Light microscopy in combination with computer image analysis for the identification of processed animal protein in feed

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Food production is a complex process, with the ultimate objective of the food industry and food safety regulators being to ensure that food reaching the consumer is safe and wholesome. For food derived from animals, the hazard may originate from a number of sources including the consumption by food producing animals of contaminated feed. This is the case of the protracted outbreak of mad-cow disease, for which a feed ban has been introduced [1]. Microscopy and polymerase chain reaction (PCR) together, are the official accepted methods for detection of animal proteins in feed [2] [5]. Nevertheless, neither of two methods fits all the requirements for the accurate identification of prohibited ingredients of animal origin. Light microscopy in combination with computer image analysis (IA), which is based on the identification of bone particles or tissues in feedingstuffs, has been also proposed. Findings in these studies have indicated that the use of the microscopic method in association with IA to identify the origin of processed animal proteins (PAPs) appears promising, especially as a complementary method to the DNA-based ones. This paper explored the potential of the use of microscopy in combination with IA measurements in distinguishing between different PAPs.

Keywords: Feed; meat and bone meal; microscopic method; computer image analysis

1. General remarks

Processed animal proteins (PAPs) is a wide category of **Animal by product** that can be used in animal nutrition with several limitations according to feed ban. A ban on the feeding of mammalian meat and bone meal (MBM) to cattle, sheep and goats was introduced as of July 1994. In order to manage the risk of presence of prohibited material in ruminant feed through cross-contamination, this partial ban was extended to a total EU wide suspension on the use of processed animal proteins in feed for any animals farmed for the production of food on 1 January 2001 [1] with some exceptions like the use of fish meal for non-ruminants. More recently, a risk-based lifting of the feed ban, has been adopted [3]. Further details about the use of processed animal proteins in feed formulation for food producing animal can be obtained elsewhere [4]. In this frame over the last decade, a feed safety program for trace and detect processed animal proteins has been implemented. An essential aspect of these programs was the adoption of EU-approved methods for detecting PAPs in feed. Microscopy and polymerase chain reaction (PCR) together, are the official accepted methods for detection of animal proteins in feed [5]. Nevertheless, neither of two methods fits all the requirements for the accurate identification of prohibited ingredients of animal origin.

The protocol for the detection of processed animal protein in feedstuffs by the microscopic examination technique is described in Commission regulation 51/2013 [5]. Using this method, treated samples are viewed under a stereomicroscope and compound microscope at several magnifications to identify bone constituents mainly. The accuracy of the qualitative and quantitative estimates depends crucially on the experience of the analyst, and the quantitative estimate is always approximate. The usual method of expressing the result is to specify whether animal material is present or absent. Thus, while the microscopic method may be adequate for enforcing the EU's total ban on MBM in ruminant feeds, and is usually able to distinguish fish from land animal material, it is unable to determine both the animal class and the species-specific origin of animal material (Figure 1).

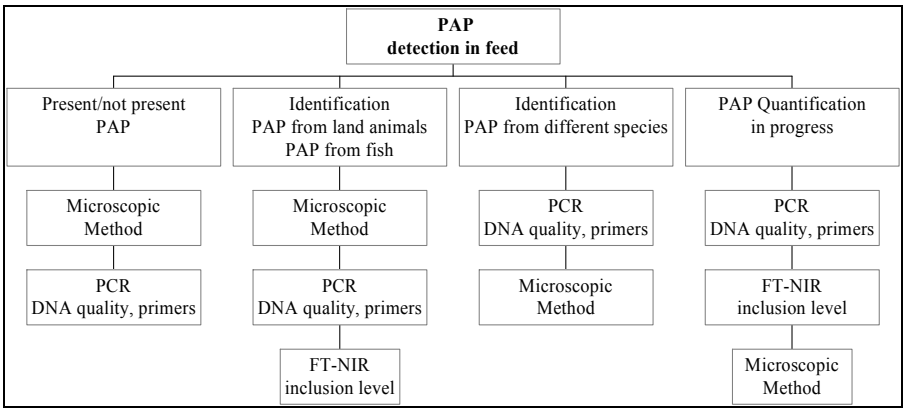

Figure 1 Diagram representing the analytical method selection criteria for detection of animal particles in compound feed.

Recent Regulations [3] [5] in fact, clearly implies that it must be possible to identify the origin of animal materials at higher taxonomic levels than in the past. Thus improvements in all methods of detecting constituents of animal origin are required, including the microscopic method. This chapter examines the problem of meat and bone meal in animal feeds, and the use of microscopic methods in association with computer image analysis to identify the source of these feedstuff ingredients/contaminants.

2. Bone lacunae features: a marker for PAP identification

In general, it has been reported that the microscopic method is capable of distinguishing between fish and land animal material based essentially on bone characteristics. Mammalian and avian bone material may also be distinguished [6] [7]. van Raamsdonk and co-workers [8] reported that the markers or characteristics of bone fragments can be examined at three different levels: i) the shape and structure of an entire bone fragment, ii) the shape, size and density of the lacunae, iii) and the visibility of the canaliculae. Specifically at high magnifications (from 10x) mammalian bone particles are generally transparent, more or less rounded, and contain elliptical to almost circular lacunae; canaliculae may be visible depending on the preservation and transparency of the particles. By contrast, bone particles from poultry are darker, have a more splintered (sharp-edged) appearance, more rounded and denser lacunae, and canaliculae are not visible [6] [8]. These characteristics were determined by examination of samples of known origin and processing history, and are consistent with histological textbooks and the literature on animal meals [6]. Nevertheless, poultry and mammalian particles remain difficult to detect and their characteristics may overlap. The situation is exacerbated in the case of fish material. In fact, most of these methods developed and implemented for PAPs identification, have been focused on the feed ban for land animal like mammalian (ruminants, pig) and poultry [9]. Therefore, fishmeal characterization, by microscopy, was limited and mainly descriptive. Moreover, several species of fish (e.g., tuna and salmon) have bone lacunae resembling those of land animals [8] making difficult the discrimination from other animal classes. As a consequence, the experience of the microscopist is a crucial factor in successful identification. In this respect, any support to the analyst that can lead to objective results should be welcomed. The benefit of combining the microscopic method with computer image analysis can be an increased accuracy of the qualitative and quantitative estimates for characterising feedstuff constituents by the microscopic method.

3. Microscopy and image analysis

When compound feed materials or raw materials are inspected by microscopy, the protocol usually adopted is that reported by the European Commission [5], in which the microscopic method for PAP is reported. The final step of the methods is to prepare several microscopic slides for each sample, prepared using Norland Optical adhesive 65 (recommended) as embedding agent. A subsequent step is the observation of the slides using a compound microscope, at several magnifications. In general the shape and structure of entire bone fragment is obtained at 10X, while size and shape, of the lacunae, and the canaliculae network can be observed at 40x. However this is a general rule, since the material under the microscope is characterised by a huge variability in term of morphology, thickness, focal points etc. In order to guarantee a randomized image acquisition, several bone fragment lacunae images have to be randomly acquired in each sample without any pre-selection. When image/picture are acquired the computer image analysis can start.

The image analysis procedure consists of a sequence of steps as follows: (i) image acquisition captures in digital form in the computer an array of pixels representing the structure and features to be evaluated. (ii) Image enhancement improves the visibility of image detail, and is a precursor to image thresholding. (iii) Thresholding selects a range of brightness or colour values that characterize the object or structures of interest so as to isolate them from the rest of the image. Automatic setting of thresholds is preferred to manual setting because it is more reproducible and permits automation. However in some cases, manual definition of the image dimension and the features to be measured and counted is useful, and can be performed with computer peripherals such as a pen tablet (see Pinotti, 2009) [10]. In either case, a binary (black and white) image is produced that captures the important structural features. Thresholder images are rarely perfect, and further processing to correct problems and measure selected parameters can be performed. Key steps of image analysis are summarized in figure 2. According to the image analysis software used, a series of morphometric variables or descriptors can be measured or obtained from each lacuna.

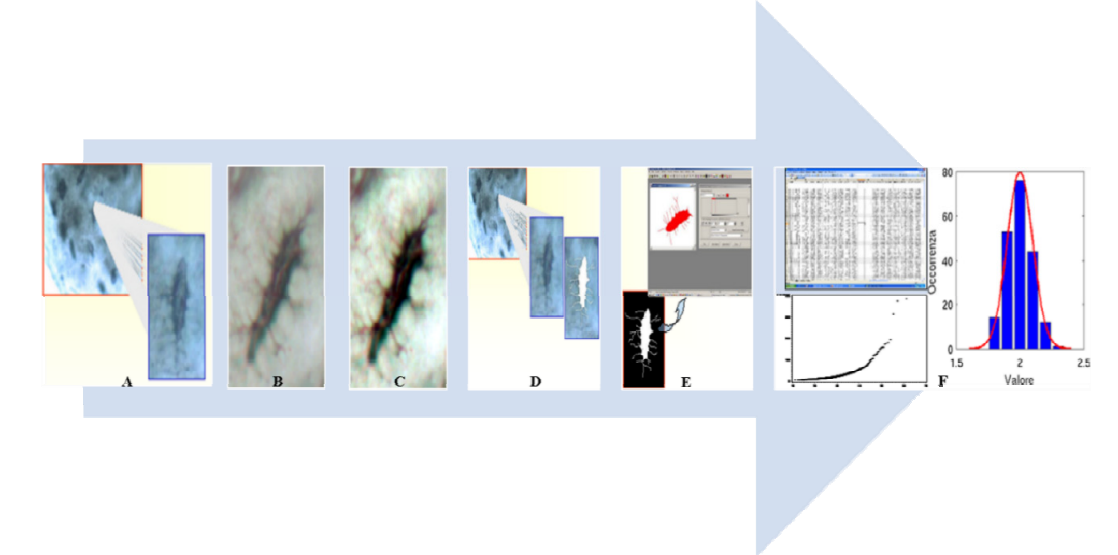

Figure 2 Key steps of image analysis: A image acquisition captures in digital form in the computer an array of pixels representing the structure and features to be evaluated; B image enhancement improves the visibility of image detail, and is a precursor to image thresholding; C thresholding selects a range of brightness or colour values that characterize the object or structures of interest so as to isolate them from the rest of the image; D a binary (black and white) image is produced that captures the important structural features; E thresholder images can be further processed to improves the visibility of image detail and measure selected parameters can be performed; F lacunae measurements are collected in Excel files and statistically analysed.

4. Microscopy and image analysis for bone markers selection

As reported elsewhere [11] [12] [13] 30 geometric variables have been considered effective in animal meal identification and characterisation. A further step in using these geometric descriptors/variables was their partition in two main groups, namely: *size descriptors* and *derived shape descriptors*. The size descriptors, also termed as dimension (primary) descriptors, represent direct measurements on bone lacunae. By contrast, the derived shape parameters are constructed by combining the various size parameters so that the dimension units are cancelled out [14]. Both groups of descriptors are listed in Table 1, for their full description Pinotti et al. 2013 [11] can be consulted.

Table 1 Morphometric descriptors divided by group: size descriptors and derived shape descriptors

Type of morphometric descriptors	
Size descriptors (primary descriptors)	Area, Axis major, Axis minor, Diameter max Diameter min, Diameter mean, Radius max, Radius min, Perimeter, Size (length), Size (width), Perimeter 2, Perimeter (convex), Perimeter (ellipse), Polygon area, Box Width, Box Height, Feret (min.), Feret (max.) and Feret mean and Convex area.
Derived shape descriptors	Aspect, Area/Box, Box X/Y, Radius ratio, Roundness, Perimeter ratio Form factor, Roundness 2, and Solidity.

A series of papers [15] [16] [17] [10] [11] [12] [13] have been published to document the potential of light microscopy in combination with image analysis in discriminating between different animal bone materials. In general what has been observed is that for all of the directly measured variables, which relate to size, the mammalian lacunae have higher means than the avian ones. For the shape variables, the picture is less clear-cut, with differences in both directions. As expected, however, the preliminary inspection of the data indicate also that the features of lacunae of mammalian and avian origins highly overlap (as indicated by figure 3 and by the d values), but the t-tests for comparing the means of variables for mammals and avian give statistically significant differences. This suggests that grouping the data and taking the means to represent the grouped data, can reduce the variability in the dataset and give higher levels of accuracy.

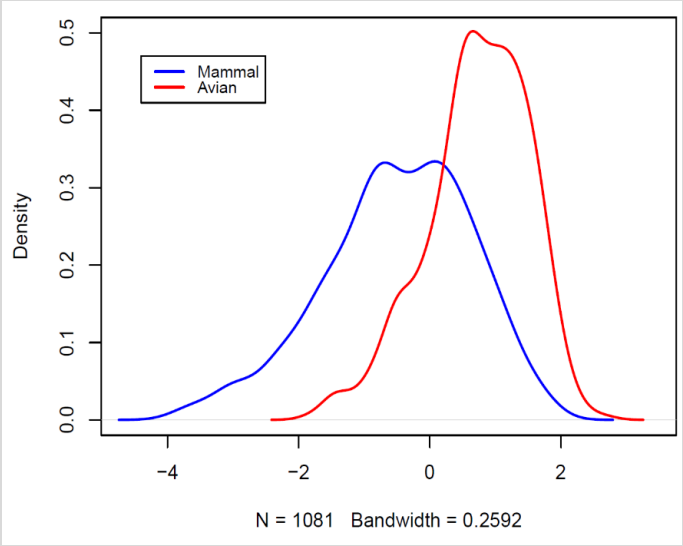

Figure 3. Kernel density estimates of mammalian and avian classes on the linear discriminant (modified from [11])

A different scenario can be observed for fish material, that selecting specific size and shape descriptors it can be distinguished from land animals meals (obtained from mammalian and poultry). Mean and standard error (SE) by class of the size and shape descriptors measured in different experiments are reported in figure 4 and 5. Most of these descriptors were closely associated with the general characteristics of mammalian, poultry, and fish bone fragments and lacunae reported in literature [6] [8]. However, selected descriptors like the aspect and axis minor, indicated that poultry lacunae were not as globular as previously reported [6]. In fact, aspect was higher and axis minor was lower in poultry than mammalian lacunae, indicating that lacunae had a tapering shape in poultry. In the case of fish materials, shape and length (included in the size group) descriptors could be valid markers for their identification. Specifically, for some of them, i.e. Aspect, Radius ratio, Roundness, Form factor and Roundness2 a large gap between fish and mammalian and avian material has been observed. Beside them, values of selected "length" descriptors (Axis major, Diameter max, Radius max, Size length, Box height, Feret max), recorded in fish were twice as big compared to those measured in land animal's materials (figure 6). These results indicated that, in general, bone lacunae are significantly longer in fish than in land animal materials. Thus, not only shape descriptors but also selected size descriptors can help in distinguishing between fish and land animals.

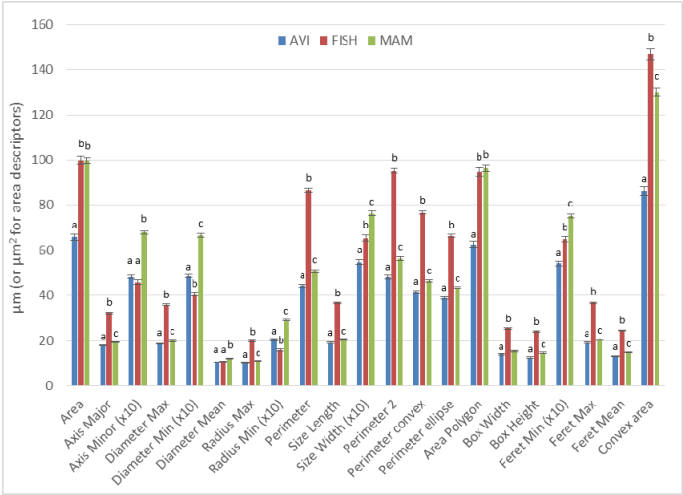

Figure 4 Graphic representation of means and standard error (SE) by class of the size descriptors. AVI = avian; FISH = fish; MAM = mammals; (*10) = measured value multiplied by 10. The means within morphometric descriptors with different letters (a, b, c) differ significantly ($P < 0.001$).

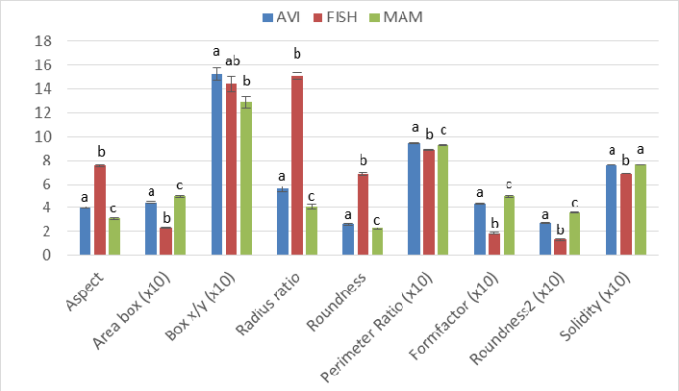

Figure 5 Graphic representation of means and standard error (SE) by class of the shape descriptors. AVI = avian; FISH = fish; MAM = mammals; (*10) = measured value multiplied by 10. The means within morphometric descriptors with different letters (a, b, c) differ significantly ($P < 0.001$).

Figure 6 Comparison of fish, poultry and mammal materials for selected morphometric descriptors: Aspect, Axis Major (µm), Axis Minor (µm), Diameter Max (µm), Diameter Min (µm), Size Length (µm), Size Width (µm), Feret Min (µm), Feret Max (µm), Roundeness 2 (measured value multiplied by 10), Formfactor (measured value multiplied by 10), and Perimeter (µm).

5. Conclusion and remarks

To conclude, the use of microscopic methods in association with computer image analysis to identify the origin of processed animal proteins appears promising although there are several limitations, particularly when trying to distinguish material from higher taxonomic level than class. In spite of that, this approach can be useful in identifying potential markers referred to bone particles and lacunae particularly in the different animal classes. Moreover it has been observed that moving from (i) the shape and structure of an entire bone fragment, to the (ii) shape, size and density of the lacunae, and to the (iii) canaliculae (i.e. the three levels proposed by van Raamsdonk and co-workers)[8] can be useful also in tracing non-target species in animal meals (e.g. sea mammals) (Pinotti unpublished results). Working on sea mammals experimental samples (sea mammals bone fragments obtained from museum and environmental biology projects)[18], it has been observed that even though most of variables measured were significantly different between mammals (both land and sea mammals) vs. poultry in term of mean (figure 7a), no differences between land mammals and sea mammals have been detected [19]. However when lacunae area/fragment area ratio was considered some differences have been observed. Sea mammals material have shown the lowest (by about 30%) the ratio lacunae area/fragment area compared to both land mammals and avian materials (figure 7b) [19]. Thus, data here presented indicate that some of the variables/descriptors provided by image analysis related to lacunae dimensions and features have some potential in distinguishing between land animal and marine animal meals.

Further progress in this area however requires the establishment of a sufficiently large and representative reference dataset, the identification of further key distinguishing descriptors (e.g. derived shape descriptors) and the use of more defined statistical methods to support the image analysis approach. Improvements in these areas will render image processing, integrated with morphometric measurements, better able to provide and accurate and reliable means for characterising feedstuff constituents.

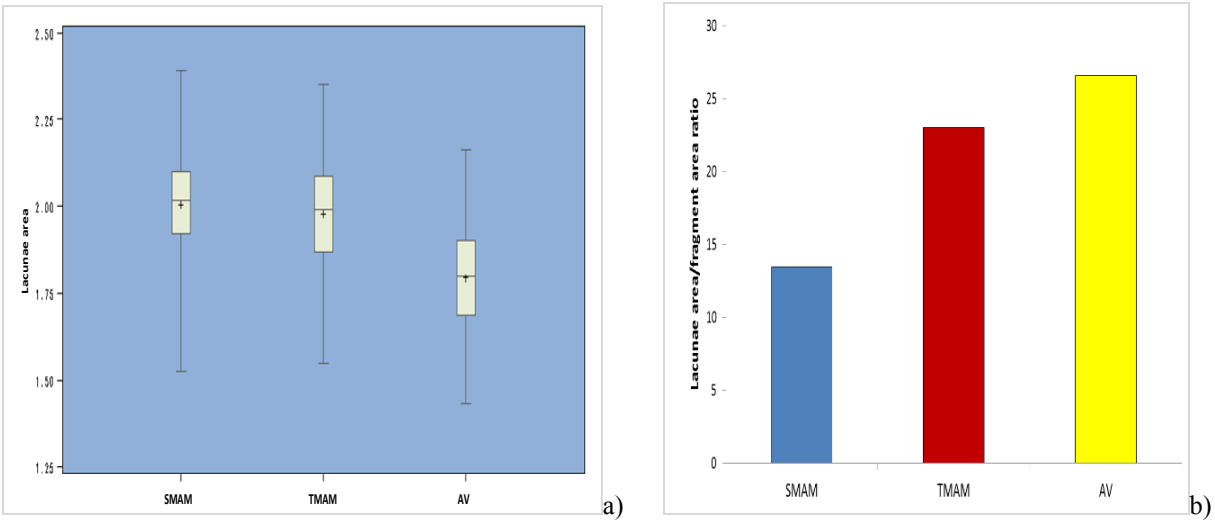

Figure 7. Lacunae area (log_{10} μm^2) box plot (on the left), and variables and lacunae area/fragment area ratio (on the right), in the different animal class. TMAM = land mammals; SMAM = sea mammals; AV = poultry.

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