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STUDY ON THE MICROSCOPE METHOD AND THE VIEW OF THE ANALYTICAL TECHNIQUES FOR IDENTIFICATION AND ESTIMATION OF THE PROCESSED ANIMAL PROTEINS IN ANIMAL FEED

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Abstract

Hypothesis universally accepted as the most likely route of infection with ESB appeared due to the consume of the animal by-products which are not intended for human consumption that contained prion protein derivative - infected led to ban the feeding of farmed animals with processed animal protein (PAP) that focused primarily feed for ruminants and later expanded to all feed for all farm animals. Entry into force of the ban on the use of the processed animal proteins (PAP) in feed for farmed animals and especially in ruminants is considered an important measure of prophylaxis to prevent BSE so the identification and the microscopic estimation of the constituents of animal origin became the official method and mandatory in all Member States.

The microscopic analysis depends upon the identification of the hsitological characters macro-and microscopic structural of the processed animal tissue added in feed mixtures.To identify the microscopic animal constituents, some technical conditions are essential: optical microscope, stereo microscope, high-density solvent (chloroform or tetrachloroethane) clarifying agents (phenol-glycerol, paraffin), microscope with digital visual images support as decision support .

The method allows the identification of bone fragments, muscle tissue, hair, feathers, shell fragments and plant and mineral components.

Nowadays four different approaches are applied to control the compliance on the prohibition of feeding with PAP: microscopic analysis, immunological analysis, infrared spectroscopy and microscopy (NIR), polymerization chain reaction (PCR). In this stage, the microscopic method is the only method validated and able to identify the nature of the animal in feed components with detection limit of <0.1%, but it cannot accurately detect the species of origin.

Key words: *feed, microscopic identification, polymerization chain reaction, processed animal proteins*, *transmissible spongiform encephalopathy.*

INTRODUCTION

So far it has been shown that animal by-products not intended for human consumption, resulting in a row processing, more or less correct, cadavers, products and by-products from animals, constituting a potential source of risk to public health and the animal. This way the probable infection with prionic protein derivative-infected, has led to a ban on the feeding of farm animals with processed animal proteins (PAP) which focused primarily on feeding stuffs for ruminants, and was subsequently extended to all feed for all farm animals. Safety use of proteins derived from animals in feed at European level constitutes an important decision factor in the prevention of transmissible spongiform encephalopathy's (TSEs). Properties of infectious agents of TSEs have proven to be very unusual. Infectious agents of TSEs, manifested in humans or in animals are actually made from a single type of protein called PrP^{Sc}, are devoid of nucleic acids.

Use of protein meat-and-bone type as an ingredient in animal feed is regulated according the European legislation (Commission Decision 97/534/EC) since 1997, and effective heat treatment to which they are subjected has been set to, 3 bar, 133^{o}C, 20 minutes (Regulation (EC) no 1069/2009). European regulations prohibit feeding of animals of farm intended for human consumption, intraspecie with proteins derived. Considering the fact that the methods of analysis for protein supplements have carried out a series of inconveniences in selectivity (in particular for the identification of protein derived from ruminants) the ban was extended for proteins derived from all

species. Extending the ban, he called for better identification methods and more accurate for effective sterilization control as well as for specific detection of meat-and-bone type in compound feeding stuffs.

At present there are applied or are under development in the following ways: microscopic analysis for the identification of particles derived from animal by-products (blood, hair, tissue, muscular, and others), the polymerization chain reaction (PCR) for the detection of species specific DNA, immunological analysis (ELISA) for the detection of specific proteins, spectroscopic methods in the near infrared (NIR) for the detection of protein flour in compound feeding stuffs.

All methods have the advantages and disadvantages of specific technique used.

Taking into account the specific proteins, nucleic acids and are still present in processed flour, have been tested and molecular biology techniques. In this context two main techniques are used which are focused on the detection of proteins with specific antibodies by ELISA (Ansfield et al, 2000) and taxonomical identification of nucleic acids based on genetic methods of amplification by PCR (Chikuni et al.,1990). Near-infrared spectroscopy (NIR) is used for a long time (Baeten et al., 2001) for identification of certain major components (water, fat, protein, etc.) of the food or feed substrates.

The scientific community's interest for NIR spectroscopy is based on the eventual possibility of accidental contamination of feeding stuffs with consistent meal, bearing in mind that this technique is one of the most used for the routine exams. By conducting comparative tests (8-STRATFEED European Project, 2000) with different substrates and different concentrations of fodder of animal protein, it was noted that the processing temperature and duration thereof constitutes a major impediment to the successful application of enzyme immunoassay methods. In cases where the processing temperature is more than 140^{o} C, ELISA techniques performances but even PCR greatly decrease with increasing percentage of false-negative results .Unlike these, NIR spectroscopy is not influenced by temperature, but the limit of detection is 10 times greater than what makes use for routine analysis is not possible.

In 1998, the Committee of Experts on Methods of Analysis (CEMA) of the Directorate-General for Agriculture, put into question the comparative performance of microscopic analysis, the immunoassay analysis (ELISA), polymerase chain reaction(PCR) and infrared spectroscopic methods (NIR). As a result of this analysis and the results of tests carried out at the time, in 2001, he was the European STRATFEED project, whose purpose was to assess the performance of the different methods described above, in a series of tests of interlaboratory comparison with the participation of several European laboratories. Our Institute, by laboratory of microscopy, participated in phase II of the project STRATFEED, in 2003-2004, which included tests of microscopic method validation (DC 126\/2003 EC).

MATERIALS AND METHODS

In this paper are presented analysis methods as development in the Institute of Hygiene and Public Veterinary Health, microscopic identification method and a proper system of visual comparison and interpretation of digital images taken under a microscope and stereomicroscope and polymerization chain reaction method.

Since 2004 has been continually developed as analytical method microscopic method, which is the method of reference at European level for the identification of animal proteins in feeding stuffs and validates alternative method based on the polymerase chain reaction (PCR) for the detection of protein derived from ruminants with the prospect of enlargement and to identify the species of origin derived from non-ruminants.

Microscopic analysis now routinely applied to our laboratory, is based on the identification of structural macro-and microscopic animal tissue derived from consistent flour added to compound feeding stuffs. In principle, the microscopic identification is based on microscopic characteristics of animal tissues. Microscopic identification of animal

derivatives is based on knowledge of basic histology. Histological structure of components that have undergone heat treatment as is the case PAP is different from the normal structures. The differences can be accentuated, such as soft structures (muscle, epithelial tissue, connective tissue, etc.) or less accentuated, some unaltered, such as hard structures (bone tissue, teeth, scales, feathers, etc.). Drying and grinding are changed not only the initial histological structure but also macrostructure, so generally available for identification only small fragments. Because these fragments are then included in complex matrices (cereals, legumes, seeds ground and their derivatives, minerals, vitamins, feed additives etc.) the extraction and separation of these fragments becomes all too important and time consuming to be identified. Bone fragments present in the mixture should be differentiated based on typical lacunae between the bones from fish and terrestrial animals (mammals and birds).

Separation of constituents is facilitated by sieving and concentrated sediment technique, which consists in suspending the sample in a solvent with high density (chloroform, tetrachloroethane) which makes the bones, minerals and other high-density fragments to settle and plant tissues and other low-density constituents, to be found in the floating. Alizarin red staining for identification is very important because only colored bone and cartilage making them easy to recognize separately. Observation of lacunae by clarifying preparation is a densely agent (paraffin, glycerol) that cannot penetrate the capillary so that they remain filled with air and appear black on a light background of the bone fragment.

Alternative, our laboratory has implemented and is in the process of validating DNA detection from ruminants through Real-Time PCR in products intended for animal feeding, aiming a genetic sequence of repetitive nuclear level 85/86 pairs of bases, test developed and validated by the reference Laboratory of the European Union for animal proteins (EURL-AP) Belgium. Target region of plasmid DNA is amplified by specific primers, which, with each cycle of amplification is evidenced in real time with the help of a marked fluorescent probes. Fluorescent signal passes a threshold (threshold) value after a certain number of PCR cycles. The value of this threshold cycle (Ct-cycle threshold) is likened to a predetermined figure after calibration of the Real-time PCR platform, which includes the equipment and reagents for PCR to determine if the result is positive or negative.

RESULTS AND DISCUSSIONS

Constituents of animal nature are identifying by macroscopic examination on stereomicroscope and by microscopic examination on compound microscope. Community methods, as determined by the European law (Regulation EU No 53/2013) indicate the scope of the technical guide for the separation and examination of the constituents of nature of these principles. The more difficult for our laboratory was establishment of a protocol and a database for Visual comparison. In this sense have been selected several methods and tests to confirm through chemical reactions of components identified. Thus was constituted a supplementary material for analysts, supplemented with a Visual support with digital images from the microscope, as decision support.

The tissues that are important to the microscopic identification are bones, teeth, muscle tissue, cartilage tissue, cornified epithelial tissue, feathers, feathers, egg shells fragments, hair and hard tissues of the hoof, guiding elements analysis and identification. The main elements for microscopic diagnosis are bone fragments.

In stereomicroscope mammal and bird bone fragments appear as irregular particles, opaque, yellowish white, pearl and fragments of fish bones appear translucent, with slightly

glazed surface, often lamellar shape, sharp (Figure 1).

On compound microscope, in embedding media (paraffin, glycerine) filled with air lacunae remain and are visible by contrast. Characteristic mammals form longitudinal lacunae size of ~ 5/30 μm, and their arrangement in the form of linear (Figure 2).

Figure1.Bone fragments on stereomicroscope

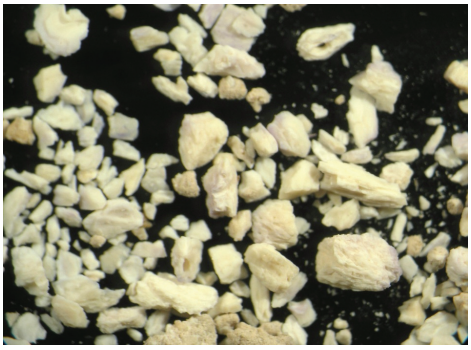
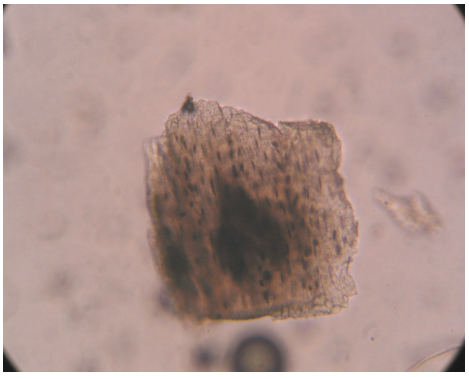
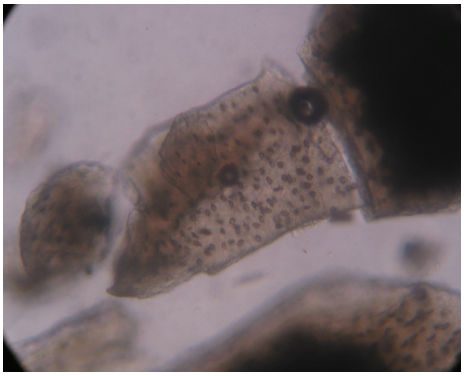


Figure 2.Mammal bone aspect on microscope



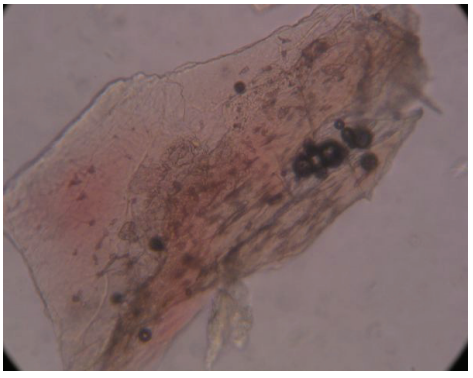
In birds histological bone structure is identical to mammals, the difference is globular lacunae with dimensions of ~ 15/25 μm and lacunae arrangement is in the form of cluster radial aspect and less linear as the mammalian (Figure 3). Other diagnostic elements present bird constituents are represented by presence of egg shell fragments, presence of feather (barbs and barbules) that shows great importance for orientation of identification.

Figure 3.Bird bone aspect on microscope



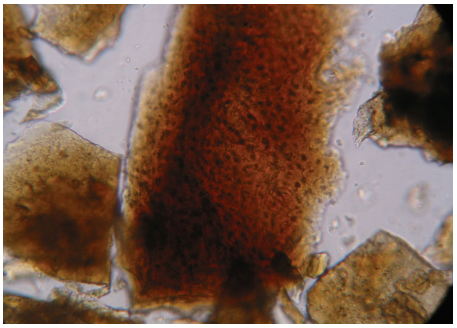
For fish are important microscopic identification of characteristic components of bones and scales. One can distinguish two categories of fish bones: bones and bone lacunae containing osteocytes without lacunae (Figure 4).

Figure 4.Fish bone aspect on microscope



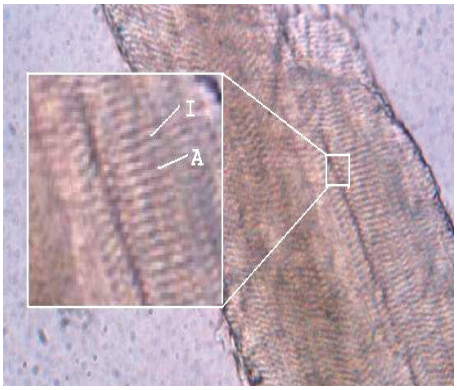
In fishmeal can be found various tissues from clams, shrimp, crabs, snails originating from marine fish harvested with or emanating from the stomach of the fish. Calcified exoskeleton of crabs and shrimps shows generally symmetrical structure and similar arrangement of cells in plant tissues (Figure 5).

Figure 5 – Exoskeleton of crabs



Microscopic examination of muscle tissue, both freshly prepared (in polarized light) show myofibrils that appear with a periodic structure, made of alternating zones or clear discs (I) called isotropic, monorefringents, dark disc (A) called anisotropic, birefringents (Figure 6).

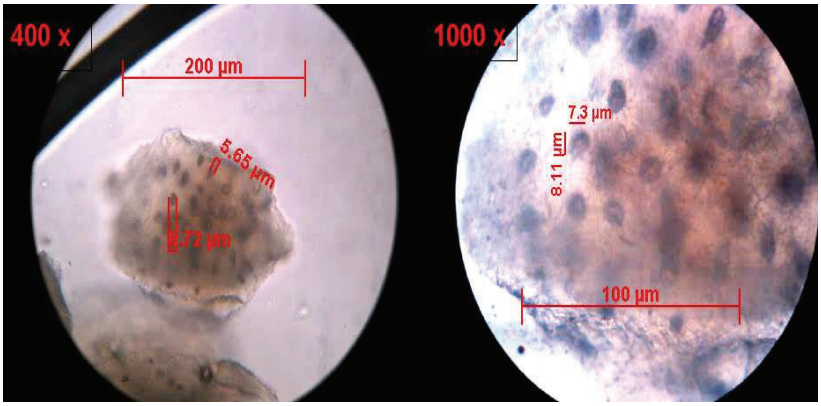
Figure 6. Muscle tissue



To support differentiation of constituents identified, was built an image database as decision support, using program acquisition AxioVision LE and editing of existing laboratory photomicrographs. For this purpose were conducted and evaluated over 1,500 photomicrographs acquired with camera fitted to the microscope, and images were selected by features and images very frequently encountered in routine examinations and were then labelled and compiled in a manner accessible for comparison during work.

If fish bones before the differentiation of the terrestrial species can be made with relative ease, differentiating between species of mammal bone or bones of mammals of the poultry involves a more complex examination. In this respect it is necessary to assess gaps form (with magnification from 400 to 1000 x) and second coordinate dimensions. In general, the size of the gaps are between 15-25/8-30 µm. The appreciation of these dimensions is possible through the treatment procedures based on digital images. For this purpose was established a procedure of evaluation and description of structures in digital image (Figure 8).

Figure 8. Morphometric measurements



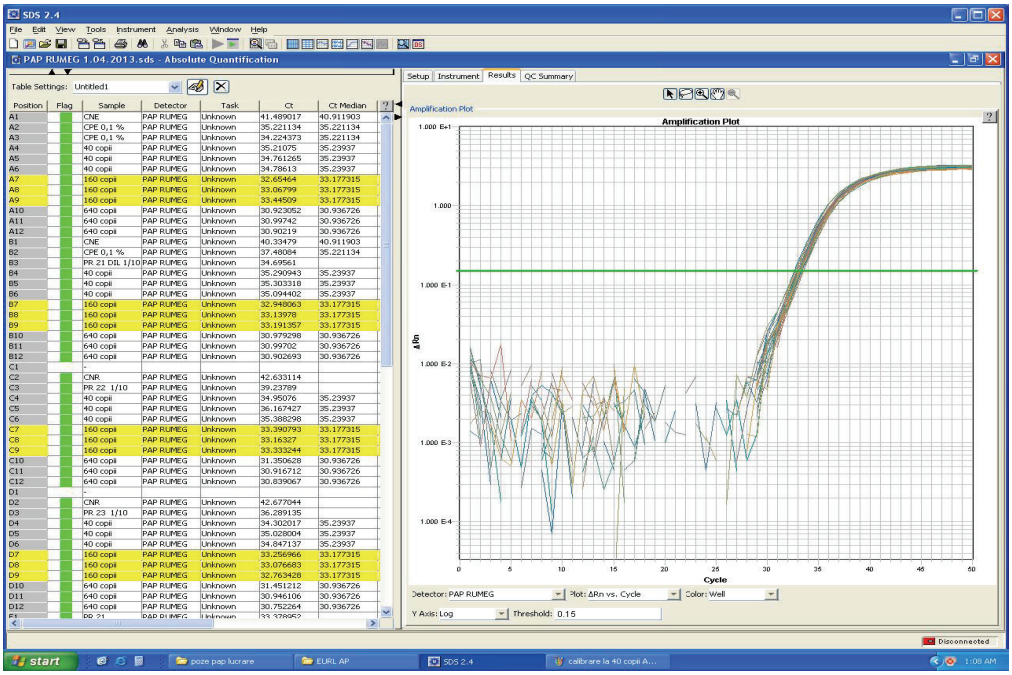
The sensitivity of the method depending on the type of constituents of animal nature can be detected very small amounts (<0.1%) of constituents in feed mixtures as assessed by microscopic identification detection capability demonstrated in the validation of the method. The size of the fragments identified on compound microscope is about 30 µm. Details included are identifiable dimensions between 2.5-5 µm.

Internal validation and accreditation methods were used samples of different mixtures to which have been added to the known applied bone flour, meat or fish. Bone fragments to differentiate between species of mammals, the Visual comparison are required. For this purpose were formed 25 packages of selected images to meet specific characters with a probability of 95%. Such characters shall be represented in the form of gaps, their layout and size of the two plans.

Laboratory records-for samples analysed are kept digital micrographs recorded in a folder with the number of the sample that is applied by editing (AxioVision LE 4.1) sample number and corresponding objective and micrometer scale that worked.

in Detection of DNA derived from ruminants through Real-Time PCR in products intended for animal feeding is a technique of 5 '-nuclease and it consists of selective amplification of DNA sequences through a succession of amplification cycles, each cycle having in turn three steps: denaturation of DNA chains, the delimitation of the amplicon with the help of very short sequences of approx. 20-25 of the complementary DNA database called sense and antisense primer that takes place at a temperature which is calculated according to the structure to develop the extension, polymerization. In accordance with the procedure of EURL-AP, working platforms (equipment and reagents) used to perform this method, must be calibrated, this means the testing of 16 times a set of 3 calibrants this tests obtaining the cut-off method in your own laboratory (Figure 9).

Figure 9. Calibration to 160 copy of DNA rumegator (real-time PCR Applied System)



The samples tested on a calibrated (e.g. with the cut-off value of 38.15) will have a qualitative result of type "present" = samples with Real-time amplification cycle PCR below cut-off or "absent" = samples with Real-time amplification cycle PCR over cut-off value.

CONCLUSIONS

Currently compliance on the prohibition of feeding with PAP, are based on four different approaches: microscopic analysis, immunoassays, spectroscopy and infrared microscopy (NIR), the polymerization chain reaction (PCR), all methods have advantages and disadvantages of technique used and performance parameter.

Testing laboratory has developed a laboratory microscopy applied to control feed with original contributions to the evaluation of digital photomicrographs, future work is focused on microscopic identification by using an expert system to correct recorded images.

In Romania, microscopic method is the only method accredited to ISO/CEN 17025 by RENAR (Accreditation Association Romania) and able to identify and the nature of animal feed components with a detection limit of <0.1%, but cannot accurately detect the species of origin.

Feed ban on the use of processed animal proteins interspecies constitutes a new challenge for analytical methodologies and identification methods will require better and more precise control.

Testing laboratory which formed the basis of this study is accredited for microscopic method and validates alternative method based on polymerase chain reaction (PCR) for detection of ruminant constituents with the prospect of enlargement and to identify species from non-ruminant origin.

Method for detection of DNA derived from ruminants through Real-Time PCR in products intended for animal feeding must be calibrated in the laboratory's own type of equipment and reagents used, in order to obtain the cut off and the result is qualitatively as “present" or “absent".

Our task for the future is focused on validation and accreditation methods for DNA detection through Real-Time PCR products intended for feeding farm animals, so that Romania can fulfill the requirements of the ban on the use of future animal proteins derived from ruminants but also and prohibition of feeding an intraspecific (ban of feeding an animal species with proteins derived from the bodies or parts of bodies, of the same species).

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