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Overview of methods for the detection of species specific proteins in feed intended for farmed animals

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Christoph von Holst[1], Vincent Baeten[2], Gilbert Berben[2], Gianfranco Brambilla[3]

[1]DG-JRC-IRMM, Geel, Belgium

[2]Walloon Agricultural Research Centre, CRA-W Gembloux, Belgium

3 Istituto Superiore di Sanita, Rome, Italy

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European Commission
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Contact information
Christoph von Holst
European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium

E-mail: christoph.von-holst@cec.eu.int

Tel.: +32 (0)14 571 221
Fax: +32 (0)14 571 787

http://www.irmm.jrc.be
http://www.jrc.cec.eu.int

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1. Introduction

The objective of this document is to describe the characteristics and performance of methods suitable for the detection of species specific proteins in feed. It represents the state of the art in September 2004. Species specific detection and detection of groups of species such as ruminants is required according to European legislation dealing with the safe use of animal by-products in animal nutrition [1, 2 and 3]. Various methods are applied to the analysis of feed samples for the presence of banned processed animal proteins (PAPs) including meat and bone meal (MBM). In this document, however, we focus on the following four methods that are applied either in routine laboratories or for research purposes, since this selection includes official methods as well methods that showed the most promising potential in research projects or intercomparison studies.

1. *Classical microscopy*:
   This technique is currently the only official method and allows the detection of constituents of animal origin in feed.
2. *Polymerase chain reaction (PCR)*
   By applying this technique well-defined DNA targets are determined to detect the presence of PAPs at various taxonomic levels
3. *Immunoassay analysis*
   The targets of this method are proteins at various taxonomic levels, depending on the design of the immunoassay
4. *Near infrared-microscopy (NIR-microscopy)*
   The principle of this technique is the measurement of the NIR spectrum of individual particles of a feed samples.

The summary in this report is mainly based on results from two intercomparison studies for the detection of PAPs in feed carried out on behalf of DG SANCO [4,5] and the outcome of the FP 5 project "Stratfeed" [6].

Though all these methods have the same objective - which is the detection of the banned PAPs in feed - it is important to note that the targets are quite different. For instance, microscopy relies partly on the presence of bones, whereas PCR detects DNA, which demonstrates the presence of PAPs in the sample. The inherent difference of the various methods in terms of their targets should therefore be taken in consideration when comparing the performance of these methods. It is also important to emphasize that this report focuses on the *qualitative* determination of banned PAPs in feed, whereas *quantitative* aspects are not taken into account, reflecting the fact that European legislation does not foresee legal limits for PAPs.

2. Classical microscopy

Classical microscopy is the only official method within the EU to detect the presence of constituents of animal origin in feed. In general, microscopy can be applied to the detection of constituents of animal origin – or MBM - at two levels: (1) the detection of MBM irrespective of the origin and (2) the detection of MBM from terrestrial animals in the presence of fishmeal. Some laboratories that are extremely experienced in microscopy showed that this method could also differentiate between MBM from mammals and material from poultry. However, the results from the DG SANCO 2003 study [4] and from the Stratfeed validation study [9] showed that the majority of the participating laboratories could not achieve the differentiation power at this taxonomic level. Therefore this report will focus on the performance of microscopy to detect MBM from terrestrial animals in the presence of fishmeal, expressed as sensitivity, which is the portion of correctly assigned positive results for feed containing MBM from terrestrial animals at 0.1 %.

2.1 Information on the microscopic methods used

The results of two intercomparison studies conducted in 2003 [4, 8] and the results from the Stratfeed project [9] led to a revision of the former protocol, introducing a new protocol [7] that specifies some important method parameters. However, there were still deviating protocols under discussion and the influence of the various protocols on detection could be assessed by evaluating the results from the DG SANCO study 2004 [5], the Stratfeed validation study [9] and the IFFO study [8].

In principle four different protocols were investigated which where (1) the IFFO protocol, (2) the Stratfeed validation protocol, (3) the Austrian protocol and (4) the French protocol. In the Stratfeed validation study the refined Stratfeed protocol was applied, which was slightly modified to cover all experimental options that are allowed according to the current European protocol [7]. In the IFFO study the laboratories applied the Stratfeed protocol, which is a subset of the method parameters allowed by European protocol [7]. The Austrian method almost complies with this protocol but allows minor deviations. In contrast the French methods represents a major deviation from the European protocol since two different halogenated solvents are applied. An overview of the different protocols is given in [5].

2.2 Results

When comparing the results from the various studies also the first DG SANCO from 2003 was included, though the laboratories applied methods that still complied with the former protocol. However we included this study to demonstrate the improvement of the microscopic method gained in the last year.

Method performance characteristics: ***Sensitivity***

*Target parameter****: Processed Animal Proteins*** (***PAPs) from terrestrial animals***:

- *Detection of 0.1 % MBM in feed.*
  - ⇒ Stratfeed study : 99 %
  - ⇒ DG SANCO 2003 study: 92 %
  - ⇒ DG SANCO 2004 study:
    - ⇒ Austrian method: 95 %
    - ⇒ French method: 93 %

⇒ IFFO study: 100 %

- *Detection of 0.1 % MBM in feed* in the presence of *5 % fishmeal*.

  ⇒ Stratfeed study : 77 %

  ⇒ DG SANCO 2003 study: 44 %

  ⇒ DG SANCO 2004 study:

    ⇒ Austrian method: 76 %

    ⇒ French method: 60 %

  ⇒ IFFO study: 98 %

- *Detection of 0.5 % MBM in feed* in the presence of *5 % fishmeal*

  ⇒ DG SANCO 2003 study: 100 %

2.3 Conclusions

The following conclusions can be drawn from the various studies:

- For the material containing 0.1 % MBM in the presence of fishmeal the values of the sensitivity obtained in the Stratfeed validation study and the DG SANCO study 2004 (Austrian method) were almost equivalent (about 77 %). The very good results from the IFFO study reflect most likely the high expertise of the involved laboratories.
- Comparing the results from the DG SANCO study 2003 and the Stratfeed validation study it can be concluded that the improved potocol [7] is significantly better than the one established by former Directive 98/88/EC.
- Three laboratories participating in the DG SANCO 2003 *and* the Stratfeed validation study obtained an overall accuracy for all materials of 100 % and identified MBM in **all** samples containing 0.1 % MBM in the presence of fishmeal. Two other laboratories obtained slightly inferior results. The results emphasise again the importance of the required expertise of the laboratory.
- Given the proven potential of the refined microscopic method, the continuous organisation of training courses, workshops and proficiency testing will further improve the ability of European laboratories to detect MBM at 0.1 % in the presence of fishmeal.
- Another important improvement is the development of a PC based expert system "ARIES" developed in the Stratfeed project that assists the analyst in identifying banned PAPs.

Further projects regarding this method should focus on the organisation of workshops and proficiency tests, whereas modifications of the method are not expected to lead to further improvement of the performance of the method.

3. Polymerase chain reaction (PCR)

3.1 General information on the PCR method

During the polymerase chain reaction (PCR) a well-defined DNA target is multiplied several millions of times to make it detectable. The hereby-produced DNA segments are called amplicons. The technique requires an extraction step to isolate the nucleic acids still present in the considered sample. The reaction of genetic amplification itself is then performed on a fraction of this extract. According to which approach is used a third step for detecting the amplified product may be required. Indeed, in classical PCR the amplicons are evidenced by agarose gel electrophoresis (a more recent alternative being capillary electrophoresis). As only the final product of the reaction is seen, classical PCR is an end-point detection method.

Another way to work is using real-time PCR where, through more complex devices, production of the amplicons can be followed during the reaction itself (generally on-line but not necessarily). This latter method gives information on the kinetics of the reaction and is more informative, that is why it can be used for quantitation purposes. However it should be stressed that basically the technique quantifies a number of copies of targets. In the case of MBM detection in feed, it is difficult to use this parameter because there is no straight relationship between the weight of MBM and its content in number of copies of a defined target as this is largely influenced by the type of rendering undergone.

3.2 Targets that can be amplified by PCR and their discrimination power towards identification of species or groups of species

The rationale behind the choice of a specific target is the fact that with the genetic information contained in each animal cell, it is possible to find DNA segments with a conserved nucleotide sequence that is specific for the considered species. Therefore results from PCR have a high forensic value, similar to classical microscopy. Moreover, as there is also conservation of the nucleotide sequences of pieces of DNA between genomes belonging to a common taxonomic level higher than species, it is also possible to have targets for groups of animals like ruminants, mammals and even up to eukaryotes. This also means that several PCR targets may be possible for a same purpose, although they may perhaps not all have the same performance.

In the framework of detection of animal DNA contained in MBM it should be stressed that there are two important parameters to consider:

- The abundance of the chosen target within the animal cell: It is of paramount importance to already have a target known to be present in large number within the animal cell. This is the case of mitochondrial targets. Mitochondria may be present, certainly in muscle, in hundreds of copies. Nevertheless some nuclear targets may also be very abundant (e.g. SINE’s = short interspersed nuclear elements).

- The size of the target is crucial too, because even if DNA is a rather strong molecule that can survive a lot of drastic processes, rendering will degrade DNA by giving rise to a certain rate of hydrolysis. This results in smaller pieces of DNA. Therefore the target should be small enough (i.e. preferably below 100 bp) to be somewhat below the mean size of the remnant DNA pieces.

On a merely technical basis there is no doubt that PCR is able to distinguish an appropriately chosen ruminant DNA target when mixed to DNA of non-ruminant DNA. In a similar way it is possible to design whatever other species-specific target.

3.3 Available targets

The Stratfeed project developed several PCR targets that might be useful for the purpose of MBM detection in feed. Among them there is a cattle target and a ruminant target. Next to the Stratfeed project there are several publications presenting targets that could be used for that purpose with sometimes excessive claims about the real performance for sensitivity. In fact the real performance of a PCR assay can only be established by subjecting the method to an intercomparison study through full validation.

Taking into account some of the up to now performed validation studies [10] done before the Stratfeed project and the intercomparison tests [4] carried out during Stratfeed, one should rather have to conclude that PCR fails in the detection of MBM in feed. One validation study however succeeded in the USA [11] but on material that was not pressure cooked.

In the DG SANCO 2003 study [4] the only acceptable PCR method was the one developed within Stratfeed but used in the laboratory of the developer and the test material for this study was prepared in the same department.

Nevertheless this supports the findings of the Stratfeed project about PCR that it should have some potential in detection of MBM in feed. It seems realistic to consider that the 0.5% level (% in weight of PAPs of the considered animal species or group of species that the assay can detect) could be reached and may be even up to 0.1% but this has still to be verified through interlaboratory assays and validation studies. Moreover these levels have to be considered for MBM having undergone a rendering through the batch process (at 133°C-134°C) with defattening after sterilisation. Applicability on all the possible processes used in Europe that are in compliance with the mandatory sterilisation parameters [1] has still to be evaluated.

3.4 Limits of the technique

The PCR approach being a DNA-based technique, detection will only be possible as long as DNA is still available (or at least DNA in pieces with a sufficient amount in the size range between 60 and 100bp) in the MBM. The research within Stratfeed showed that PCR tests using targets above this level likely fail when detecting traces of MBM in feed sterilised according to European legislation.

In this respect, the sterilisation temperature of the rendering process may be a limiting factor for the sensitivity of PCR assays. It should however be stressed that it is not only the temperature that is important but also the whole process of rendering. A process with a sterilisation temperature at 133°C may result in greater damaging effect of DNA than another one operating sterilisation at an even higher temperature.

Although it is not well known at what temperature DNA will completely disappear (normally higher than 140°C) it is clear that there is a temperature limit for the technique but this should however be balanced by the fact that the nutritional value of the meal undergoing such a heat treatment may also be affected. It seems however that for detection of low levels of MBM (< 0.5%) the 136-138°C threshold range should not be exceeded.

Special treatments with acids or bases, if used during rendering, can also have a great damaging effect on DNA although it seems that the DNA in bone particles is better protected (e.g. in fossils too DNA has been kept for very long periods).

Another limit of the PCR approach is the fact that presence of animal DNA (belonging to a species or a group of species) does not necessarily come from MBM or PAPs, it may come from milk, blood, fat or egg products. The practical impact of this limitation is not well known as some of these products are rather expensive so that they are not that widely used in feed (except perhaps when there are incentives for it, what may be the case for milk powder). Nevertheless it may be a cause of false positive results. It should be stressed however that presently blood-derivatives are also forbidden in feed. So for blood it is not really a problem unless its re-introduction in feed would be considered but probably this would be limited to non-ruminant blood. For fat too, the problem is probably limited because with high quality fat no DNA will be detectable, while with lesser quality fats containing impurities, DNA may still be found but this type of fat may also represent a greater risk for transmission of TSEs.

A last possible point of concern is the fact that the test portions of feed used for extraction are generally very tiny. One generally speaks of less than one gram of feed per test portion. It is not completely sure that these amounts are sufficiently representative when levels of 0.1% MBM are to be detected. However it seems that in new extraction protocols the first extraction steps could handle up to 10 g of feed [12].

3.5 Progress made in recent years

The following objectives were **achieved** within the Stratfeed project:

- The impact of sterilisation has been clearly established with meat and bone meal processed in a batch-type commercial rendering plant at different temperatures up to 141°C. At that temperature DNA is still detectable from the pure MBM. It should be remembered that formerly some scientists thought this was even not possible. However, MBM that was previously sterilised at elevated temperatures above the legal requirement and present at low levels (0.5%) in feed is presently not detectable. With the batch process the temperature should not exceed 136°C-138°C to be detectable below the 0.5% level.

- Appropriate targets were selected for various animal species or groups of species like ruminants, cattle, pig, poultry and sheep.
- In-house validation with Stratfeed assays performed on feedstuffs fortified with MBM showed that next to the influence of the content in MBM and the sterilisation temperature of the MBM there may be also a clear influence of the feed matrix itself on the capacity of detection.

3.6 Work in progress

The feasibility of PCR analysing the sediment of the samples such as in the case of classical microscopy has been demonstrated [13] but the evaluation of the full potential of this approach needs much more research. In theory it would have the advantage to minimize the false positives due to animal material not considered as PAPs. Focusing on the sediments would also offer the advantage of reducing the impact of matrix. However it might also lead to false negative results on MBM of soft tissues that are not present in the sediments. This item requires further investigation during at least one year to give a reliable statement.

A small laboratory intercomparison study has been initiated using the the PCR assays developed in Stratfeed and showing good results in the DG SANCO 2003 study. The trial would be done on already extracted material to focus on the PCR itself. New preliminary studies on the extracts to be provided are being done and seem encouraging because a first attempt of transferability done in summer 2003 was not fully convincing. If all goes well the trial is planned for mid-autumn 2004 by focussing the 0.1 and 0.5% levels.

3.7 Future activities

It is also planned to conduct a validation trial with European control labs but this depends on the results of the previous step. If this former step succeeds well, then such a validation could be prepared for 2005 depending also on available budget for it (FP6). However, specific topics within FP 6 for this application have not been published yet. If the tests fails on the lowest level (0.1%) improvements on extraction should be considered.

The proposal of combination of Near Infrared Microscopy (NIRM) with PCR to be done on particles that were selected by NIRM analysis is a promising approach that could combine advantages of both techniques [14]. Practical results are not to be expected before a three to five year period.

Special PCR techniques [15] might also be helpful in detection of presence of specified risk material in the MBM but the proposed technique has been tested up to now at LGC (Teddington, UK) only on raw material.

3.8 Conclusions

Although probably not that convincing up to now, PCR as technique has certainly a place next to other techniques like microscopy or immunology in the control of presence of MBM in feed. Even if it will not solve all problems because it has its own limits, the technique has a real potential certainly if species-identification becomes an important issue.

Concerning routine application of the technique, this should not be a problem as throughout Europe a lot of labs are equipped to detect GMOs in a quantitative manner. The requirements concerning equipment and skills are completely comparable.

4. Immunoassay analysis

The principle of the immunoassay technique is the interaction between the antibody of the test and the antigen in the sample which are in this case processed animals proteins at various taxonomic levels. Various designs for the detection of this interaction have been developed but in this field only the ELISA technique and lateral flow "dip-stick" technology are used. The former method requires the use of typical equipment of an immunoassay laboratory such as a microplate reader whereas dip sticks can be used without specific equipment.

In a recent intercomparison study [5] four commercially available tests have been evaluated regarding their capability to detect MBM in feed at 0.1 %. Each of the laboratories had to analyse a set of 50 samples that contained various feed mixtures in replicates. 16 out of 50 samples contained ruminant MBM at 0.1 % to detect the sensitivity of the methods assessed in this study. The sample set also contained feed with fish meal and porcine MBM to evaluate the specificity of the tests.

4.1 Immunoassays used in MBM feed analysis

Two dip stick techniques measured exclusively PAPs from ruminant and a third dip stick detected two parameters which where PAPs from all animals and mammalian PAPs. The study also included an ELISA method that measured separately PAPs from (1) ruminants, (2) pig, (3) poultry and (4) terrestrial animals. The method also differed in terms of the target tissue (e.g skeletal muscle vers. connective tissue).

4.2 Results from the recent study

Focusing on the parameter “ruminant PAPs” one of the four methods obtained very good results since almost all samples were correctly identified as positive. Also a second method reported quite convincing result regarding the parameter “ruminant PAPs” as demonstrated by a high sensitivity. It is also important to note that both methods turned out to be selective enough for the intended purpose, since the presence of fishmeal did not result in a larger number of false positive results. One test also demonstrated sufficient *specificity* of the parameters PAPs from pig and PAPs from poultry, since the vast majority of the samples that did not contain PAPs from either animals were correctly classified as negative.

Compared to the results from the former study [1] the sensitivity has been significantly improved. This can be partly explained by the fact, that the results from the former study stimulated laboratories to develop tests that are keyed to the specific needs of present and upcoming European legislation.

In contrast to former studies, the result did not hint at any influence of the sterilisation temperature on the response of the immunoassay.

4.3 Conclusions

Two of the currently available tests showed very good results and should therefore be included in further research programmes.

Major aspects that still need to be evaluated are the impact of the following factors on the response of the tests:

(1) the temperature reached during the sterilisation process

(2) the tissue composition of the PAPs

(3) feed process conditions (e.g. pelleting or not)

(4) different feed compositions

(5) Inclusion of material containing PAPs from other animals such as pigs and poultry

This work can be carried out in 2005 followed by a validation study that should be organised as intercomparison study in which European control laboratories participate.

5. Near infrared-microscopy (NIR-microscopy)

5.1 General information on the NIR-Microscopy method

The near infrared microscopic method is based on the use of the infrared spectra of individual particles to discriminate the origin of the feed compounds making up the samples. The NIR-microscopy method follows exactly the same protocol for the sample preparation as classical microscopy. Hundreds of particles from the raw fraction or the sediment fraction are analysed in order to detect the presence of animal by-product in the sediment. This method has been developed since 1998 and has been tested in the framework of the Stratfeed project. The method has been also successfully used to test the homogeneity of the samples prepared for several intercomparison studies.

5.2 Pro and cons of the method

This method has not been evaluated yet in an intercomparison study. However, there is sufficient information from the STRATFEED project available to evaluate the applicability of this technique to the intended purpose. The pros and cons of the method can be summarised as follows.

The method allows the detection of animal by-products at a level ≤ 0.1 %. This limit of detection can be achieved on the raw fraction or the sediment fraction. The analysis of the raw fraction being too slow, and therefore an alternative instrument, which is the IR camera, can be applied, since the measurement of the spectra of the various particles is much faster. The routine analysis by NIR-microscopy is concentrated on the sediment fraction requiring the application of a sedimentation protocol.

Compared to classical microscopy a less experienced person may conduct the analysis, since the knowledge how to identify particles from animal origin is in the discriminant functions.

5.3 Potential of species identification

The discrimination of terrestrial PAPs from fish by-products can easily be accomplished by this method. Discriminant equations that are already available have to be used to distinguish the source of the particles coming from the raw fraction or the sediment. For the discrimination of the different species of terrestrial animal origin, the results of various studies indicated that the discrimination is possible. However, since there are some overlapping of the NIR spectra between the different groups, the technique can only give an indication from which terrestrial animal the detected PAPs derive.

The percentage of false positive and false negative is below 1 and 0.5 % respectively. In addition no statistical difference has been observed between the NIR-microscopic method and the classical microscopy method.

Another aspect is the non-destructive characteristic of method thereby allowing to select the suspicious particles and to perform additional analysis (e.g. PCR or classical microscopy) on them.

5.4 Current situation

Within the Stratfeed project the method was significantly improved. Major aspect are summarised as followed :

- Development of a protocol by focusing on the sediment part of the sample which contains mainly heavier particles such as bones
- Comparison of the performance with classical microscopy
- Transfer of the method in another laboratory which used a different instrument but the discriminant function established in the CRA-W laboratory

The following work still needs to be conducted

- Small intercomparison study, planned in 2004-2005
- Transfer of the method to the IR camera technology (imaging technology allow to speed the system)
- Discrimination of the terrestrial species in the sediment fraction

6. Overall conclusion

The performance of methods improved significantly within the last years. For classical microscopy we conclude that future activities should focus on the organisation of training courses and proficiency tests. The current European protocol [7] is considered as fit for the purpose.

PCR, in principle, should be the future cornerstone of feed analysis, since it combines both requirements that are high species specificity and high forensic value of the result. The research of Stratfeed resulted in a significant improvement of a specific PCR protocol that was also confirmed in an intercomparison study. However there is still work to do, especially in terms of validation.

Also the immunoassay technique improved significantly and is ready for the final validation studies. Given the simplicity of conducting the test and the capability of high sample throughput and the proven sensitivity and selectivity, immunoassays will play an extremely important in an overall feed safety strategy.

NIR-microscopy and the more advanced instruments such as IR camera offer a non-destructive method also have a potential in feed, given their potential for a higher number samples to be analysed and the proven performance characteristics of this method. However, also this approach needs still to be thoroughly validated, before application by other laboratories can be considered.

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