PAPER

Microscopy and image analysis based approaches for the species-specific identification of bovine and swine bone containing material

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Abstract

The aim of this study was to evaluate the potential of image analysis measurements in combination with the official analytical method for the detection of constituents of animal origin in feedstuffs, in distinguishing between bovine and swine (bone containing) material. Authentic samples of controlled origin containing bovine or swine meat and bone meals were analysed by the microscopic method, in accordance with the official analytical method. Sediment fractions of each sample were observed with a compound microscope at X40. A total of 362 bone fragment lacunae images were recorded and processed through image analysis software, deriving 30 geometric variables for each lacuna. Results indicated that not only were most variables significantly ($P<0.001$) different between bovine and swine samples, but also that two thirds of the same variables were bigger in bovine than in swine. This information, however, does not seem to be so effective in practice since bovine and swine features and measurements overlapped. It can be concluded that the microscopic method even when combined with image analysis does not fit all the requirements for accurately identifying prohibited ingredients of animal origin. A combined approach with other methods is therefore recommended.

Introduction

Since 1st June 2013 (European Commission, 2013b) processed animal proteins (PAPs) from non-ruminants have been re-authorised for use as feed or feed ingredients in aquaculture. This is clearly a first step towards the re-introduction of non-ruminant PAPs in feed (IFFO, 2013), which could also enable the EU to decrease its dependence on other sources of proteins (European Commission, 2010). However, recent advances in feed ban regulations have also imposed some amendments to sampling and analyses for the official control of feed, which are reported in Annex VI of Regulation (EC) No 51/2013 (European Commission, 2013a) replacing Regulation (EC) No 152/2009 (European Commission, 2009a). This regulation suggests microscopy and/or polymerase chain reaction (PCR) as methods for the determination of constituents of animal origin for the official control of feed.

The microscopic method, as described in the literature (Makowski *et al.*, 2011; Vermeulen *et al.*, 2012; Charoud-Got *et al.*, 2012; Van Raamsdonk *et al.*, 2012a, 2012b), was the first method officially accepted for detection of animal proteins in feed by the European Commission. This method distinguishes between constituents derived from terrestrial animals and those derived from fish, but is unable to quantify with sufficient accuracy the amount of animal constituents present in feed, and therefore should not be used for this purpose.

The detection of PAPs using PCR has been widely investigated (Fumière *et al.*, 2012). Thanks to the good stability of DNA to high temperatures and rendering processes, several PCR methods using small multi-copy targets have already proved their efficiency in the detection of PAPs in animal feed at low levels. This indirect method of targeting DNA is the most promising analytical approach to complement microscopy with information on the protein origin at a species level. In fact, neither of the methods (microscopy or PCR) fits all the requirements for the accurate identification of prohibited ingredients of animal origin, *i.e.* for verifying the correct implementation of feeding prohibitions laid down in Regulations (EC) No 999/2001 and (EC) No 1069/2009 (European Commission, 2001, 2009b). This suggest that an approach that combines all the methods should be used. In a recent work (Pinotti *et al.*, 2013) it was suggested that computer image analysis could represent an additional tool for the identification of processed poultry and mammals proteins containing bones. Key lacunae features for animal classification were those related to size, the lacuna area for example. The aim of this work was to investigate the use of microscopy in combination with image analysis measurements in distinguishing between bovine and swine bone material.

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Materials and methods

For this study, 10 samples of controlled origin and processing were used, containing bovine (5 samples) or swine (5 samples) meat and bone meal (Walloon Agricultural Research Centre, Gembloux, Belgium; Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare, Università di Milano, Italy). The aim was to combine the official analytical method with image analysis measurements in order to detect constituents of animal origin in feedstuff, in order to discriminate between bovine and swine lacunae. In each experiment the samples were analysed using the microscopic method (European Commission, 2013a). Sediment fractions of each sample were observed with a compound microscope (Olympus BX41; Tokyo, Japan) at several mag-

nifications, in order to obtain several bone fragment lacunae images at X40 for each sample. Using a digital camera and image analysis software (Image-Pro Plus 7.0; Media Cybernetics Inc., Rockville, MD, USA), 362 bone fragment lacunae images at X40 were obtained (Figure 1). Images were acquired according to Pinotti (2009). The images were then processed in order to obtain a monochrome mask for each lacuna (Figure 2). On each lacuna, 30 geometric variables were measured as previously described (Pinotti *et al.*, 2013). Using this method, size descriptors and derived shape descriptors can be identified. The size descriptors, such as area, perimeter, axis minor and major, radius min and max, *etc*. (Table 1) represent direct measurements on bone lacunae, and are also termed as dimension (primary) descriptors. On the other hand, the derived shape parameters (Table 2) are constructed by combining the various size parameters so that the dimension units are cancelled out (Russ, 2005). Derived shape descriptors are represented by V2, V3, V4, V20, V21, V34, V55, V56 and V58. All lacunae measurement data were collected in Excel files and used for dataset assembly. Tables 1 and 2 report the full list and description of all the 30 geometric variables used.

Bovine and swine lacunae measurements were analysed using one-way ANalysis Of VAriance (ANOVA) in order to compare means of the two species (GLM procedure of SAS statistical software 9.3). The analysis has been performed using the following model:

$$y_{ij}=\mu_j+e_{ij}$$

where y_{ij} are the observations (measurements), μ_j is the mean of the observations for the j^{th} group (species) and ε_{ij} is the random error. Differences with P=0.001 were considered significant. Furthermore, since considerable overlap of species distributions of the sizes of individual lacunae was expected (Pinotti *et al.*, 2013), a graphic test (box plot) for mean and median comparisons has been done. Accordingly, the box plot procedure was performed in order to display the mean, median, quartiles, minimum and maximum observations and outliers for each single species. For brevity only selected box plot data has been presented, *i.e.* lacunae axis major, lacunae perimeter, lacunae aspect and roundness2.

Results and discussion

The results obtained (Tables 3 and 4) indicated that out of 30 variables/descriptors measured on each lacuna, only 15 variables/descrip-

Table 1. Primary descriptors used in the experiment.

ID	Variable	Description
V1	Area, µm^2	Area of the object, includes area of the hole if fill holes is turned on
V11	Axis major, µm	Length of major axis of ellipse
V12	Axis minor, µm	Length of minor axis of ellipse
V13	Diameter max, µm	Length of longest line joining two points of the object's outline and passing through the centroid
V14	Diameter min, µm	Length of shortest line joining two points of the object's outline and passing through the centroid
V15	Diameter mean, µm	Average length of diameters measured at 2 degree intervals and passing through the object's centroid
V16	Radius max, µm	Maximum distance between object's centroid and outline
V17	Radius min, µm	Minimum distance between object's centroid and outline
V19	Perimeter, µm	Length of the object's outline. More accurate than previous version. Old version now called perimeter2
V28	Size (length), µm	Feret diameter (*i.e.* caliper length) along major axis of object
V29	Size (width), µm	Feret diameter (*i.e.* caliper length) along minor axis of object
V30	Perimeter2, µm	Chain code length of the outline. It also includes any outlines of holes. Faster but less accurate than perimeter
V32	Perimeter (convex), µm	Perimeter of the convex outline of the object
V33	Perimeter (ellipse), µm	Perimeter of the equivalent ellipse
V35	Polygon area, µm^2	Area included in the polygon defining the object's outline. Same polygon as that used for perimeter
V40	Box width, µm	Width of the object's bounding box
V41	Box height, µm	Height of the object's bounding box
V42	Min feret, µm	Smallest caliper (feret) length
V43	Max feret, µm	Longest caliper (feret) length
V44	Feret mean, µm	Average caliper (feret) length
V57	Convex area, µm^2	Area of a polygon which has major axis and minimum axis for sides

Table 2. Derived shape descriptors used in the experiment.

ID	Variable	Description
V2	Aspect	Ratio between major axis and minor axis of the ellipse equivalent to object
V3	Area/box	Ratio between area of object and area of its bounding box
V4	Box X/Y	Ratio between width and height of object's bounding box
V20	Radius ratio	Ratio between max and min radius
V21	Roundness	(Perimeter2)/(4π area). It uses perimeter2 and area by default. Select perimeter and area for more accurate roundness
V34	Perimeter ratio	Ratio of convex perimeter to perimeter
V55	Form factor	4π area/perimeter2
V56	Roundness2	4π area/axis major2
V58	Solidity	Area/convex area

tors were significantly (P<0.001) different between bovine and swine in terms of overall mean. Of these, 10 were primary descriptors including major axis, maximum diameter, maximum radius, perimeter, size length, perimeter2, perimeter convex, perimeter ellipse, maximum feret, and mean feret. Five, on the other hand, were shape derived descriptors: aspect, area/box, radius ratio, form factor, and roundness. By contrast area, box X/Y, minor axis, minimum diameter, minimum radius, roundness, size width, perimeter ratio, area polygon, box width, box height, minimum feret, convex area and solidity did not differ between bovine and swine. These findings are very close to those observed in other studies on the same type of material from avian and mammalian by-products (Pinotti *et al.*, 2007, 2013; Campagnoli *et al.*, 2009; Van Raamsdonk *et al.*, 2012a).

The results also indicated that 11 variables were bigger in bovine than in swine, except for area/box, form factor and roundness. Thus, values for all variables/descriptors measured in bovine were higher (+11% in terms of mean; P<0.001) than in swine. On the other hand, area/box, form factor and roundness were 11% smaller in bovine than swine. Our data therefore indicate that not only are lacunae in bovine generally bigger than in swine, but also that lacunae in this animal species differ slightly in shape. In fact, several shape descriptors, such as aspect, roundness and form factor suggest that swine lacunae are more globular than in bovine.

However, probably 11% of differences are not detectable in routine lab practice, indicating that only differences can be detected with an image analysis approach/support. In the case studied in this paper, area and other primary descriptors that have been recently (Pinotti *et al.*, 2013) proposed as key descriptors in distinguishing between animal classes (poultry and mammals), were not so effective, confirming that species identification needs

Table 3. Mean and standard deviation by species of all lacunae primary descriptors measured.

ID	Variable	BOV		SUS		P
		Mean	SD	Mean	SD	
V1	Area, μm^2	102.15	33.64	93.85	27.77	0.0105
V11	Axis major, μm	18.24	3.30	16.35	3.71	<0.0001
V12	Axis minor, μm	7.33	2.12	7.50	1.67	0.3826
V13	Diameter max, μm	18.85	3.54	16.74	3.89	<0.0001
V14	Diameter min, μm	6.74	1.89	6.91	1.46	0.3396
V15	Diameter mean, μm	11.30	1.50	10.86	1.37	0.0043
V16	Radius max, μm	10.04	1.89	9.04	2.17	<0.0001
V17	Radius min, μm	3.01	0.94	3.09	0.76	0.3364
V19	Perimeter, μm	49.91	10.82	45.14	11.15	<0.0001
V28	Size (length), μm	19.15	3.58	17.11	3.97	<0.0001
V29	Size (width), μm	8.30	2.39	8.23	1.90	0.7549
V30	Perimeter2, μm	53.79	11.93	48.62	12.19	<0.0001
V32	Perimeter convex, μm	44.53	7.10	40.91	7.79	<0.0001
V33	Perimeter ellipse, μm	42.24	6.27	38.97	6.87	<0.0001
V35	Polygon area, μm^2	96.28	32.75	88.48	26.88	0.0134
V40	Box width, μm	14.65	4.80	13.78	4.61	0.0822
V41	Box height, μm	14.18	4.83	12.68	4.23	0.0017
V42	Feret (min), μm	8.17	2.33	8.10	1.80	0.7446
V43	Feret (max), μm	19.20	3.56	17.18	3.95	<0.0001
V44	Feret (mean), μm	14.30	2.26	13.14	2.48	<0.0001
V57	Convex area, μm^2	132.80	43.93	121.96	36.66	0.0109

BOV, bovine; SUS, swine; SD, standard deviation.

Table 4. Mean and standard deviation by species of all lacunae derived shape descriptors measured.

ID	Variable	BOV		SUS		P
		Mean	SD	Mean	SD	
V2	Aspect	2.74	1.06	2.31	0.86	<0.0001
V3	Area/box	0.54	0.11	0.58	0.09	<0.0001
V4	Box X/Y	1.26	0.80	1.28	0.77	0.7802
V20	Radius ratio	3.76	1.68	3.18	1.46	0.0005
V21	Roundness	2.27	0.69	2.00	0.97	0.0033
V34	Perimeter ratio	0.88	0.07	0.90	0.06	0.0623
V55	Form factor	0.53	0.14	0.60	0.14	<0.0001
V56	Roundness2	0.41	0.15	0.48	0.15	<0.0001
V58	Solidity	0.77	0.01	0.77	0.02	0.4597

BOV, bovine; SUS, swine; SD, standard deviation.

an integrated approach (*i.e.* a combination of methods). Furthermore, in the studies in which mammalian and avian materials (distinguishing between class) have been tested (Pinotti *et al.*, 2007, 2013; Campagnoli *et al.*, 2009), the differences between variables were bigger than those measured between swine and bovine. This is supported by the more extensive investigation of the present dataset, which was performed using a box plot procedure. When for each variable mean, median, quartiles, and outliers were considered, the dataset showed a considerable overlap between species as expected. In this respect a few variables are presented in Figures 3 and 4. An analysis of these selected means, medians, and box plots clearly indicated that even though most of the variables measured were significantly different between bovine and swine in terms of overall mean, none of them is able to discriminate between species material (*i.e.* bovine *vs* swine) *per se*. These results therefore confirm other findings (Pinotti *et al.*, 2013) in the field, in which no clear indication of species differences within classes has been reported.

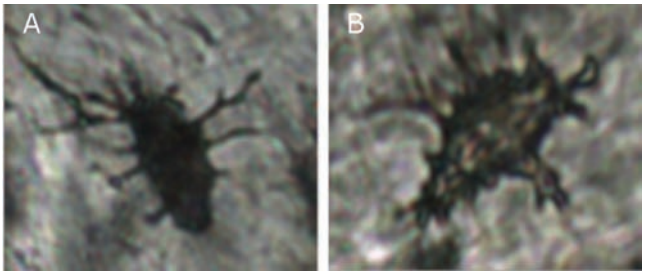

Figure 1. Examples of swine (*Sus scrofa*) (A) and bovine (*Bos taurus*)(B) bone lacunae at X40 magnification.

Figure 2. Key steps in lacunae measurements. Reproduced with permission of Prof. L. Pinotti (Pinotti, 2009).

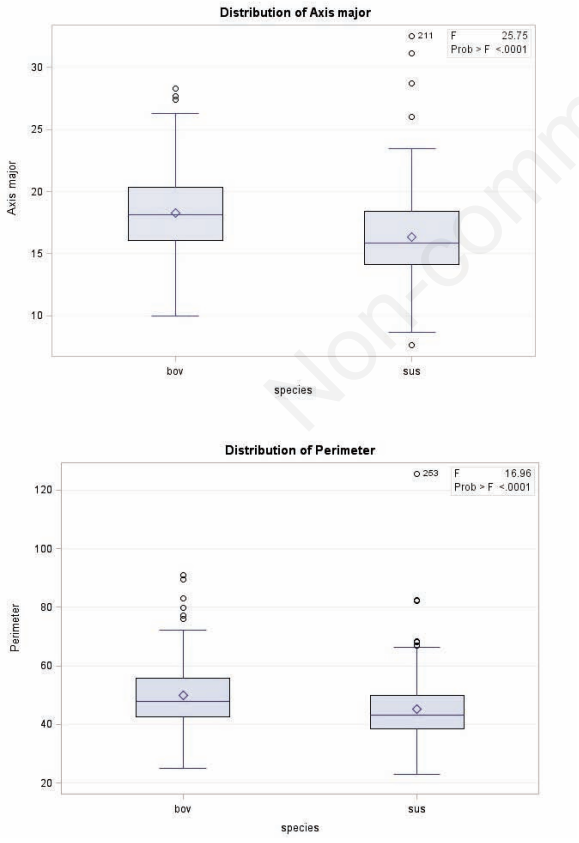

Figure 3. Box plots showing selected primary descriptors (lacunae axis major and perimeter) in bovine (bov) and swine (sus) species. Diamond represents mean; line within the box is the median; circle stand for the outlier.

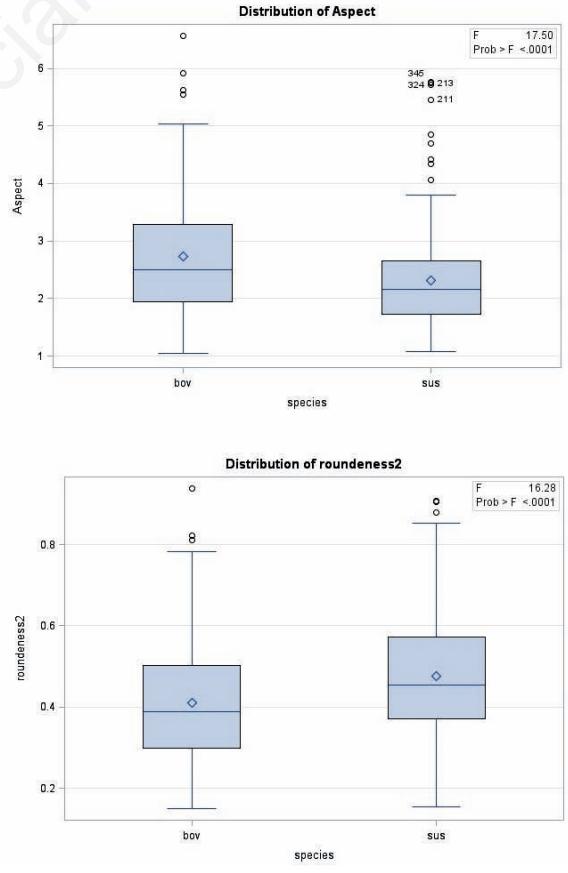

Figure 4. Box plots showing selected derived shape descriptors (lacunae aspect and roundness2) in bovine (bov), and swine (sus) species. Diamond represents mean; line within the box is the median; circle stand for the outlier.

an integrated approach (*i.e.* a combination of methods). Furthermore, in the studies in which mammalian and avian materials (distinguishing between class) have been tested (Pinotti *et al.*, 2007, 2013; Campagnoli *et al.*, 2009), the differences between variables were bigger than those measured between swine and bovine. This is supported by the more extensive investigation of the present dataset, which was performed using a box plot procedure. When for each variable mean, median, quartiles, and outliers were considered, the dataset showed a considerable overlap between species as expected. In this respect a few variables are presented in Figures 3 and 4. An analysis of these selected means, medians, and box plots clearly indicated that even though most of the variables measured were significantly different between bovine and swine in terms of overall mean, none of them is able to discriminate between species material (*i.e.* bovine *vs* swine) *per se*. These results therefore confirm other findings (Pinotti *et al.*, 2013) in the field, in which no clear indication of species differences within classes has been reported.

Conclusions

From this study it can be concluded that the microscopic method is not able to discriminate between bovine and swine material, even when improved via image analysis. However, the method may be useful in indicating the best approach (*e.g.* PCR) for further analysis and investigation, excluding for instance the presence of poultry material. In fact, neither microscopy nor PCR is able by itself to meet all the requirements for the accurate identification of prohibited/authorised ingredients of animal origin (milk powder). A combined approach in which both methods are merged is therefore recommended.

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