Chapter 6
Markers for microscopic detection

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MARKERS FOR MICROSCOPIC DETECTION

SUMMARY

A range of markers can be used to detect or to identify animal proteins in animal feed. The presence of hairs and teeth, of feather filaments, and of scales, gills and otoliths will point to the presence of material of the vertebrate classes of mammals and birds, and to the superclass of fish, respectively. The structure of bone fragments of different groups of the class of ray-finned fish is clearly distinguishable, and an identification on the level of ray-finned fish orders can be achieved. The difference between mammalian and avian bone fragments is more complicated. The collection of information on a range of markers of bone fragments might give a first indication of the source of the material. It is important to synthesize all available information; the application of a single marker, especially used for discriminating between mammalian and avian material should be discouraged. The use of programs such ARIES allows to interpret the collected information in a reliable way. A range of confusing particles in a feed complicates the correct detection and identification. This range includes material of invertebrate animals, minerals, plant hairs and certain plant structures (seed epidermis).

Keywords. Animal proteins, meat and bone meal, fishmeal, microscopy method, identification.

6.1. INTRODUCTION

After a suitable application of the microscopic method as presented in the previous chapter (chapter 5), a series of observations has to be made to reach a final conclusion on the type and identity of the materials. Investigations of morphological descriptors to distinguish animal material at the taxonomic level of classes (i.e. fish, birds, mammals) have been carried out recently, concentrating on several features that can be used as potential markers. Key distinguishing descriptors such us features of bone fragments, hairs for mammals, feather filaments for birds, scales, gills and otoliths for fish have been proposed as promising markers for distinguish between the most frequently occurring animal materials. In this context the distinction between terrestrial animals and fish will be particularly emphasised, because of the current legislation (chapter 3). Some general indications for the distinction between mammals and birds will be discussed, since future legislation (species-to-species ban) needs support. This chapter will present a first outline of the identification of key distinguishing descriptors of meat and bone meals in animal feeds, and the use of microscopic methods to identify the source species of these feedstuff contaminants/ingredients.

6.2. DESCRIPTION

The following paragraphs present possible observations of fractions of material, which are prepared according to the method described in the previous chapter. A correct application of the method is necessary for a proper application of the information in these paragraphs.

6.2.1. Presence and structure of principal ingredients

All bone fragments, muscle fibres, hairs, feather filaments, scales, gills, otoliths and cartilage indicate material that is subjected to the ban of animal proteins. These particles provide information at several systematic levels for a proper detection and identification (figure 6.1). In the case of **ruminant feeds**, these are all prohibited. Besides a proper documentation of the findings in terms of presence, further examinations are basically not necessary. One exception exists for young ruminant feeds where fish material is allowed as milk replacer (European Commission, 2008). In the case of all other **non-ruminant feeds**, fish meal is allowed. Feed plants may hold and process fish meal parties only if they have a permit for these activities, but from the point of view of monitoring this does not make a difference.

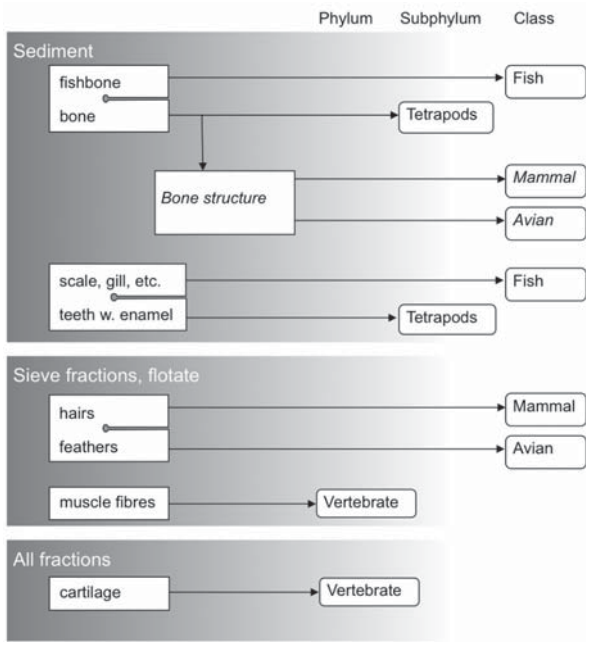

Figure 6.1. Overview of particles, their source from the microscopic procedure, and the identifications resulting from their presence. In italics: identification depends on a range of features of bone particles, and the conclusions indicate only tendencies.

6.2.2. Sediment

Bone fragments

As illustrated in figure 6.2, the markers or characteristics of bone fragments can be examined at three different levels: the shape and structure of an entire bone fragment, the shape, size and density of the lacunae, and the visibility of the canaliculae.

The first step of the analysis of sediment material is the examination at lower magnifications using a stereomicroscope. The advantage is that the entire sediment can be examined at one glance in the petri dish or in a watch glass. In this way all the sediment material representing the recommended amount of 10 grams used for sedimentation is considered, which is virtually impossible if a series of slides containing all the material have to be produced and examined. Especially the portion with larger particles can give a first indication of the type of material; the portion with the smaller fragments is more suitable for preparing slides for examination at higher magnifications. The outline of bone particles can be smooth (figure 6.2a top: mammal and some fish groups), edged (figure 6.2a bottom: birds) or parallel sided (some fish groups). Fish bones might show a (semi-) transparent and more or less glossy view. Bone fragments of terrestrial animals show a whitish or cream colour and look worn out. They differ from minerals showing crystals structures. The bone fragments of poultry material are crispy. In some occasions they can be crushed to powder when pressed between a pair of tweezers (Gasparini et al., 1994; Gizzi et al., 2003; ARIES, 2010). The intensity of the colour of the bone fragments of land animals might depend on the severity of the temperature during the sterilization process. As a result of this circumstance, poultry bones might show a whiter colour, since these bones are normally not sterilised. The application of Alizarin Red staining is especially useful for examination at lower magnifications. The red colouring is apparent and might help in the discrimination of the particles.

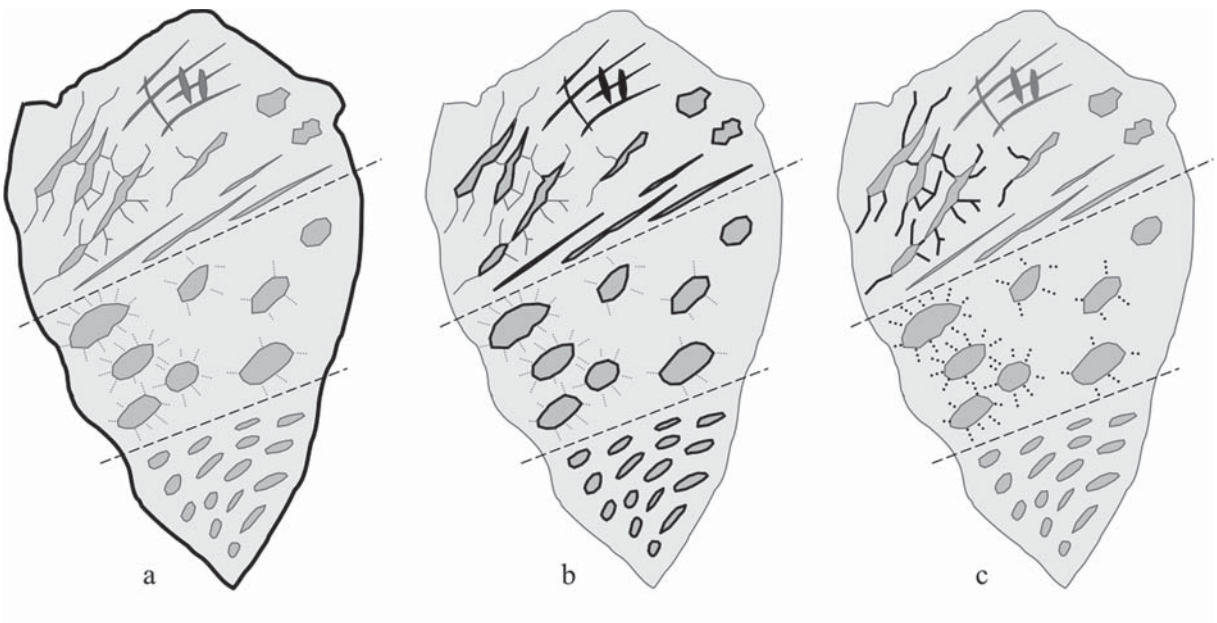

Figure 6.2. Overview of different descriptors of bone fragments at the level of the entire fragment (left), of the lacunae (centre), and of the canaliculae (right). Explanation: the following characteristics are included, and translated to the situation in practice:
General shape of fragment (left),
Area of lacunae (centre),
Shape of lacunae (centre),
Width of lacunae (centre),
Density of lacunae (centre),
Visibility of canaliculae (right).
The top part of each fragment depicts the fish, the central part the mammalian, and the bottom part the avian variability.

The examination of a slide at higher magnifications will reveal information on the inner structure of the bone fragments. The lacunae in bone fragments can be elliptical or elongated (figure 6.2b top: fish), or oval to elliptical (figure 6.2b centre and bottom: terrestrial animals).
With respect to fish material, herring and sardine have elliptical or elongated lacunae with clearly visible canaliculae, with irregularly shaped bone fragments (figure 6.3). Fish bones of cod and relatives are normally parallel-sided, and show nearly linear lacunae without visible canaliculae, which are orientated parallel to the sides of the bone fragment. Tuna also shows a dense pattern of clear lacunae, randomly orientated in the bone fragment. Salmon fragments show irregular oval lacunae with hardly visible canaliculae. This group of fish species shows sometimes an appearance that looks like that of land animals. Canaliculae might be completely visible, forming spider-like structures (figure 6.2c top-left: herring), or might not be visible (figure 6.2c top-right: some fish families).

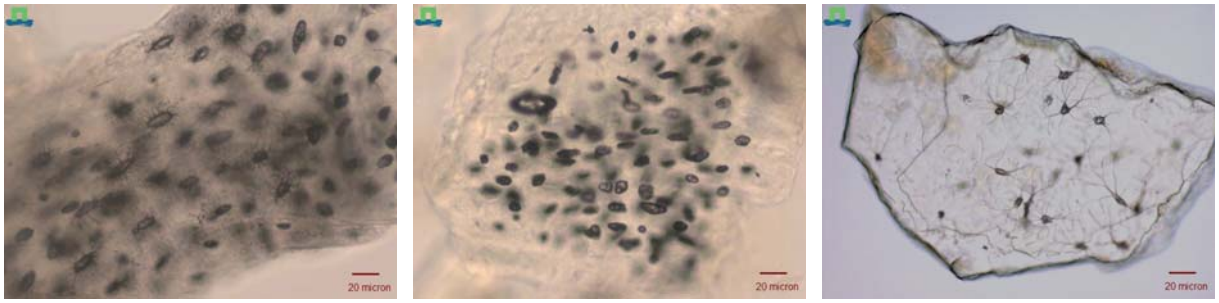

Figure 6.3. Bone particles from different animals. From left to right: mammal, chicken, herring. Images abstracted from the expert system ARIES. The scale bar is 20 micron.

The differences between avian and mammalian bone fragments might be more difficult, because overlap between species groups exist for all relevant markers. Recent investigations (see State of the art; cf. Figure 6.3) revealed that on average lacunae from mammalian bone fragments are wider (larger width and diameter; figure 6.2b centre) and larger (larger area) than lacunae from avian bone fragments (figure 6.2b bottom).

Canaliculae might be faintly visible around some lacunae (figure 6.2c centre: mammals), or never visible (figure 6.2c bottom: birds). The visibility of the canaliculae depends on the viscosity of the embedding agent. Canaliculae (and lacunae) remain filled with air for a longer time in an embedding agent with a high viscosity, thus enhancing their visibility. The lower the viscosity, the sooner the air is removed from the bone structure and replaced by the embedding agent, which makes it more difficult to do the observations. The differences between mammals and birds are gradual and overlap exists (figure 6.4). Colouring of the fragments (Alizarin Red staining) is indicated as being supportive for the recognition of calcium containing material, i.e. bones (Veys and Baeten, 2008), but staining might hinder the observations of the fine bone structures (van Raamsdonk et al., 2009). It could be recommended to examine the sediment material at least partly without any further treatment.

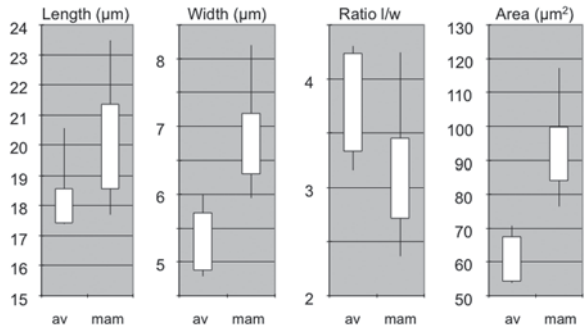

Figure 6.4. The distribution of four parameters of lacunae in bone fragments of birds (av) and of mammals (mam). A length/width ratio of 1 indicates a circular shape; the higher the ratio the more elliptical or elongated the shape. Each box represents the 25-75% of the total range of variation (line).

These descriptions are very general and without an indication of intra-group variation. It is very difficult to recognise the entire variation which can occur in practice and a large experience is needed from the microscopist. Furthermore confusing particles such as plant hairs can be present. Extensive use of knowledge systems, image databases and reference books is highly recommended.

Other (animal) particles in the sediment

The sediment may contain other types of fragments if animal proteins are present in the sample. These particles may include gills, scales, teeth and otoliths (ear bones) of fish, and tooth fragments of mammals. Cartilage, recognised by a dense pattern of generally large circular lacunae, may also show up in the sediment. Cartilage can not be distinguished for their source, either fish, birds or mammals.
Egg shells will show up in the sediment when present. These ingredients are legally applied as calcium source. They can be best identified at low magnifications. Additionally a chloric acid spot test for calciumcarbonate will give a clear effervescence.

6.2.3. Flotate or entire sample

Muscle fibres

Important indications of the presence of animal proteins in general is the presence of muscle fibres. Skeletal muscles show a cross striation formed by the sarcomere structure of the content of the fibre. Depending on the severity of the heat treatment during rendering this cross striation is more or less visible, and other heat damage can be visible, such as brown colouring and dark brown or even black regions. The smooth muscle tissue shows no cross striation, which reduces the visibility of these fibres. The application of a sodium hydroxide solution (Fehling's reagent) can help to improve the visibility. It should be noted that the share of smooth muscle might be larger than the share of skeleton muscle, since the latter category is predominantly used for human consumption. The muscle ratio, as published in previous papers (van Raamsdonk et al., 2004; 2005), proved in more recent investigations to have very large overlaps between different groups of animals. No further value is attributed to this feature.

Hairs

The presence of hairs in the sieve fractions or in the flotate points to the presence of mammalian proteins in the sample. Their occurrence is rare, as can be concluded from long term monitoring practices. This rare occurrence fits in the requirements as set in Regulation 242/2010/EC (European Commission, 2010). If present, two different areas should be examined from the guard hair fragments (Figure 6.5): the shield and the shaft, each from two structural parts: the medulla and the cuticle. The medulla is the inner region of the hair, which is best observed with paraffin oil as mounting medium. Distinction can be made based on the structure (amorphous appearance) and distribution of cells (a one-row versus a multi-row distribution of cells). The appearance of the medulla depends on the area (shaft/shield) and on the original species of the guard hair. The cuticle is the outer layer of the hair, composed by overlapping scales. As for the medulla appearance, these scales of the hairs show a specific pattern depending on the area and on the original species. The ratio cuticle width / medulla width can also provide information on species-group identification, but it is less valuable. With respect to farmed animals (e.g. cattle, pig) versus species groups that might show up unintentionally (rodents, hares and rabbits included accidentally during harvest or present in the storage places of feed), the distinction between these groups is especially informative (figure 6.6). Further reading can be found in Brunner and Coman (1974) and Teerink (1991).

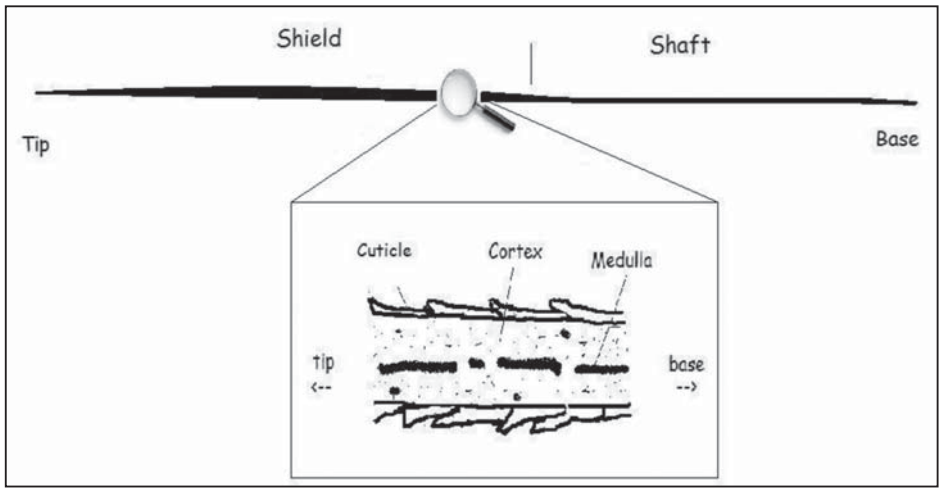

Figure 6.5. General schema of a guard hair: For hair studies, two areas (shield and shaft) and two structural parts (cuticle and medulla) should be examined.

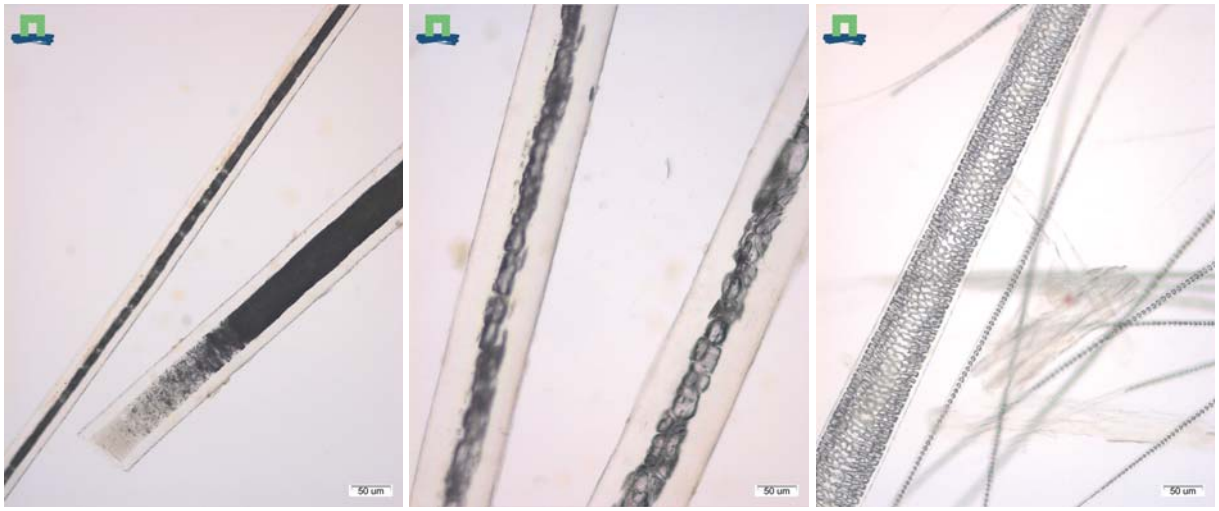

Figure 6.6. Hairs of different mammals. From left to right: cattle, rat (fur and guard hairs), dog. Images abstracted from the expert system ARIES. The scale bar is 50 micron.

It should be noted that the heat treatment during incineration can (severely) damage the structure of hairs. The application of cysteine reagens (lead acetate solution, chapter 5) can ease the visibility of hairs.

Feather, non- hydrolysed and hydrolysed**.**

The presence of feather filaments can be an important indication of avian material. However, the detection is not easy, since feather meal is often hydrolysed before applying it in a feed for increasing the digestibility. Hydrolysed feather meal might appear as amber-like particles, which often show the remnants of feather filaments on their surface. The cystein reagent test might give extra information on the identity of the material. It should be noted that small particles of hoofs (mammalian material) might appear in a comparable way as hydrolysed feather meal, while a cystein reaction is similar, due to the high keratin content in hoofs as well.

Blood and other animal related products

Blood meal, milk powder, and gelatine are animal products that can be legally added to animal feeds, at least for non-ruminant purposes. Also over due materials such as bakery by products have a legal application in feed. If the blood meal and the milk powder is spray dried these substances might be visible by light microscopy as small globules containing air bursts. Gelatin and bakery by products can not be identified by classical microscopy. DNA detection might give a positive signal when these ingredients are present in the feed. In the (proven) absence of meat and bone meal the detection of ruminant DNA might point to some of these (partly) legal substances. In the presence of meat and bone meal, milk products can be a confusing ingredient.

6.2.4. Further reference

It has to be emphasized that a manual for evaluating the observations made by microscopic examinations can not be presented on a few pages. In addition to the initial information presented here, extensive documentation needs to be available for support of the evaluation. Possibilities are:

- Decision support system ARIES, providing several galleries, information on method modifications and additions, legislation and several identification trees (Vermeulen et al., 2003). A new web application of ARIES is launched in 2010 (ARIES, 2010).
- Micrograph collection and Identification key for rodents, on the website of the Community Reference Laboratory for animal proteins: www.crl.cra.wallonie.be. This collection initially dedicated to the members of the network of National Reference Laboratories for Animal Proteins in feedingstuffs and now available for all members of the IAG working group Feeding Stuff Microscopy: www.iag-micro.org .
- Hand books and atlases: there is no dedicated hand book for the detection of animal proteins. Some general text books on microscopic detection of feed ingredients provide a chapter on animal proteins: Gassner et al.,

1989; Klein & Marquard, 2003; Hohmann, 2006.
- Manual of the section Microscopy of the American organisation AOAC (Bates et al., 1992). A new version is scheduled for 2010.
- General updated information on feed safety is provided by : www.feedsafety.org .

These sources also help to recognise series of parts of plant and mineral sources in order to avoid confusion with structures such as punched hulls, plant trichomes, starch, invertebrate shells and minerals of organic origin.

6.3. STATE OF THE ART

The recognition of different groups of animal material can be interpreted in several ways. Incineration plants are obliged to comply with legislation, which differentiate between different categories of material. These categories do not comply to biologically recognisable species groups, but to the background of the materials. Three categories are recognised based type of organ (brain tissue, tonsils, eyes, back bone vs. other organs), age of animal, fallen stock, gut and gut content, and intention of human consumption (Regulation 1774/2002/EC, Annex 1). With respect to the species-to-species ban (Regulation 1774/2002/EC, article 22: Ban of cannibalism), other distinctions have to be made. The ability to distinguish between animal groups depends on the level of the systematic category where the distinction should be made: the higher the systematic category, the more prominent the differences are. As an example, pigs as well as ruminants are both part of the mammalian order of the even-toed ungulates. Making distinction on the basis of morphological features is expected to be impossible, but at higher levels (e.g. mammalian vs. avian) a distinction might be less difficult.

Furthermore, the detection of animal proteins in a feed might apply to a single species or to a mixture of different species. Considering the situation that for several markers overlap exists, even between mammalian and avian material, the possibility of the presence of a mixture complicates further the monitoring. Fish meal parties sometimes consist of small amounts of “terrestrial animals”. Besides the possibility that contamination with material of farmed animals can occur, it is likely to be assumed that sea mammals or sea birds sometimes show up in caught fish meal parties.

Considering bone fragments as the main targets for the detection of animal proteins in feed, some prerequisites have to be made for the monitoring of these animal proteins. At first, the different bones in an animal body show different characteristics with respect to their structure, lacunae, osteons, Haversian canals, etc. (Bacha and Bacha, 2000). Since bones are smashed randomly in an incinerator, the relationship between the view of bone fragments in feed and their original orientation in the original bone is lost (Domenis et al., 2009). Furthermore, large variations might exist. The second point is the daily practice of the monitoring. A few erratic bone fragments in a slide of a sediment might present a different appearance compared to bone fragments in optimally prepared slides based on pure animal meals. Nevertheless, recent analyses of a range of characteristics of bone fragments reveal that combinations of markers might be useful to collect information on the source of the material. In the following paragraphs background information on markers which might be evaluated in the detection of animal proteins will be presented and discussed.

6.3.1. Multivariate analyses of bone particles

Several studies have been carried out to analyse the diversity within and between the groups of birds and mammals. A range of different markers are being used for the characterisation of bone fragments (e.g. shape, size and density of lacunae, visibility of connecting canals) (Pinotti et al., 2004; Pinotti et al., 2008; Campagnoli et al., 2009; Paltanin et al., 2009).

In an elaborate study within the SAFEED-PAP project a dataset of 1128 randomly collected and observed lacunae, examined in eight poultry samples and 17 mammalian samples have been evaluated with respect to a total of 10 descriptors. Additionally, analyses have been carried out for investigating the visibility of the canaliculae and the density of the lacunae in a bone fragment. The details of these studies and the detailed results will be presented in other publications. Here it is important to discuss the possibility to translate these scientific results to markers to be used in the situation of the laboratory practice.

A principal component analysis of the 10 variables indicated a clear distinction between the groups of poultry and of mammalian materials (figure 6.7; unpublished results). The major discrimination is along the first principal component (x-axis). By contrast there is no apparent discrimination within the two major groups when the second principal component (y-axis) is considered. Of note in this study is that rabbit samples have high factor scores for the second principal component, whereas a very high score at the x-axis was calculated for a cattle sample specifically produced for the former European project STRATFEED (cattle MBM sterilised at 133 °C; Garrido-Varo et al., 2005). Although frequently used for several tests (cf. van Raamsdonk et al., 2008) this sample material might not be fully representative for average mammalian material.

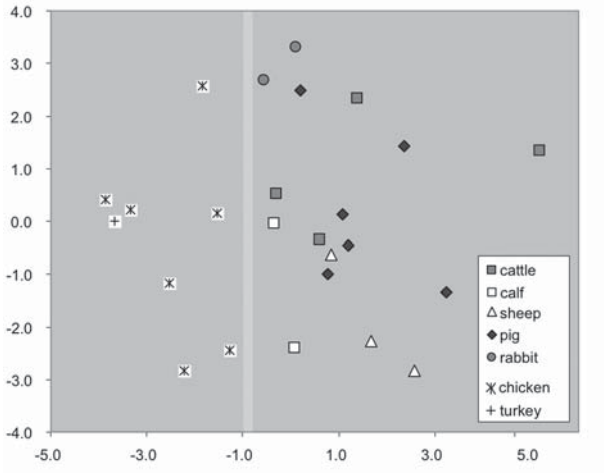

Figure 6.7. Principal component plot of the factor scores of 25 samples of animal proteins plotted against the first PC (x-axis) and the second PC (y-axis).

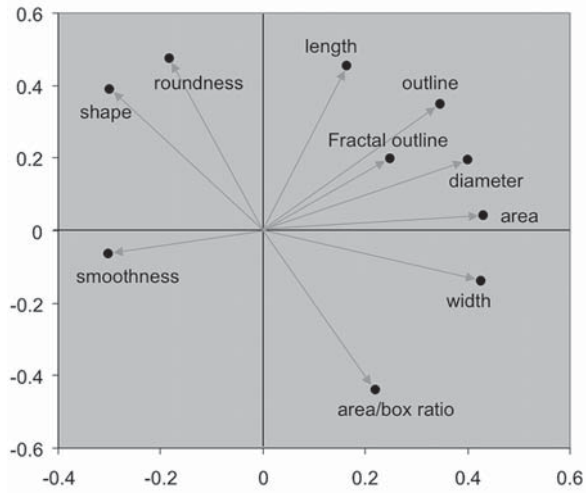

Figure 6.8. Principal component plot of the factor loads of all 10 variables plotted against the first PC (x-axis) and the second PC (y-axis).

The factor loadings of the 10 variables on the first and second principal component are shown in figure 6.8. The first principal component is primarily directed by the original variables shape, smoothness, area and width according to the length of arrows as measured along the x-axis. This indicates that these variables are more informative than the others. Accordingly, the results of this principal component analysis allow to draw two main conclusions. At first, it appears possible to discriminate gradually between the major classes birds and mammals based on morphological features of bone material, i.e. meat and bone meal, even if a clear relationship with histological specified body parts is not available. Secondly, the major variables supporting this gradual distinction can be recognised. It is also true that only a combination of these variables can be considered valuable for discrimination.

In a further study (Paltanin et al., 2009) performed on 574 lacunae equally distributed among poultry, swine, bovine and ovine, bone particles were examined for the number of canaliculae and for the density of the lacunae. This study focused primarily on those bone fragments with visible canaliculae. Provisional observations reveal that the number of canaliculae per lacuna is higher in pig (26.70 canaliculae per lacuna) than in ruminants [(bovine plus ovine) 19.15 canaliculae per lacuna] and in poultry (15 canaliculae per lacuna). An important factor in this study was the extensive application of image processing: lacunae images have to be processed with different filtering procedures of image analysis software in order to facilitate the count of canaliculae in each lacuna. Data from practice indicate that usually no canaliculae are visible in poultry material, whereas mammalian material might show a few canaliculae. This trend is now supported by this detailed study. It should be noted that the visibility of the canaliculae depends on the viscosity of the embedding agent. With regard to lacunae density (i.e. lacunae area per bone fragment area), recent investigations (Pinotti et al, unpublished results) indicate that density in porcine fragments is higher than in bovine and ovine species, while an intermediate position is possessed by poultry material. The use of these two variables as reliable markers in distinguishing ruminant and non-ruminants from avian material merit further investigation.

A series of papers have been published to document the markers necessary for discriminating between different categories of animal proteins (Voehringer, 1979; Gasparini et al., 1994; Mondini et al., 1999; Frick et al., 2002; Maret,

2002; Gizzi et al., 2003). More recently Domenis et al. (2009) analysed the relationship between histological identified bone structures and smashed bone fragments of poultry material. The parallel fibred structure of the diaphysis of long bones might cause the predominant occurrence of elliptic shape of lacunae in bone fragments of that area. Random fragments of other bones and of the epiphysis of long bones, however, will contain circular or oval lacunae in considerable frequencies. It is interesting to note that the lacunae pattern in the smashed bone particles is rather dense (Domenis et al., 2009).

The collected information should be carefully translated to markers for the different types of MBMs, including the ranges in variability (figure 6.4), since time consuming measurements are difficult to make in practice. Considering the prerequisites, the purposely at random collection of data in the SAFEED-PAP experiments reflects the situation in practice in its aspect that any information on orientation of bone fragments is lacking. Also the visibility of fine structures depends on the quality of bone particles and on e.g. the embedding agent or colouring method (chapter 5). All the information from all the markers is normally pooled by the analyst in order to obtain an overall impression of the material investigated for a sample in practice, and in that way a final choice could be made. Situations might frequently occur that the observed value for one marker deviates from the appropriate marker's variability range, whereas the observations for the other variables do fit. In those cases that not all observations will point in the same direction, computer applications such as ARIES will provide the tools to evaluate the set of examined marker states and give indications of the reliability of a possible conclusion.

6.3.2. Value of hair examination

In normal practice hairs are usually not detected in samples. This might be due to absence in meat and bone meals, or to the situation that they are simply overlooked. Previous results from the monitoring activities of several partners indicate that hairs are rarely present in samples. Regulation 242/2010/EC states that "The product must be substantially free of hooves, horn, bristle, hair and feathers, as well as digestive tract content." Presence of low amounts of animal proteins in feeds can, however, be due to the situation that occasionally rodents enter the production facilities and the product flow. In these cases hairs can be expected as well and they can be used to discriminate between these unintentional side effects and the presence of processed animal proteins in the sense of the European legislation.

It is known that the major groups of mammals (i.e. ungulates including ruminants, predators including fur animals and pets, and rodents) can be distinguished using hair characteristics (Brunnan and Coman, 1974; Teerink, 1991). In the occasions that a feed sample in practice contains one or a few particles of animal origin, it would be an advantage to discriminate at least between ruminant and rodent material. Identification is now documented based on recent studies carried out in the framework of SAFEED-PAP project and due to the request to the European commission.

Staining of a part of the sieve fractions of the whole feed sample with cystine reagent, in order to enhance the visibility of keratin as major component of hairs, is only a facultative step in the procedure as described in Regulation 152/2009/EC (European Commission, 2009; see also chapter 5). The different types of hairs as indicated in literature can also be found after heat treatment, but it is likely to expect a damaged appearance.

Currently hairs are found in samples from monitoring programs at a very low frequency. It has to be investigated whether these rare occurrences are due to the situation that a special colour reaction is normally not applied, or that the frequency of occurrence is really low. A further analysis is recommended.

6.4. CONFUSING PARTICLES

6.4.1.Animal ingredients of non-vertebrates

A series of products with an animal origin are allowed in animal feeds, such as foraminifers, bivalve shell particles, krill, shrimp and crab parts. Some of these ingredients are basically applied as source of calcium. Tentacles of krill, shrimps and crab might look like feather filaments, but they will not give a colour reaction after applying cystine reagent, since the chitin is a polycarbonate instead of a (sulphur rich) protein.

6.4.2. Plant parts

Several plant parts, which are the by products of oil processing such as hulls, might look like bone particles. A first difference is the situation that these plant particles usually show up in the flotate and are only very rarely found in the sediment. Lacunae in bone fragments, especially when they are still filled with air, look like darker spots in an almost white matrix. The hulls of rapeseed have an opposite appearance: penta- or hexagonal light brown coloured palisade cells surrounded by dark brown walls. The cells of soya hulls are black and look like having canaliculae to other adjacent cells. This appearance is caused by the structure of the secondary cell wall. The primary cell wall is normally visible as well, which provides a good opportunity to distinguish these hulls from animal material.

Further documentation of confusing plant parts is provided by the knowledge system, websites and text books.

6.4.3. Plant hairs

A special type of plant particles consists of plant trichomes, which can be confused with animal hairs. Plant trichomes are usually short and tapering, with a blunt end. Identification is not difficult when the plant hairs are still connected to the original tissue, they are easily differentiable with polarization technique in microscopy. Cotton also consists of special types of hairs of plant origin, which are long, flat and curled. Alizarin Red staining is also helpful in this case, teeth are slighty stained but not the plant trichomes.

6.5. DISCUSSION AND CONCLUSIONS

The detection of species specific elements (e.g. scales for fish, feather filaments for birds, hairs for mammals, or the hair differences for the orders of ruminants, rodents and carnivores) does not imply that all the other muscle fibres and bone fragments that might be present, belong exclusively to the same species. There is always a chance that mixtures are present. Mixtures of fish and terrestrial animal material are well recognisable in most cases (see Chapter 5), but the proper recognition of a mixture of mammalian and avian fragments is much more difficult, or impossible. This situation might result in the conclusion that microscopic characters are not feasible for any identification at all, but the same premise does apply to all other identification methods. If, for example, an antibody for ruminant muscle protein is applied with a positive result, the presence of muscle material from other mammals, birds and fish can only be ruled out if antibodies to those other groups are applied as well, and give a negative signal. The same situation applies to DNA detection.

In general it is recommended to develop markers for the most frequently occurring animal groups. In the case of visually detectable markers, e.g. hairs or fish bone structure, the information for discrimination is readily available.

It can be concluded that no specific marker can be based on muscle fibre statistics. Moreover, the research indicates that a part of the fibres is treated to such an extent that the cross striation can not be or can hardly be examined. These considerations indicate that further analysis for visible descriptors for muscle fibres is not appropriate with the exception of potential immunochemical parameters (chapter 13). However, the analyses of bone particle parameters show possibilities to differentiate species groups, e.g. classes or orders. It is recommended to analyse the now available materials in more detail in order to extend the basis for valuable descriptors.

Several extensive studies in the framework of SAFEED-PAP have provided information on the variability of a range of descriptors, and on the applicability of them. It might be concluded that some of those descriptors, especially the shape of the lacunae, is depending on the orientation of the fragment in the original bone. A larger variability should be expected for those descriptors. In normal practice, however, microscopists would not examine single lacunae and draw conclusions from those individual observations. Instead, a bone fragment will be considered as a whole and all the information from different aspects will be considered, including an average impression of the visibility of the lacunae and of the canaliculae. It is important to emphasise that only first indications can be given with respect to the origin and nature of the encountered materials in a sample from practice, and that other supplementary methods, e.g. DNA identification methods, immunochemistry or Near Infrared Microscopy, or combination thereof, need to be applied to provide further evidence.

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