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Microscopic method in processed animal proteins identification in feed: applications of image analysis

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Processed animal proteins (PAP) detection and identification in feedstuffs can be difficult in distinguishing among land animals, i.e. poultry and mammals. Thus, the aim of this study was to evaluate the potential application of image analysis in PAP identification. For this purpose four reference samples containing poultry meals and four reference samples containing mammalian meat and bone meals were used. Each sample was analyzed using the microscopic method (98/88/EC). Bone fragments are characterized by similar morphological features (colours, shape, lacunae shape, lacunae distribution, etc.) that make it difficult to distinguish between poultry and mammals. Through a digital camera and an image analysis software a total of 30 bone fragment lacunae images at X400 were obtained. For each image 29 geometric parameters related to the lacunae and 3 geometric parameters related to the canaliculae of lacunae, were measured using the image analysis software obtaining 960 observations. Of the 32 descriptors used two, the area of the lacunae and their perimeter, were able to explain 96.15% of the total variability of the data, even though their contribution was different (83.97% vs. 12.18%, respectively). Through these two descriptors it was possible to distinguish between mammalian and poultry lacunae, except in two cases (6.6%), in which poultry lacunae were wrongly classified as mammalian. This latter can be related with higher variability in the lacunae area recorded for mammals compared to poultry. On the basis of the present study, it can be concluded that image analysis represents a promising potential tool in PAP identification, that may provide accurate and reliable results in feedstuffs characterisation, analysis and control.
Keywords. Processed animal proteins (PAP), official microscopic method, image analysis.

1. INTRODUCTION

Following the outbreak of BSE, processed animal proteins (PAP) were banned in animal feedstuffs in the EU and each individual member state was required to implement a feed quality programme to enforce this ban. An essential aspect of these programmes was the adoption of EU-approved methods for detecting PAP in feed. The official analytical method for the detection of processed animal proteins in feedstuffs is the microscopic examination technique described in Commission Directive 98/88/EC. At the present, however, a simple PAP detection in feedstuff is not enough, and an improvement of the microscopic method is required (Gizzi *et al.*, 2003; Moretti *et al.*, 2003; Pinotti *et al.*, 2003). PAP differentiation, not only between classes of vertebrates, but also at higher taxonomy levels, in fact, has become mandatory with Regulation 2002/1774/EC. This Regulation does not relax the total ban on feeding PAPto ruminant species, but for other livestock simply prohibits the use of feed containing processed proteins from the same species (prohibition of cannibalism). Therefore, while the microscopic method may be adequate for enforcing the EU's total ban on MBM in ruminant feeds, and it is usually able to distinguish fish from land animal material, it is often unable to distinguish between land (terrestrial) animals (i.e. poultry and mammals). Origin of animal material in feed is based on the observation of bone fragments and their morphological features. Thus, although it is usually possible to identify the animal class from bone fragments characteristics, several of these features in land animals materials (i.e. poultry and mammals) are not always distinguishable. Starting from these assumptions the aim of this study was to evaluate the potential application of image analysis for distinguish among land animals in PAP identification and characterization.

2. MATERIALS AND METHODS

For this purpose four reference samples containing poultry meals (ECB s.p.a., Bergamo, Italy; VSA, University of Milan) and four reference samples containing mammalian meat and bone meals (Agricultural Research Centre of Gembloux, Belgium, STRATFEED Project;

VSA, University of Milan) were used. Each sample was analyzed using the official microscopic method (98/88/EC). The obtained sediment samples were viewed under a stereomicroscope (Olympus SX9, Germany) and compound microscope (Olympus BX41, Germany) at several magnifications to identify bone constituents. Bone fragments characterized by similar morphological features (colours, shape, lacunae shape, lacunae distribution, etc.) that made difficult to distinguish between poultry and mammals were analysed. Through a digital camera and an image analysis software (Image-for Plus 4.5.1, Media Cybernetics Inc., Silver Springs, USA) a total of 30 bone fragment lacunae images at X400 were obtained. Images have been elaborated/manipulated obtaining for each lacunae a monochrome "masks" (**Figure 1**), on which several measurements were performed. In detail, for each image 29 geometric parameters related to the lacunae (**Table 1**) and three geometric parameters related to the canaliculae of lacunae (dendritic lenght, dendrites end point), were measured to provide 960 observations. Obtained data were analysed using the PRINCOMP, ANOVA procedures and BOXPLOT of SAS (2001).

3. RESULTS AND DISCUSSION

Results of Principal Component Analysis (PRINCOMP) are shown in **figure 2**. The first principal component (Prin 1) was the descriptor "area polygon" measuring the area of the lacunae (83.97% of the total variability of the data), while the second principal component (Prin 2) was the descriptor "perimeter" indicating the perimeter of the lacunae (12.18% of the variability of the data). As a consequence, of 32 descriptors used, two principal components were able to explain 96.15% of the total variability of the data, while the sum of all the other 30 descriptors, covered the remainder 3.85% of the total variability. Through these two descriptors it was possible to distinguish between mammalian and poultry lacunae, except in two cases (6.6%), in which poultry lacunae were wrongly classified as mammalian.

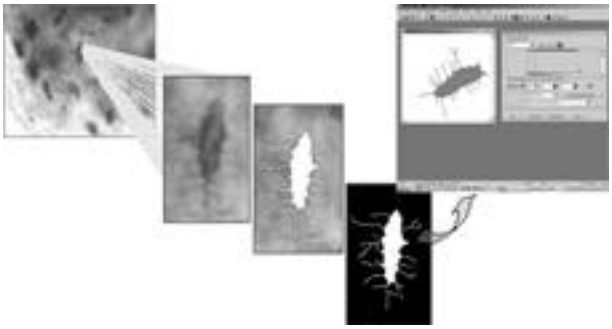

Figure 1. Sequence of images processing and analysis.

Table 1. Measurement performed by image analysis software.

Parameter	Description
Aspect	Ratio between major axis and minor axis of ellipse equivalent to object
Area/box	Ratio between object area and object bounding box area
Axis	
major	Length of major axis of ellipse with same moments of order 0, 1 and 2 as object
minor	Length of minor axis of ellipse with same moments of order 0, 1 and 2 as object
Area polygon	Area included in the polygon defining the object outline. Same polygon as that used for "perimeter"
Box	
x/y	Ratio between width and height of object bounding box
width	Width of the object bounding box
height	Height of the object bounding box
Center	
-x	X coordinate of object centroid intensity weighted centroid X-position
-y	Y coordinate of object centroid
Diameter	
min	Length of shortest line joining two points of object outline and passing through the centroid
max	Length of longest line joining two points of object outline and passing through the centroid
mean	Average length of diameters measured at 2 degree intervals and passing through object centroid
Fractal dim	Fractal dimension of the object outline
Feret	
min	Smallest caliper (feret) length
max	Longest caliper (feret) length
mean	Average caliper (feret) length
Perimeter	Length of the object outline
Perimeter	
conve	Perimeter of the convex outline of the object
ellip	Perimeter of the equivalent ellipse
ratio	Ratio of Convex Perimeter to Perimeter
Per-area	Ratio of area of object to total area of image
Radius	
max	Maximum distance between object centroid and outline
min	Minimum distance between object centroid and outline
ratio	Ratio between "Max Radius" and "Min Radius"
Size	
length	Feret diameter (i.e. caliper length) along major axis of object
width	Feret diameter (i.e. caliper length) along minor axis of object

These results were also supported by the variance analysis (ANOVA) for the two variables, that showed how descriptor "area poly" ($P<0.001$) was more informative than descriptor "perimeter" ($P<0.0165$). Additional information, was provided by quartiles BOXPLOT

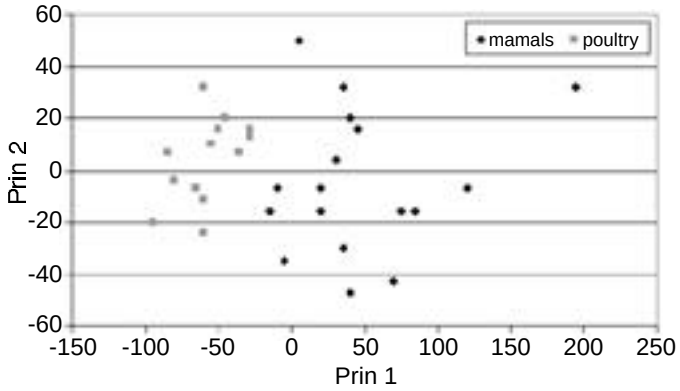

Figure 2. Results of Principal Component Analysis. Prin 1, lacunae area; Prin 2, lacunae perimeter.

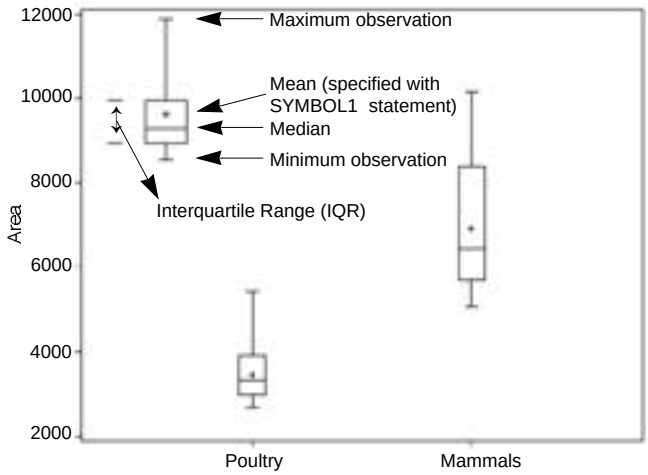

Figure 3. Quartiles BOXPLOT. The boxes represent the 2nd and 3rd quartiles.

(**Figure 3**), which indicate that data variability was higher for mammalian lacunae area compared to poultry one. Mean values for mammals and poultry lacunae area were 155.67±37.5 μ and 82.72±16.8 μ, respectively. However, despite mammals mean area values were double compare to poultry, 1st and 4th quartiles indicate that in several cases there was an overlap that can also explain why two poultry lacunae were wrongly identified as mammals. Collectively these results indicate that image analysis offers new and interesting applications in morphological and histological characterization. Image processing, integrated with morphometric measurements (area, radius, diameter, and their structural relations) can provide accurate and reliable results that can be very useful to the analyst for feedstuffs characterization, analysis and control. Generally traditional microscopic feed inspection performed by human analyst does not generate precise descriptive data, quickly and in objective manner (Gizzi *et al.*, 2003; Pinotti *et al.*, 2003). As a consequence, the accuracy of the microscopic method depends crucially on the experience of the analyst, and any kind of quantitative estimate is always approximate. The usual method of expressing the results is to specify whether animal material is present or absent. On the contrary, image analysis approach provides several benefits and drawbacks that can support and/or facilitate the analyst in an objective assessment of the sample.

4. CONCLUSIONS

It is evident, that even if the microscopic method may be adequate for enforcing the EU's total ban on MBM in ruminant feeds, it is often unable to distinguish between land animal material. For this reason suitable techniques for routine feed control that distinguish, not only between classes of vertebrates, but also at higher taxonomy levels are required. In this field image analysis represents a promising potential tool for determining the origin of animal material in feedstuffs.

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