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The microscopic detection of animal proteins in feeds

Leo WD. van Raamsdonk [1], Jeroen Vancutsem [2], Jos Zegers [3], Geneviève Frick [4], Jan-Sten Jorgenson [5], Victor Pinckaers [1], Jaume Bosch [6], Inge Paradies-Severin [7]

[1] Institute of food safety (RIKILT). P.O. Box 230. NL-6700 AE Wageningen (The Netherlands).
E-mail: Leo.vanraamsdonk@wur.nl
[2] Agence fédérale pour la sécurité de la chaîne alimentaire – Federal feed and food laboratory (AFSCA-FVLT) of Tervuren. Leuvensesteenweg, 17. B-3080 Tervuren (Belgium).
[3] Masterlab (NUTRECO). Veerstraat, 38. NL-5831 JN Boxmeer (The Netherlands).
[4] Agroscope Liebefeld-Posieux. Station fédérale de Recherches en Production animale et laitière (ALP). Tioleyre, 4. CH-1725 Posieux (Switzerland).
[5] Danish plant directorate (DPD). Skovbrynet, 20. DK-2800 Lyngby (Denmark).
[6] Laboratori agroalimentari de la Generalitat de Catalunya (LAGC). Carretera de Vilassar de Mar a Cabrils s/n. SP-08348 Cabrils, Barcelona (Spain).
[7] Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA). LUFA Nord-West. Finkenborner Weg 1A. D-31787 Hameln (Germany).

In the framework of the European Union funded research project STRATFEED for detection and identification of animal proteins in feeds, the microscopic method was optimized at several key steps and better documented. A check list was developed for uniform reporting. Characters of bone fragments, hairs, muscles and gills are fully documented. A so-called muscle ratio has been developed for the identification of muscle fibers at the level of vertebrate classes (mammals, birds and fishes). Both the improved protocol and the entire range of characters which can be observed, are documented in a Decision Support System called ARIES (Animal Remains Identification and Evaluation System). A second internet-based system called STRATFEED-DSS exclusively assists in identification of animal particles in feeds. A new strategy with microscopy as screening technique and either DNA or protein identification as confirmation technique is proposed. The advantages of this combination are the extremely low level of false negatives, low detection limits and the heat-resistant nature of microscopic detection, together with the possibility of a very specific identification of particles by one of the other methods.
Keywords. Animal proteins in feed, microscopy, decision support system, ARIES, muscle ratio.

1. INTRODUCTION

The diseases indicated in general as Transmissible Spongiform Encephalopathy are connected to the production and presence of prions. Several specific types are found in different groups of animals, e.g. BSE in bovine, scrapie in sheeps, variant Creutzfeld Jacob disease and kuru in humans, FSE in cats, and chronic wasting disease in elk and deer, whereas no variant has been found so far in pigs and poultry (Lasmézas, 2003). The presence of BSE in an important group of farmed animals poses a very serious risk in the animal food production chain. The general opinion is that BSE in cattle is caused by the presence in the feeds of animal proteins containing prions (Prince *et al.*, 2003). The only validated and hence accepted method by the European Union to detect a possible presence of animal proteins is classical microscopy (EC, 2003). Microscopic analysis can detect animal proteins in the form of meat-and-bone meal (MBM) at sufficiently low levels (< 0,1%) with hardly any false negative (Engling *et al.*, 2000). However, a reliable distinction between mammalian and avian material is not possible in the current practice; distinction between terrestrial animals in general and fish material offers generally no problem (Gizzi *et al.*, 2003b). The currently proposed species-to-species ban (EC, 2002) requests a method for identification; for the control of ruminant feeds, which need to be free of any animal protein, detection is sufficient.

In the framework of the European Union funded project STRATFEED research was carried out at three different fields of research: DNA detection (PCR), near-infrared detection and classical microscopy. For the latter the existing protocol was improved, new characteristics were developed and the construction of an expert system was initiated. In this paper the results of the experiments and procedures are presented.

2. PROTOCOL FOR MICROSCOPIC DETECTION

EU legislation from 1998 on (EC, 1998) defined the basic guidelines for the identification of constituents of animal origin in animal feedingstuffs by microscopic analysis. Fine structures originating either from mammals, poultry or fish are visible on microscopic inspection at various magnifications (Frick *et al.*, 2002). Modifications have been allowed when necessary provided that the results are comparable to those obtained by the advised method. A modification of the sedimentation procedure is the so-called French method, using two different solvents (TCE/TBE), which results in the separation of the sediment in two parts. The amount of the bone sediment after using the French method is even smaller than the standard sedimentation procedure, which makes the results of the two methods incomparable. This situation changed recently by the publication of a new Directive (EC, 2003) which is far more strict and primarily based on the protocol as developed in the framework of STRATFEED.

2.1. Optimisation experiments

Some experiments have been carried out for the optimisation of the sedimentation procedure. First the effect of grinding on the resulting particle size was analysed using three different mesh sizes: 6.0 mm, 2.0 mm and 0.75 mm. Furthermore the effect of different action during the sedimentation procedure was examined: 1) shaking in a separating funnel and 2) stirring in a sedimentation beaker. The amount of material used for sedimentation (after grinding) was ten gram for feed and 25 gram for raw material. In total six samples were examined, all containing bone particles; two pelleted cattlefeeds and four pelleted raw materials (bakery by-products). The results are presented in **figures 1** and **2**. The amount of sediment for the two cattle feeds differ from 0.4% for the 2.0 mm grinding after using the sediment beaker to 1.28% for the 0.75 mm grinding after using the separation funnel. The number of bones depends largely on both the grinding size (**Figure 1**) as well as the separation method (**Figure 2**). In general more bone particles might be expected after grinding to smaller particles.

The maximum amounts found are no exclusive indication of the best results. Particles with a size smaller than 0.75 mm are difficult to examine by a stereo microscope, whereas grinding with a mesh size of 6 mm might result in a size of the fine sediment fraction, which is too small for a suitable examination by the research microscope. The results obtained indicate that grinding at a mesh size of 2.0 mm and a subsequent separation by using a separation funnel will yield optimal results. These findings have been used for the optimisation of the protocol.

In a proficiency test carried out in 2003 with 23 laboratories participating (Gizzi *et al.*, 2003b), a prime group of ten labs was able to detect 0.1% mammalian MBM in the presence of fish meal with hardly any false negative. This group of laboratories used shaking in a closed separation funnel as predominant method to obtain the sediment. The average percentage of sediment obtained after shaking was 1.38% (n=7), whereas stirring in an open funnel resulted in 1.11% (n=6) using the same samples (Gizzi *et al.*, 2003b). These results comply with the previously conducted experiments. Both laboratories, involved in Gizzi *et al.* (2003b), using the French sedimentation method (n=2) failed for the detection of MBM in the presence of fish meal in most cases.

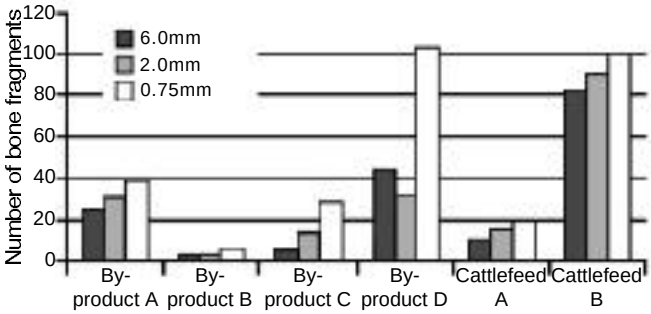

Figure 1. Effect of mesh-size during grinding on the amount of bones achieved.

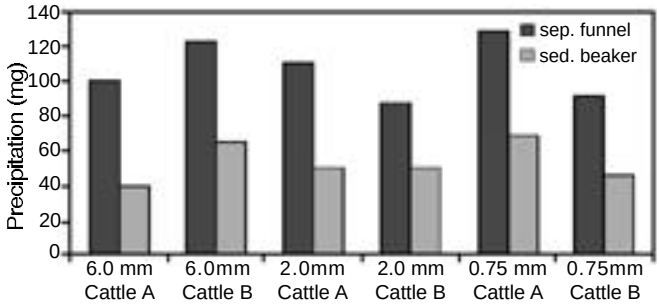

Figure 2. Amount of sediment obtained after shaking in a closed separation funnel and after stirring in an open sedimentation beaker.

2.2. Optimized protocol

The several experiments and considerations resulted in a protocol that is primarily based on EC 88/1998 (1998) but which is optimised for several key steps: the procedure starts with a higher amount of material, an electric mill should be used for grinding, and an

exclusive use of a close separation funnel for sedimentation with only one solvent (tetrachloroethylene) during five minutes. The general flow chart is presented in **figure 3**. The evaluation of several different fractions at both lower and higher magnifications (by using a stereo microscope and a compound microscope, respectively) is required. Slides should be made by using an embedding agent with a specified viscosity.

Two advantages of the sedimentation procedure are the concentration and selection of bone particles from the feed, and the defatting of the material in the tetrachloroethylene, which results in a clear view of the particles.

It is important to collect and document the observations effectively. A checklist was developed including parameters, and primary parameter states with tick boxes, which can be checked during the evaluation of a sample. In this way a uniform way of reporting can be established among the partners. The different states per characters are illustrated by referring to sample pictures that are included in ARIES. During the project several improvements have been achieved, leading to the currently used version 2.1.

The protocol together with the results of a proficiency test (Gizzi *et al.*, 2003b) and a ring trial (van Raamsdonk, van der Voet, 2003) based on the STRATFEED protocol have been used as a basis to set up a modified and improved EU guideline for detection of animal proteins (EC, 2002). A major extension to the STRATFEED protocol is the acceptance in this Directive of the application of an open beaker as is in use in some EU member States. It was argued that acceptable results can be obtained in practice provided a sufficient level of expertise in handling the beaker, and cleaning time is shorter compared to the cleaning of a funnel. As in the Stratfeed protocol, the French method is not accepted for the time being according to EC 2003/126 (2003), whereas it fitted in the modifications allowed in EC 88/1998 (1998). The acceptance of five grams of material to start with (STRATFEED protocol: ten grams) can be expected to influence the level of detection. The chance that a low level contamination of a feed will show up in a sediment in the form of a few bone particles will be lower.

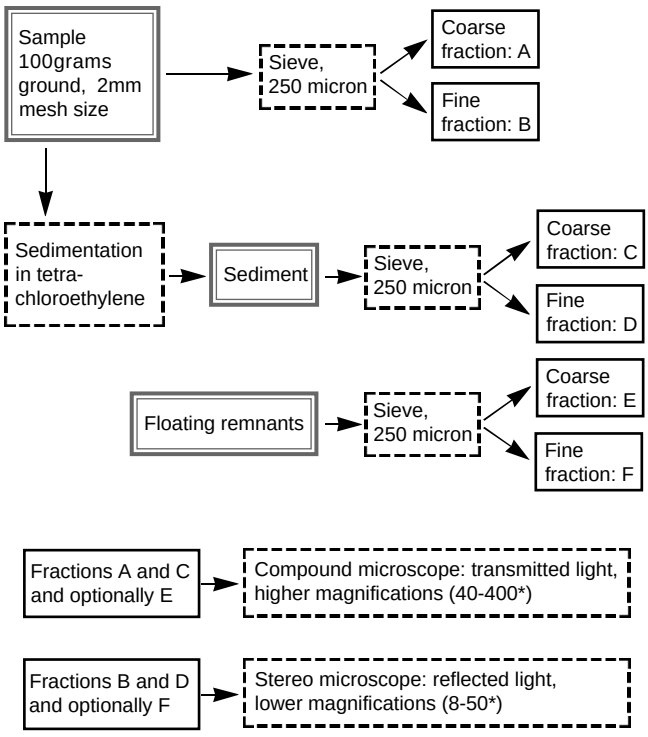

Figure 3. Flow diagram of the procedure for microscopic examination of animal proteins.

3. MUSCLE FIBRE CHARACTERISTICS

Among all different types of particles in a typical animal meal, bone particles appear to show the most extensive information characteristics. Other particles such as hairs, teeth, feather filaments, egg scales, fish scales, gills, etc. can be used as additional evidence only when present. Muscle fibres are basically used to state the presence of animal proteins in general.

Muscle tissue can be present as single fibres, which are broken to relatively short fragments. The width of the fibres depends largely on the state of nutrition of the animal and the treatment during slaughter (Devine *et al.*, 2002; Kang, Sams, 1999). The fibres are character-poor and an attempt has been made to find additional characteristics. It can be assumed that the muscle proteins will retract to a standard situation during the rendering process and especially after the heat treatment, which will result in different parameters at least at the level of vertebrate classes, i.e. mammalian versus avian versus fish material. A possibility to characterise the differences might be a so-called muscle ratio between number of striae per one unit of the fibre width, or in other words the quotient fibre-width / sarcomere-length (**Figure 4**).

From a set of six pure animal materials, processed in a dedicated pilot plant, 20 fibres per sample were measured by counting the number of sarcomeres per unit fibre width. In addition ten fibres each were measured from five samples of terrestrial animals and three fish samples, selected from the STRATFEED sample bank (set B). All labels of these eight samples indicated pure meals; besides a conformation of the specially processed pure materials, a control of being a mixture of these extra samples was supposed to be achieved. The results are summarised in **table 1** and

Table 1. Ratio between the number of striae along a segment of muscle fibre as long as the width of the fibre for some samples of set B.

Animal sample	Examined bone contents	Average	SD	*n*
Cattle				
8141	pure M[1] (pilot plant)	34,9	10,1	20
84839	pure M (pilot plant)	35,5	10,5	20
1511	M, few P[2]	42,4	13,3	10
Pig				
8181	pure M (pilot plant)	36,9	12,1	20
91628	pure M (renderer)	32,0	11,3	20
1406	M, few P	47,8	**19,6**	10
1412	M, few P	46,1	10,2	10
Poultry				
8221	pure P (pilot plant)	25,3	8,5	20
53744	pure P (renderer)	24,5	9,6	20
150	P, few M	21,6	8,5	15
1107	P + M	30,8	12,6	20
Fish				
1097	F[3] (cod, tuna, crustacea)	34,4	12,1	10
203	F (cod, herring, tuna)	39,0	8,1	10
288	F (cod, herring), few M	47,4	10,8	10

[1] M = mammalian material; [2] P = poultry material; [3] F = fish material.
Standard deviations indicating mixtures are shown in bold.

figure 5. It is clear that on average the muscle ratio is lower for poultry material compared to mammalian material. The standard deviations, however, indicate that this difference is not statistically significant. A trend towards different ratio's and structures can be concluded, which can support the identification using bone characteristics. The basic information as obtained from the pure materials (with n=20 fibres observed per sample) was approved by the eight samples of set B (with n=10 fibres per sample). The trend that samples with predominant mammalian material (1511, 1406 and 1412) show higher ratio's than samples with mainly poultry material (150) supports the results obtained from the proven pure meals. The few bones in sample 150 that have been identified as mammalian material could originate from some special bones that occur in poultry animals with an appearance like a mammalian bone structure (Mondini, 1999). So, accepting the occurrence of this deviating type of poultry bones, sample 150 could be considered to consist of pure poultry material. There seems to be a relationship between types of fish and the ratio. The standard deviation for one mixed sample is considerably higher than for pure materials, which can be expected from a mixture, e.g. Pig 1406 (see also **figure 5**). For this sample the mixed origin was not found after bone inspection. Some other standard deviations (e.g. 1511, 1107) are questionably high for pure materials. Besides the presented trends, high standard deviations might occasionally help to recognise mixtures, which can support the identification of (deviating) bones types.

In addition, the samples of set B were classified by all partners (n=6) in different classes for their muscle ratio: 15–25, 20–35, and 30–45. The presumed pure mammalian samples (1511, 1406 and 1412) were predominantly classified in the class 30–45, and the samples labelled as poultry (150 and 1107) were mainly indicated to have a muscle ratio between 20–35, with occasional indication for the class of 30–45. Fish material appeared to be classified in both classes 20–35 and 30–45. These results provide a further support to the gradual difference between mammalian and avian muscle tissue.

The muscle ratio should be investigated in more detail. The actual width of the fibres and sarcomere length as basic data should be considered as well.

4. DEVELOPMENT OF DECISION SUPPORT SYSTEMS

The majority of the knowledge that is used in the process of handling samples and identifying the particles of animal proteins is non-numerical, i.e. descriptive (text) or illustrative (images). Nevertheless, some sort of decisive process is followed when suspected particles are detected and identified. This step-wise process and the supporting knowledge need to be documented. The representation of a process of decision making can be effectuated in the form of decision rules or in the form of a matrix with parameter states. The latter representation allows to calculate the fit between the observations and the parameter states of an identified type (e.g. mammal meal). Computer programs exist which can provide the structures to develop identification trees with related information. These programs are a special type of expert systems, named decision support systems. The goal of developing such a system in the framework of STRATFEED was twofold: the availability of a support tool for identifying animal material in feeds in the daily practice of control laboratories, as well as a training tool for new feed microscopists.

The intention was to make advantage of existing program packages rather than developing a completely new system. An existing computer program can facilitate the development of a dedicated system by primarily including the required knowledge. In order to develop a decision support system a survey was carried out for checking the usability of a range of different expert systems for their compliance with the

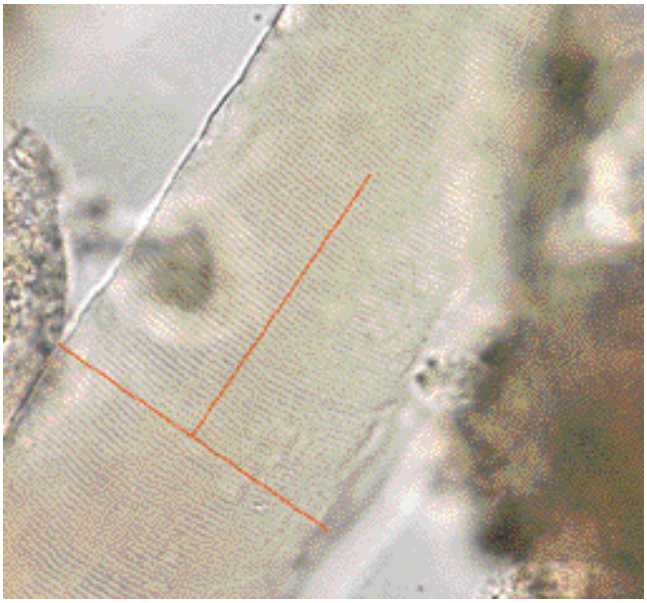

Figure 4. Muscle ratio. The longitudinal bar is as long as the fibre width (transverse bar). The muscle ratio is the number of striae along the longitudinal bar.

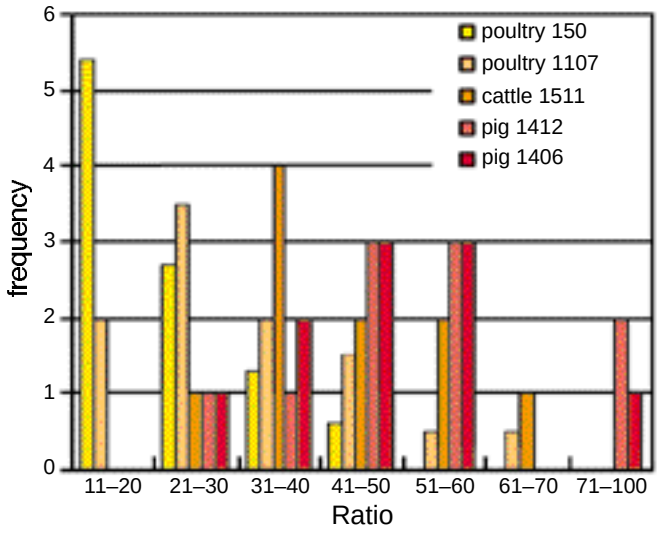

Figure 5. Frequency distributions of muscle ratio data for five samples of terrestrial animals of set B.

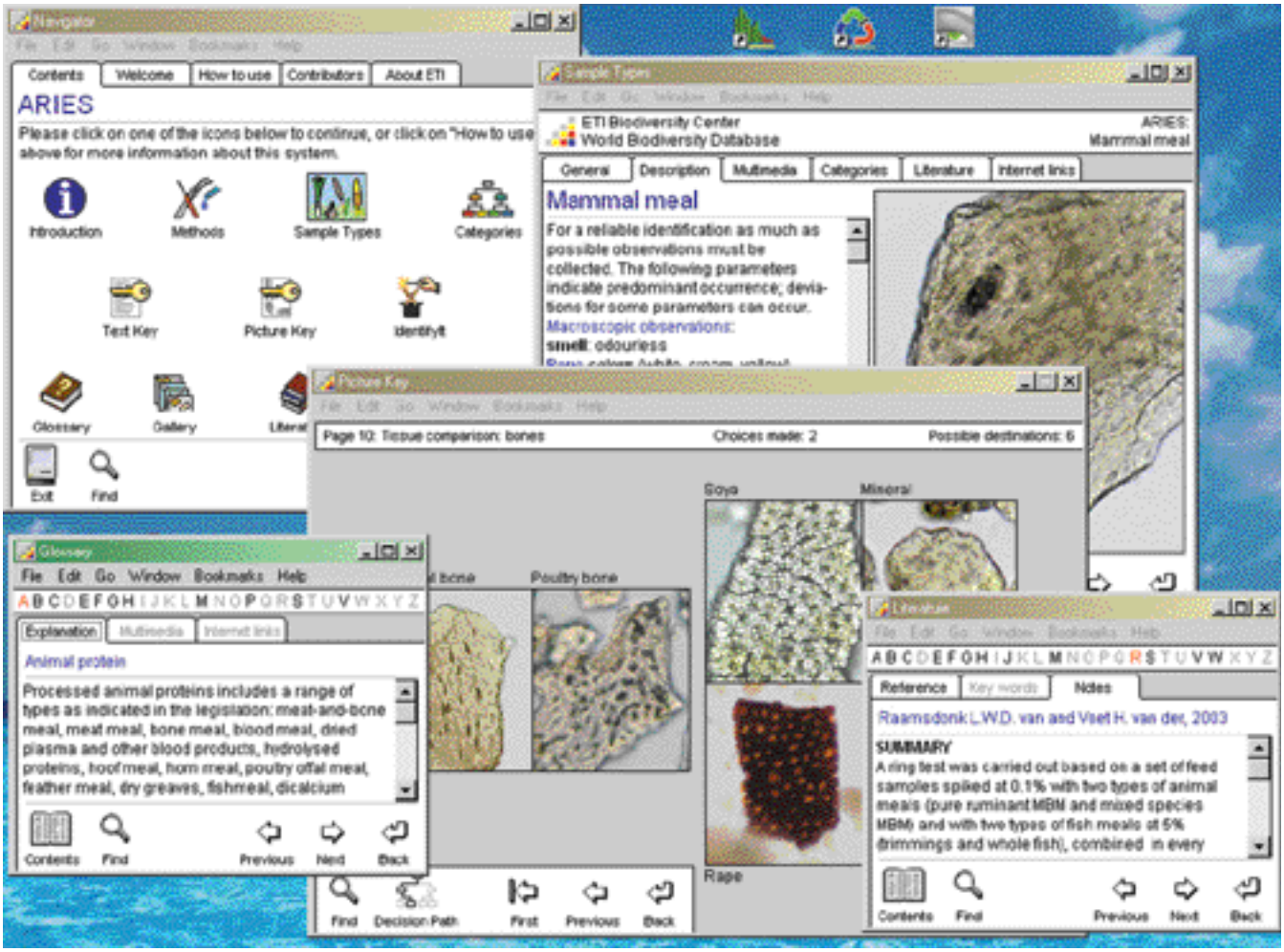

Figure 6. Screen dump of ARIES with the main navigating screen containing the icons to the modules (upper left) and several modules opened.

needs in the STRATFEED project. A set of ten computer packages was designed, including computer based training systems, free-text databases, and two types of decision support systems, i.e. matrix-based and rule-based. A list of about 15 requirements was drawn up and every demand was checked for each of the computer packages. In this way a priority listing was established, which provided the basis for the decision on the final choice of the package to be used in STRATFEED. The system Linnaeus II (ETI, 2004) got first priority, although several drawbacks of this package have been identified. However, the advantages of this package include a free-text database, modules containing different approaches for identification, and modules for building a picture gallery and a glossary. For the major disadvantages customisation was achieved.

The developed system called ARIES (Animal Remains Identification and Evaluation System) (Vermeulen *et al.*, 2003) provides a full range of animal meal descriptions as developed in STRATFEED, including shell fish and a range of plant parts and minerals that can be confused with animal material. Three different modules for step-wise identification are being developed, and a glossary, a gallery with additional series of images, a range of literature and information on legislation is included (see screen dump in **figure 6**). The package can be used to support and document the actual identification in common practice and is capable of being used as a training system. ARIES will be made available at first as a stand-alone system (CD-ROM) and depending on the result of a market analysis in second instance as web-based system.

A quick identification system called STRATFEED-DSS has been developed for internet application (Vermeulen *et al.*, 2003; van Raamsdonk *et al.*, 2004). It is based on 15 parameters divided in four different categories: macroscopic *vs.* microscopic and bone *vs.* additional features. A selected set or all four categories can be chosen to be answered for a final identification: mammalian, avian or fish as source. The match of the investigated material with the profiles of each of these vertebrate classes is given, together with descriptions of the materials of these possible sources. STRATFEED-DSS provides a subset of the possibilities that are included in ARIES. It shows the potential power of decision support systems as well the possibility of identification of animal protein contamination in feeds.

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