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Selection of new markers for animal by-products characterization by classical microscopy

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ABSTRACT: The aim of this study was to identify possible markers to distinguish differences between land animals by using the microscopic method in association with computer image analysis. For this purpose bone fragments from poultry and mammals were obtained and analysed by microscopic method. Through a digital camera and an image analysis software 85 bone lacunae images have been processed and elaborated in order to obtain for each lacuna a monochrome mask on which several measurements were performed. Data were analysed by ANOVA and LDA. Results obtained in the present study indicated that of 32 descriptors processed by image analysis software, only 12 were significantly ($P<0.001$) different between mammalian and poultry. However, when morphometric measurements were analysed by LDA, 86% of lacunae were correctly classified into the animal class of origin (i.e. mammalian as mammalian and poultry as poultry). By contrast 14% of lacunae were incorrectly classified. In conclusion, data here presented indicate that some of descriptors used by image analysis appears promising not only for a reliable distinction between the different origins of animal meal at the level of vertebrate classes, but also for further characterisation and identification of processed animal proteins in animal feeds.

Key words: Animal by-products, Microscopic method, Image analysis.

INTRODUCTION – The TSE Roadmap published in 2005 (DG for Health and Consumer Protection, 2005) indicates within of amendments in the short and medium term (2005 – 2009) "a relaxation of certain measures of the current total feed ban when certain conditions are met". In the same document has been reported that the starting point when revising the current feed ban provisions should be risk-based but at the same time taking into account the control tools in place to evaluate and ensure the proper implementation of this feed ban. In this scenario, implementation of analytical method for the detection of constituents of animal origin in feedstuff is required. The official analytical method for the detection of constituents of animal origin in feedstuffs is the microscopic examination technique as described in Commission Directive 2003/126/EC of 23 December 2003 [OJ L 339, 24.12.2003, p. 78.]. Although the microscopic method is usually able to distinguish fish from land animal material, it is often unable to distinguish between land animals (i.e. poultry and mammals). Fulfillment of Regulation 1774/2002/EC requirements, means that it must be possible to identify the origin from which the animal materials are derived, at higher taxonomic level than in the past. As consequence, an improvement of the methods including the microscopic one is required. Starting from these assumptions, the aim of this study was to identify possible markers to distinguish differences between land animals by using the microscopic method in association with computer image analysis.

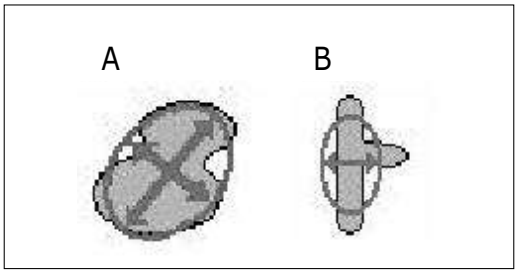
MATERIAL AND METHODS – Samples used in the experiment were prepared at the VSA laboratories. Bone fragments from tibia diaphysis of poultry and mammals were treated with NaOH, then heated in vacuo oven at 130°C at 1 bar for 1 h. Obtained dried samples were milled in order to obtain a powder for microscope observation. Samples after embedding in glycerol were observed with a compound microscope (Olympus BX41, Germany) at several magnifications. Through a digital camera and an image analysis software (Image-for Plus 4.5.1, Media

Cybernetics Inc., Silver Springs, USA), we obtained a total of 85 bone fragment lacunae images at X40. Images have been processed and elaborated in order to obtain for each lacuna a monochrome mask on which several measurements were performed. Image analysis software provides several geometric parameters related to the lacunae measurements. In detail, parameters name and description used were the follow: Area: Area of the object; 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; Axis 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; Box Width: Width of the object bounding box; Box Height: Height of the object bounding box; Diameter min: Length of shortest line joining two points of object outline and passing through the centroid; Diameter max: Length of longest line joining two points of object outline and passing through the centroid; Diameter mean: Average length of diameters measured at 2 degree intervals and passing through object centroid; Feret min: Smallest caliper (feret) length; Feret max: Longest caliper (feret) length; Feret mean: Average caliper (feret) length; Perimeter: Length of the object outline using a polygonal outline; Perimeter conve: Perimeter of the convex outline of the object; Perimeter ellip: Perimeter of the equivalent ellipse; Perimeter ratio: Ratio of Convex Perimeter to Perimeter; Perimeter2: reports the length of the outline of the object; Radius max: Maximum distance between object centroid and outline; Radius min: Minimum distance between object centroid and outline; Radius ratio: Ratio between "Max Radius" and "Min Radius"; Roundness: Reports the roundness of the object, as determined by the following formula (perimeter2)/(4*pi*area); Size length: Feret diameter (i.e. caliper length) along major axis of object; Size width: Feret diameter (i.e. caliper length) along minor axis of object. Total observations used in the present study were 2720. Data were analysed by ANOVA (GLM procedure of SAS statistic software) and LDA (Linear Discriminant Analysis, DISCRIM procedure of SAS).

RESULTS AND CONCLUSIONS – Results obtained in the present study indicated that of 25 descriptors processed by image analysis software, only 12 were significantly ($P<0.001$) different between mammalian and poultry. These last twelve parameters are reported in table 1.

Mostof these descriptors are strictly associated to general description of mammalian and poultry bone fragments and lacunae already reported in literature (Gizzi *et al.*, 2003). In detail it has been reported that bone particles from mammals at higher magnifications show a more or less globular appearance with elliptical to almost globular lacunae. Canaliculae may be visible depending on the quality and opaqueness of the bone particle. Particles from poultry usually show a more splintered (sharp edged) appearance, which is caused by the different structure of the air filled bones. Lacunae are more globular and denser compared to mammal bone particles. Canaliculae are not visible (Gizzi *et al.*, 2003). However, few descriptors, namely aspect (figure 1A) and axis minor (figure 1B), seem indicate that poultry lacunae not always appear globular. In fact, in poultry lacunae the higher values of aspect (3.67 *vs* 2.86, in poultry and mammalian respectively) as well as the lower measure of axis minor (4.78 vs 6.86, in poultry and mammalian respectively) suggest a tapering shape in these animals.

Figure 1. Informative descriptors as reported by Image-for Plus 4.5.1, (Media Cybernetics Inc., Silver Springs, USA); A – Aspect ; B – Axis (minor).



In the present study a further approach was to test poultry *vs.* mammals discriminant method by Linear Discriminant Analysis (LDA), in which a cross validation was adopted. When morphometric measurements were analysed by LDA, 86% of lacunae were correctly classified into the animal class of origin (i.e. mammalian as mammalian and poultry as poultry).

Table 1. Geometric parameters which consent to distinguish mammalian lacunae from poultry ones.

Variable	Class	Mean	Std Dev	P
Area, μ^2	Mammalian	96.87	33.40	<0.001
	Poultry	61.56	19.92	
Aspect	Mammalian	2.86	0.93	<0.001
	Poultry	3.67	1.12	
Axis (minor), μ	Mammalian	6.86	1.84	<0.001
	Poultry	4.78	1.02	
Diameter (min), μ	Mammalian	6.71	1.69	<0.001
	Poultry	4.73	1.00	
Diameter (mean), μ	Mammalian	11.75	1.75	<0.001
	Poultry	9.76	1.67	
Radius (min), μ	Mammalian	2.92	0.82	<0.001
	Poultry	2.07	0.50	
Perimeter, μ	Mammalian	46.97	7.98	<0.001
	Poultry	39.40	8.17	
Size (width), μ	Mammalian	7.60	2.15	<0.001
	Poultry	5.29	1.12	
Perimeter, ratio	Mammalian	0.94	0.04	<0.001
	Poultry	0.97	0.03	
Area (polygon), μ^2	Mammalian	93.68	32.96	<0.001
	Poultry	59.05	19.34	
Feret (min), μ	Mammalian	7.50	2.05	<0.001
	Poultry	5.23	1.08	
Feret (mean)	Mammalian	13.94	2.24	<0.001
	Poultry	12.10	2.37	

By contrast 14% of lacunae were incorrectly classified: 5 of mammalian lacunae were identified as poultry and 7 vice versa. However this can be due to the fact that in LDA analysis all descriptors were used, independently of their significance in distinguish between animal classes. Based on this result it can be speculated that discriminant analysis may have some potential in taxonomic discrimination between animal classes, even though data set dimension, as well as a correct approach to the sample analysis (evaluation of more than one lacuna for each bone fragment) seem to be essential in determining the robustness of the approach.

In conclusion, data here presented indicate that some of the descriptors used by image analysis appears promising not only for a reliable distinction between the different origins of animal meal at the level of vertebrate classes, but also for further characterisation and identification of processed animal proteins in animal feeds.

REFERENCES – Commission Directive 2003/126/EC of 23 December 2003 [OJ L 339, 24.12.2003, p. 78.]. **Directorate-General for Health and Consumer Protection** (2005) "The TSE Roadmap", European Commission, Brussels, COM (2005) 322 FINAL. **Gizzi, G.,** van Raamsdonk, L.W.D., Baeten, V., Murray, I., Berben, G., Brambilla, G., von Holst, **C.** 2003. "Rev. sci. tech. Off. int. Epiz." 22 (1): 311-331. **Regulation** 1774/2002/EC. **SAS**, 1999. User's Guide: Statistics, Version 8. SAS Institute Inc., Cary, NC, USA. 1999.