreover, the user does not need to learn to use a new software interface.

Our plugin offers two commands: **BinariseSEM** and **ComputeStats**. We chose to separate the computation in order to allow the user to employ a different thresholding technique without compiling the whole plugin. **BinariseSEM** is called to enhance and binarise SEM images, as described in Section 3.1. Taking a greyscale image as input, it computes a binary image partitioned into pores and protein matrix pixels. It is possible to choose whether the bandpass filter must be used or not; since it is mainly domain knowledge we prefer to leave the final decision to the user, as stated in Section 3.1. After enhancing, the final threshold is automatically determined using the Otsu algorithm.[14] However, the final choice is again left to the user, using a dialog window in which the automatically determined threshold is used as a default value. Although we experimented extensively different threshold values, we found that the default value is the best choice among global thresholds in almost all the cases we tested. Figures 2 and 1 show the output of this command.

ComputeStats implements the statistical measures described in Section 3.2. It takes as input a binary image, such as the images generated by the **BinariseSEM** command. The user is asked to introduce the magnification factor of the SEM during the acquisition. The distribution of values is visualised as a histogram for each measure or descriptor. Moreover, *directionality* is visualised using a rose plot, as shown in Figure 1(b).



(a) Histogram of pore directionalities. (b) Rose plot showing pore directionalities.

Fig. 1. Distribution of pore directionalities.

While experimenting with the first implementation of our tool, we observed that all of our test images showed a strong preferred direction at an approximate angle of 45 degrees, despite the true directionality could appear different. This depended on the great number of small pores. Namely, pores made by few pixels appear as tiny squares. Thus, their direction is parallel to their diagonal, i.e., about 45 degrees. The problem is resolved either by cutting off small pores form the computation of directionality statistics, or by weighing pore contributions by their area.

5. Experimental Results

Using the software tool we developed, we experimented on a number of samples with several different parameter settings. We found that the proposed algorithm is little sensitive to the value of most of them. The following parameters are involved in the computation

- The radius of the median filter used for despeckling
- The radius of the Gaussian used for denoising and smoothing
- The number of iterations of the morphological closure operation
- The radius of the structuring element employed for closure
- The threshold value for binarisation
- The bandpass filter radii and whether to use it or not

From the tests we run, it is clear that the only parameter that has a critical influence on the results is the bandpass filter. Basically, depending on the microstructure of the cheese and on the methodology used for sample preparation, different results can be obtained by enabling bandpass filtering or not. Hence, in this case, we leave to the user the decision whether to use it or not. Similarly, the user has the final decision on the threshold value for binarisation, although we have observed no case in which the user was able to get a better value than that suggested by the Otsu algorithm. Table 1 shows the typical settings for the other parameters involved.

Table 1. Typical settings for the parameters involved in the binarisation.

Parameter	Value
Radius of the median filter	2
Radius of the Gaussian filter	2
# iterations of closure	1
Radius of the structuring element	2
Threshold value for binarisation	default
Bandpass filter radii	3 and 40

Results of porosity obtained for the nine traditional Sicilian cheeses in the above mentioned experiment were statistically analysed using a general linear model. Statistical differences were found among the different cheese types within each group, i.e., *pressed* vs. *pasta filata* cheeses. Least square means are showed in Table 2. Furthermore, as expected from chemical and qualitative cheese microstructure analysis, pressed cheeses

Table 2. Least square mean values for porosity of 9 Sicilian traditional cheeses (see[2]).

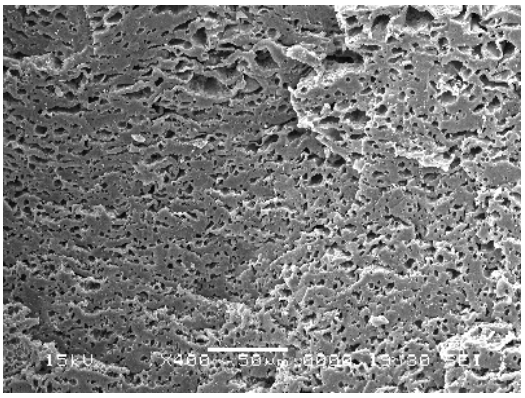
Cheese	Pasta	Ripening age (months)	Porosity	SE
Fiore Sicano	Pressed	1	0.390	0.036
Piacentinu Ennese	Pressed	4	0.345	0.029
Pecorino Siciliano P.D.O.	Pressed	4	0.325	0.025
Tuma persa	Pressed	8	0.319	0.025
Maiorchino	Pressed	16	0.254	0.025
Vastedda del Belice	Filata	7	0.213	0.029
Provola dei Nebrodi	Filata	3	0.175	0.035
Palermitano	Filata	6	0.192	0.020
Ragusano P.D.O.	Filata	9	0.196	0.015
Mean for pressed cheeses			0.327	
Mean for pasta filata cheeses			0.194	

showed higher overall porosity (p-value < 0.01, see[2]) than pasta filata cheeses (0.327 vs 0.194), while no significant differences were found between the two magnification factors used, 500× and 1000×.

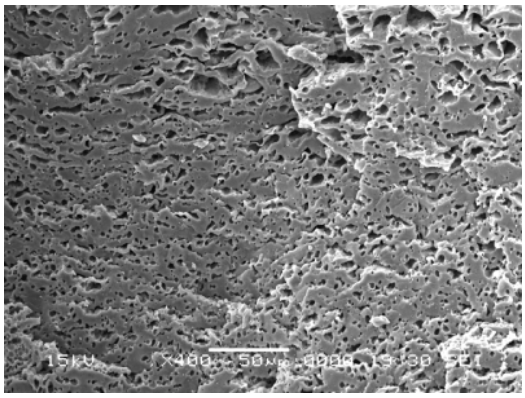
Due to the lack of space, we do not show similar tables for the shape descriptors used. As an example, in Figure 1 we plot the directionality distribution of the sample in Figure **??** and the corresponding rose plot. Two peaks are clearly visible, about the two diagonals (45 and -45 degrees). Further work and new data is needed to investigate the correlation between these descriptors and the microstructural characteristics of cheese.

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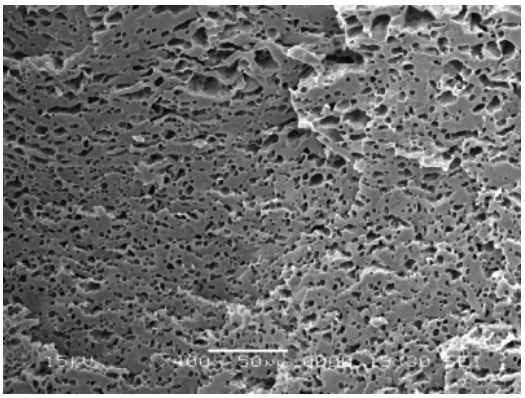
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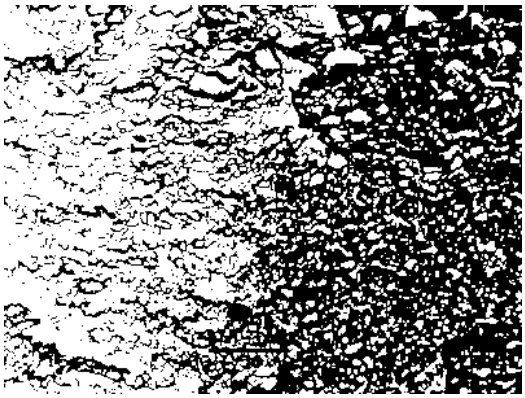
(a) SEM scan of a sample of Ragusano cheese.



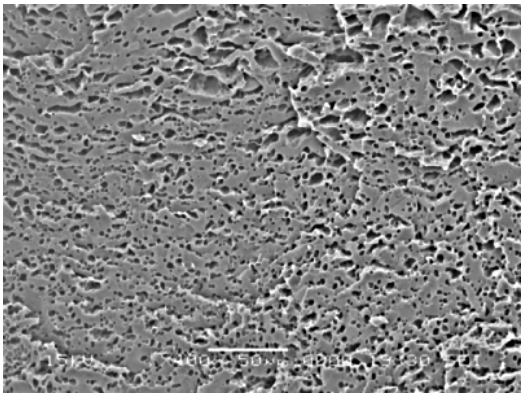
(b) Removal of noise.



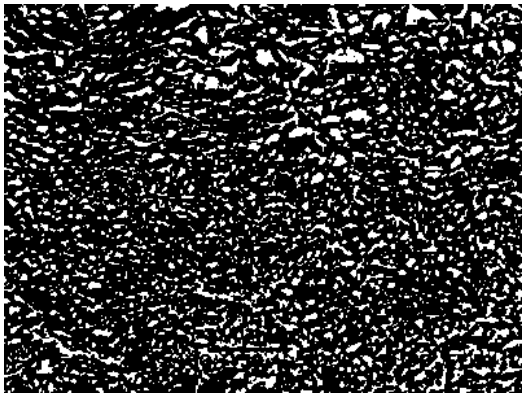
(c) Morphological dilate.



(d) Automatic thresholding. Wrong result due to electron reflections.



(e) Bandpass filtering.



(f) Automatic thresholding. Correct result after bandpass filtering.

Fig. 1. Example of electron reflections and bandpass filtering in a sample of Ragusano cheese.

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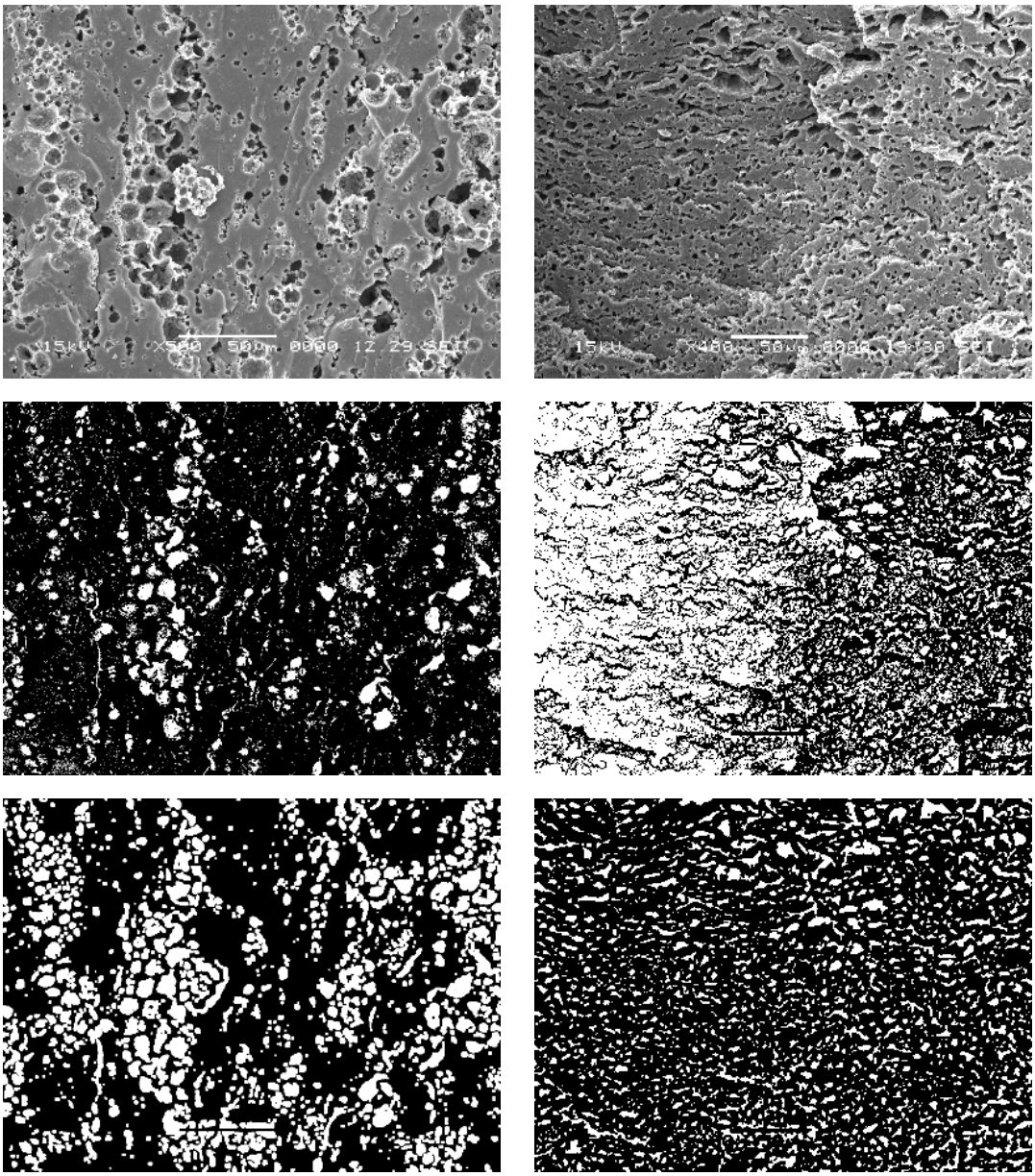


Fig. 2. Comparison between a simple manual thresholding technique and our ad-hoc automatic binarisation. The sample in the left column is of Provola dei Nebrodi cheese, while in the right column a Ragusano sample is shown. We show, from top to bottom, the original images, the images obtained by simple manual thresholding, and those obtained using the proposed method.