Chapter 3: Animal proteins in feed: legislative aspects and analytical consequences

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Abridged title: Feed/PAP legislative aspects and analytical consequences

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PROCESSED ANIMAL PROTEINS IN FEED: LEGISLATIVE ASPECTS AND ANALYTICAL CONSEQUENCES

Abridged title: Feed/PAP legislative aspects and analytical consequences

Abstract:
The purpose of this chapter is to give an overview of European legislation related to the conditions of use of protein containing processed animal by-products and in particular meat and bone meal in the feed chain over the last 15 years. Given the fact that corresponding provisions changed over time we will also elaborate on the consequences for the performance profile of analytical methodology required to enforce legal requirements. The chapter will also give an overview of some National and European initiatives launched to contribute to the implementation of the European legislation. Through those projects, the expectations and limitations of the analytical methods to meet the legislative requirements are presented. The chapter focus on three main periods of the feed ban legislation and in the development of corresponding analytical methods. A special attention is given to the possibility of the quantification of the presence of processed animal proteins (PAPs) in feedingstuffs. The consequences of the compulsory and strict rendering conditions on the analytical methods for the detection of PAPs are discussed as well.

Keywords: legislation, analytical methods, feed, PAPs, ABP, MBM.

3.1. INTRODUCTION

Since the first case of bovine spongiform encephalopathy (BSE) in 1986, the European Union (EU) issued a number of legislative acts in order to prevent, control and eradicate transmissible spongiform encephalopathies (TSEs). In this paper we focus on the legislation related to the use of animal by-products (ABPs) for the nutrition of farmed animal. ABPs represent the portion of slaughtered animals that are not intended for human consumption and make up about 50% of the animal. The description of the legal situation of the use of processed ABPs within the EU is complex, because there have been constantly adaptation since 1994, when the use of animal protein to feed ruminants have been restricted for the first time. In addition, two different legislations specify the conditions of use of processed ABPs in feed, namely (1) Regulation (EC) No 999/2001 focussing on the control of TSEs in animals and (2) Regulation EC No 1774/2002 (replaced in 2009 by Regulation EC No 1069/2009) dealing with the safe use of animal by-products not intended for human consumption.

Adaptation of legal requirements regarding ABPs in feed had a distinct impact on the "fitness for purpose" criteria of the analytical methods that have been developed until now. For instance, the required specificity for the analytical methods is different when implementing the rule that feed for pigs and poultry must not contain processed ABPs from ruminants compared to the current situation where ABPs from whatsoever terrestrial animal are banned.

This chapter gives an overview of some National (e.g. FARIMAL) and European initiatives (e.g. STRATFEED, SAFEED-PAP and CRL-AP) launched to contribute to the control of the Union legislation regarding the use of ABPs in feed. Through those projects the limitations and expectations of the analytical methods to fit to the legislative requirements are presented. This chapter focus on 3 main periods corresponding to 3 main steps in the feed ban legislation and in the analytical methods' development. Firstly, the ban on the use of protein derived from *mammalian* tissues in ruminant feed was applied from 1994 to 2001 and the corresponding development of analytical methods focused on the detection of mammalian tissues and their distinction from tissues of other group of animals. The ban was significantly extended in 2001 by introducing a total ban on feeding processed animal proteins (PAPs) to farmed animals kept for food production and method development focused on the discrimination between animal and vegetal particles. In 2002, Regulation (EC) No 1774/2002 introduced the concept of sorting ABPs into three categories according to their health risk and where only material from category 3 can be used in animal nutrition. In particular this Regulation specifies that (1) PAPs representing the protein containing fraction of ABPs exclusively derives from category 3 material and (2) animals must not be fed with PAPs which contain proteins from the same species. In consequence, the analytical methods should be capable of detecting specifically PAPs from pigs in the presence of PAPs from poultry and vice versa.

Quite different methods are used to detect PAPs in feed, namely optical microscopy which is the only official control method so far, polymerase chain reaction (PCR), immunoassays analysis and near infrared spectroscopy (NIRS), -microscopy (NIRM) or -imaging system (NIR Imaging). Other methods are less utilised such as those based on high performance liquid chromatography (HPLC), mass spectrometry (MS) or the electronic nose. A full description of those methods is given in the parts II and III of this book. In this chapter, only the impact of Union legislation on the fitness for purpose criteria for analytical methods is described. A special attention is given to the PAPs quantification aspects in case of introduction of tolerance level on the presence of PAPs in feedingstuffs. Full information

on those quantification methods is presented in chapter 6. The analytical consequences of the legislation, regarding the rendering process conditions and the use of ABPs, on PAPs detection methods are discussed as well. Additional control tools applied in feed industry are presented in chapter 1.

This chapter had to address the following goals:

1. To give an overview on the existing legislation regarding the PAP detection in feed,
2. To give an overview on the analytical methods developed to control the existing legislation,
3. To give an overview of the National and European initiatives launched to contribute to the implementation of the legislation related to the conditions of use of protein containing processed animal by-products in the feed chain over the last 15 years.

3.2. THE BAN ON MAMMALIAN PROTEINS IN RUMINANT FEEDINGSTUFFS: PERIOD 1994-2001

In order to prevent the further spread of BSE and its human form (i.e. new variant Creutzfeldt-Jakob disease), and to prevent the introduction of potentially contaminated animal proteins in the feed chain, various pieces of Union legislation have been introduced. The first piece of legislation (Commission Decision 94/381/EC), introduced a ban on the use of protein derived from mammalian tissues in ruminant feed (EC, 1994). In 1996 the Commission issued the Decision 96/449/EC (EC, 1996) requiring that mammalian animal by-products which was called animal waste in this legislation had to be steam pressure-cooked achieving minimum 133 °C at 3 bar for a minimum period of 20 minutes, prior to their potential use in animal nutrition. Animal fats were exempted from this requirement. The proper treatment of ABPs have further been specified in Council Decision 1999/534/EC), setting standards for hydrolysed protein and fat (EC, 1999), thereby repealing Decision 96/449/EC. For instance, rendered ruminant fat must not contain more than 0.1% insoluble impurities.

The only official analytical method to enforce the ban on mammal tissues in feedingstuffs for ruminants was the microscopy technique as described in Directive 98/88/EC establishing guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs (EC, 1998). This directive was based on the method developed by the International Association of Feedstuff analysis (IAG) - Section feedstuff microscopy. It was only a guideline and gave certain degree of flexibility when applying the method. This method consisted of the microscopic identification and estimation of sieved and decanted fractions of particles of animal origin in feed and depends on the experience of the analyst. This method is capable of distinguishing between fish and terrestrial animal material based essentially on bone characteristics. Inside the terrestrial group, mammalian and avian bones remain difficult to be distinguished.

Other techniques as NIR analyses, PCR and immunological tests were assessed as well for this purpose. The first NIRS studies carried out in 1998 (Garrido-Varo A. and Fernandez V. , 1998) showed the potential of the technique to predict the presence of meat and bone meal (MBM) in feedingstuffs. Since 1998, the CRA-W has been pioneer in the development of near infrared microscopic methods to detect MBM in feedingstuffs. The NIRM instrument combines a near infrared spectrometer and a microscope. With this instrument, spectra of up to hundreds or thousands of particles can be obtained from the analysis of one feed ingredient or one compound feed. Determining whether these particles are MBM particles or not, is done by comparing their spectra with reference libraries. The first NIRM method using a NIR microscope to recognize animal particles in a ground compound feedstuff was developed in 1998 within the framework of a project financed by the Belgian Ministry of Agriculture (Piraux et al., 1999). The great advantage of this technique is that recognition does not depend on the analyst's expertise and that it is possible to automate all the procedures. The polymerase chain reaction (PCR) is the most popular method to detect a well defined DNA target. The first PCR methods were developed in 1998 in the framework of national projects. The Italian national institute of health (ISS) has firstly proposed the PCR-based approach to detect bovine mitochondrial DNA as marker for bovine-derived proteins in ruminants feeds (Tartaglia, 1998). RIKILT, in close cooperation with the University of Utrecht, developed a ruminant-specific PCR test to be used in feedingstuffs containing MBM (Aarts, 1998). Immunological tests are based on specific recognition through the antibody-antigen affinity. These methods have been successfully applied for a long time for the analysis of presence of raw and moderately cooked meat in food. Kits have been compiled to detect MBM in compound feeds (Ansfield, 1994).

The detection of mammalian by-products needs methods where the targets are still detectable, even after the severe heat treatment as Commission Decision 96/449/EC. Nevertheless, antigens are partly denaturated and DNA is damaged at this high temperature. That involves very low responses of the immunoassays and polymerase chain reaction tests respectively. Ansfield was the first in 1994 to propose a method using antibodies against thermo-stable antigens resistant to such rendering process conditions (Ansfield, 1994). Other approaches using immunological tests include, a method using antibodies raised against heat-

treated albumin (Gizzi et al. 2002) , and the dip stick method based on a lateral flow assay using antibodies to target heat-stable muscle protein (i.e. troponin I) (Neogen, 2009). For the PCR technique, considering the degraded state of the DNA, the studies were orientated on targets with a high number of copies per cell (i.e.mitochondrial targets) and on short amplicons (<100bp) in order to increase the probability to detect them (Fumière et al., 2006). In contrast, microscopy and NIR techniques are less affected by the impact of the heat treatment of the samples.

This partial ban (mammal ban) created the risk of cross-contamination of ruminant feed with mammalian feed, as evidenced by the dates of birth of BSE cases detected at the end of the 1990s (also called the 'second BSE crisis'). This was observed in various Member States, including several that formerly thought they had no significant BSE problem. In particular, cross-contamination between ruminant feed and feed containing PAPs intended for other species frequently occurred as a consequence of an inadequate monitoring of the feed ban (lack of a validated species-specific method to test the presence of ruminant MBM, insufficient sensitivity of the applied methodology, and small number of samples analysed) (Vermeulen et al., 2005).

3.3. THE TEMPORARY TOTAL FEED BAN: PERIOD 2001-2003

Because of the difficulties to enforce the ban on mammalian protein in ruminant feed as, the feeding of PAPs - regardless of its origin - to all animals farmed for the production of food was prohibited (total feed ban). This restriction was implemented in January 2001 via a temporary ban (Council Decision 2000/766/EC). This legislation contained some exemptions from the total feed ban, such as the use of fishmeal in the feed for non-ruminants, which was still permitted.

In 2001, Regulation (EC) No 999/2001 of the European Parliament and of the Council has been issued establishing general rules for the prevention, control and eradication of certain TSEs. Regarding the use of ABPs in animal nutrition, this Regulation adopted the former and permanent provision that feed for ruminants must not contain proteins from mammals as specified in Article 7 of this Regulation. In addition, it allows extensions of this ban by modifying the Annexes of this Regulation. This option was applied, when the temporary total feed ban – as established by Council Decision 2000/766/EC - was included in the annexes of this Regulation via Commission Regulation (EC) No 1234/2003. Including the total feed ban into to the Annexes of this Regulation – and not in the main body of the legislation – would allow swift adaptation of the restriction on the use of PAPs, provided appropriate tools for control were available. In addition, Decision 2000/766 has been repealed by Regulation 1234/2003.

In this context we need to clarify that Council Decision 2000/766/EC had introduced the expression "processed animal proteins" (PAPs), which included various products such as MBM, meat meal, bone meal, and blood meal. However, this definition has been modified via Regulation (EC) No 1774/2002, where PAPs are confined to certain material from Category 3 as specified in that Regulation. Since the modified definition has been adopted by Commission Regulation (EC) No 1234/2003 as well, we always refer to the definition of the latter Regulation, when mentioning PAPs.

To contribute to the implementation of Union legislation it was clear that a research project at European level had to be built: STRATFEED answered that need. The objectives and results of this project summarised here below are largely described in the EC book *"Strategies and methods to detect and quantify mammalian tissues in feedingstuffs"* (Dardenne et al., 2005). That involved the development of analytical methods to detect the animal presence in feedingstuffs with a limit of detection close to 0.1% MBM in feed, comparable to the microscopic official method.

The microscopic method had limitations in terms of, for instance, detecting MBM in the presence of other species (e.g. fish, poultry) or other ABPs (e.g. feathers). The first objective of the STRATFEED project was to harmonise and improve the efficiency of the control using the official microscopic method (EC, 1998). The work performed in the STRATFEED project has led to the setting up of a Decision Support System (ARIES) involving decision-making rules for the recognition of ingredients by classical microscopy as well as to the new Directive 2003/126/EC for the implementation of the improved protocol for classical microscopy. Several institutes (CRA-W, RIKILT, PDIR and the JRC-IRMM) have collaborated and have worked on the preparation, negotiation and correct implementation (conferences, workshops and trainings) of this Directive.

The second objective of the STRATFEED project was the development and validation of new methods in support to the official method based on alternative techniques providing the speed (NIRS), the reliable quantification with little expertise (NIRM) and the species group identification even with MBM heat-treated at 141 °C (PCR).

Several studies testified the ability of NIR spectroscopy (NIRS) to identify animal ingredients in feed mixtures (Baeten and Dardenne, 2001; de la Roza et al., 2004). The STRATFEED project led to the conclusion that the detection

limit is, at best, 1-1.5% (Murray et al., 2005). Therefore this LOD is too high for being used in official control laboratories.

By NIRM, if the MBM proportion in a compound feedstuff is low, we need to analyse a large set of particles if we want to observe at least one MBM particle with a high probability. For example, if there is 0.1% MBM in a compound feedstuff and if we want to observe at least one MBM particle with a probability of 95%, about 3000 particles should be analysed. (Dardenne, 2002). However, the principal limitation of this technique is the sequential collection of the spectra (particle by particle) that, together with the need to examine large quantities of particles, makes NIR microscopy time consuming and costly. To reduce the analysis' time, one option is to work only on the sediment in order to reduce the amount of sample to be analysed. Another option is to use the NIR imaging technology with much faster sample analysis, since the spectral data are acquired in parallel. Within the STRATFEED project the NIRM method was significantly improved. Major aspects of the improvement were the development of a protocol focused on the sedimented part of the sample, which contains mainly particles with higher densities such as bones. This method can be used to detect the presence of MBM at concentrations as low as 0.05% mass fraction. When results from the NIRM method were compared with the classical microscopic method, a coefficient of determination (R^2) of 0.87 was obtained. The results of this study demonstrated that this method could be proposed as a complementary tool for the detection of banned MBM in feedstuffs (Baeten et al., 2005).

Since 2001, In the framework of Belgian research project (NIR Imaging, 2005), CRA-W has been developing a method based on the use of an NIR camera (Michotte Renier et al., 2002) to detect the presence of MBM in compound feed. An NIR camera (also called a NIR imaging system) takes pictures sequentially of a pre-defined sample area at different wavelengths. It enables about 500 particles to be analysed in 5 minutes. Combined with the new chemometric method (involving Support Vector Machines - SVM) as a classification algorithm, the NIR imaging method showed very promising results. The technique gives a detection limit of about 0.1% (depending on the number of analysed particles), allowing for a differentiation between fish and terrestrial animal sources. The simultaneous analysis of hundreds or thousands of spectra using an NIR imaging system has the advantages of speed and sensitivity that are required in a screening method (Dardenne et al., 2002, Fernandez et al., 2004).

Beside the constraints on the targets to be analysed by PCR (multi-copy, short size) linked to the severe sterilization conditions of PAPs required by the European legislation, the specificity of those targets has to be checked. The STRATFEED project showed that contamination levels between 0.5 and 1% of MBM are detectable with targets for ruminants and mammalian respectively (Brambilla et al., 2005). The Veterinary Laboratories Agency (VLA) has also successfully validated a PCR method for the mammalian and ruminant DNA detection with a LOD of 0.5%.

Beside the methods developed in the framework of the STRATFEED project, other methods were developed through other projects and laboratories to detect animal presence in feedingstuffs.

Immunological dipstick kits have been compiled for detecting MBM in feed ingredients (with a LOD of 5%) and in compound feed (LOD of 1%). An intercomparison study for the determination of MBM in feed (Gizzi et al., 2004) comparing the performance of different analytical methods when applied to same samples revealed that the sensitivity of the, at the time, available methods was significantly improved. In particular, the sterilisation temperature of the MBM that was considered until 2000 a very critical factor does not pose a problem anymore in 2005. Depending on the target analyte and the applied immunoassay the achievable detection limit was between 0.1 and 0.5 % MBM in feed.

3.4. SETTING CRITERIA FOR THE SAFE USE OF PAPS IN ANIMAL NUTRITION: THE PERIOD SINCE 2003

At the beginning of this period a key legislation regarding the safe use of PAPs in animal nutrition has been issued, which is the Regulation (EC) No 1774/2002 from the European Parliament and the Council laying down health rules concerning animal by-products not intended for human consumption (ABP Regulation). Focusing on the criteria for feeding farmed animals with PAPs, there are two main factors, namely (1) the classification of ABPs into three categories and (2) the ban of intra-species recycling, i.e. animals must not be fed with proteins from the same species. Regarding the classification, Category 1 includes ABPs of high risk, e.g. animals suspected of being infected by transmissible spongiform encephalopathies (TSE) or killed in the context of TSE eradication measures and products derived from animals to which prohibited substances have been administrated. Category 2 includes ABPs with an intermediate risk, e.g. animal killed to eradicate an epizootic disease or ABPs containing residues of veterinary drugs. Finally Category 3 includes ABPs with a low risk which are fit but not intended for human consumption for commercial reasons such as bones and offals. The Regulation specifies that products **only** from Category 3 can be utilised to feed

animals, defining protein derived from this category as processed animal proteins (PAPs). For the implementation of this classification, Commission Regulation No 1432/2007 (EC 2007) requires that processed ABPs from Categories 1 and 2 processing plants are permanently marked with glyceroltriheptanoate (GTH). GTH is an artificial fat not found in nature and meets the requirements of the legislation and in particular it withstands extreme sterilisation conditions as applied by the rendering industry (Boix, 2007).
Also the technical requirements for the treatment of ABPs and in particular the steam sterilisation of mammalian proteins were included in this Regulation, thus repealing Council Decision 1999/534/EC.
Furthermore we need to stress, that since 2003 the restrictions on the use of animal proteins in animal nutrition are specified by two legislations which are (1) the ABP Regulation (EC) No 1774/2002 and (2) the TSE Regulation (EC) No 999/2001.

In 2005 the European Commission (EC, 2005a) issued document entitled "The TSE Roadmap", elaborating on the next steps in the BSE policy in the short and medium term (2005-2009). According to the TSE Roadmap relaxation of certain measures of the current total feed ban could be envisaged without endangering the health of the consumer or the policy of eradicating BSE, provided that the positive trend continues and scientific conditions are in place.

A major modification regarding the use of PAPs in animal nutrition has been introduced by Regulation (EC) No 1923/2006 of the European Parliament and of the Council, including a permanent ban on animal proteins in the diet of ruminants in the main body of Regulation (EC) No 999/2001 by adapting Article 7 accordingly. In 2008 (EC, 2008), a derogation was included for young ruminants allowing milk replacers to contain fishmeal. In addition, Regulation (EC) No 999/2001 allows for introducing tolerance levels of insignificant amounts of animal proteins in feedingstuffs, in order to facilitate the use of such feedingstuffs.

Enforcing these regulations requires that analytical methods are capable of specifically detecting PAPs coming from ruminants, from pigs or from poultry.

The reliability of analytical method to detect traces of bones particles in feedingstuffs as recently demonstrated by the Community Reference Laboratory for the detection of animal proteins in feed (CRA-W, CRL-AP) lead to another amendment (EC, 2009b) of the Annex of Regulation (EC) No 999/2001 allowing that feedingstuffs of plant origin where such traces have been detected may be fed to farmed animals.

In this frame, the SAFEED-PAP project aimed to complete the scientific conditions that should allow the repealing of the extended feed ban (EC, 2003b). The three main objectives are: (i) the development of suitable validated methods for the species specific detection and quantification of animal protein in compound feed in order to allow the amendment of the extended total ban; (ii) the development of tools and analytical kits for the correct implementation of the methods in the labs; (iii) the set up of the appropriate environment for the optimum application of the methods.

Regarding the microscopic method, following several studies (Gizzi et al., 2003a; 2004; van Raamsdonk et al., 2004; 2005), some laboratories have still problems in terms of, for instance, (i) detecting 0.1% of terrestrial animal particles in presence of 5% of fishmeal and (ii) discriminating poultry bones from other terrestrial bones. In order to improve the differentiation at the species level, a workgroup of the SAFEED-PAP project, aimed to improve the official method and to combine the benefits of microscopy (e.g. very low LOD, no false positives or negatives above LOD, relatively fast) with the advantages of other identification methods. The species identification involves for the microscopy method a better knowledge of the bones (Domenis et al., 2007) and a deeper exploration of other particles than bones (i.e. muscles) or new markers. In order to analyse more bones descriptors, computer image analysis was used to perform lacunae morpho-metric measurements in poultry and mammal by-product (Pinotti et al. 2004; 2009; Campagnoli et al., 2009). Another way to overcome the difficulty in microscopy to differentiate the species is to combine the microscopic method with the possibilities to identify specific targets by immunochemical or PCR detection (proteins or DNA specific for bovine, ruminant or other material). This strategy should allow the *in situ* identification of particles already detected by microscopy. Preliminary promising results showed that PCR could be a useful complementary tool to microscopy for determining whether mammalian material is present in a sample containing poultry meal (Fumière, 2004).

Regarding the immunogical kits, one of the strategies of the SAFEED-PAP consortium is to take into account the last developments made in the field of the routine detection for the presence of PAPs at species level in animal feeds and to improve the different existing commercial kits or those in development.

The Real Time PCR technology for the species-specific DNA target detection has the interesting characteristic to be a very sensitive method. Many PCR methods for the detection of PAPs from various animal species are largely described in the literature. PCR often gave disappointing results in most of the studies. However, CRA-W, in the framework of the STRATFEED project, developed a PCR method with a LOD of 0.1-0.5% for the species detection of cattle, sheep, pig, chicken and fish (Brambilla et al., 2005). An Inter-laboratory study carried out by the JRC IRMM in 2006 (Prado et al., 2007) showed that three labs applying their PCR methods were able to detect 0.1% of cattle MBM either alone or in

mixtures with fishmeal. In the other hand, some research teams worked on methods of detection of small animals like cat, dog, rat, mouse (Martin et al., 2007) that could explain very low traces of terrestrial animal particles found in some feed materials.

Beside the immunoassays and PCR, other techniques like high performance liquid chromatography (HPLC) and mass spectrometry (MS) are used to develop confirmatory methods for the species-specific detection of animal proteins. Those methods are based on animal proteins or peptides detection. Results from an intercomparison study (Gizzi et al., 2004) indicated that the achievable detection limit by HPLC is very low (about 0.1%) but the potential for species specificity is very low. Species identification using HPLC seems possible if only one species is the source of animal origin material. Regarding the mass spectrometry, the major drawback of this approach is the lack of species specificity as well (Fernandez Ocaña et al., 2004). Studies using those instruments are going on in the framework of the SAFEED-PAP project. The main analytical challenges to be addressed are the identification of protein markers that could be used to distinguish PAPs from different species in some feed samples and the development of a sample extraction method for the target proteins from the feedingstuff.

Regarding the NIR technique, the species differentiation involves the development of database and equations at the species level. The macro NIRS methods can discriminate the higher taxonomic groups of species (i.e. terrestrial animal vs fish). So, NIRS can have a role to play only as screening technique. Therefore, for the species differentiation purpose, research focused on NIR microscopic technology. For the discrimination of the different species of terrestrial animal origin, the first tentative studies by NIRM indicated that the discrimination seems possible at least for some groups of species (Baeten et al., 2005; Fumière et al., 2007; De la Haba et al., 2007). SAFEED-PAP project aimed to establish the potential of the NIR microscopic methods for the discrimination of the source of the animal particles detected using pure MBM from single animal species. The first models built on relevant NIR discriminant bands (NIR markers) led to improve the specificity potential of the NIR method for the discrimination of the source of the animal particles. As showed before, the NIR imaging method allows differentiation between fish and terrestrial animal sources. Recent developments in NIR imaging technology offer possibilities to analyse samples on-line with a linescan camera. Spectra are acquired line by line. This technology should allow the analysis of larger amount of particles with a reduced acquisition time.

Another aspect of the NIR is the non-destructive characteristic of the method thereby allowing additional analysis (e.g. PCR or classical microscopy) in suspicious particles. In the framework of the Belgian research project FARIMAL (FARIMAL, 2008) NIRM and PCR were combined in an original procedure to develop authenticated species specific spectral databases (cattle, sheep, pig, chicken, fish). One analytical challenge was to develop the DNA extraction protocol to perform PCR on a single particle. Based on these libraries, the results showed that 80% of the cattle particles and 90% of the fish particles can be correctly assigned by NIRM to their species of origin (Fumière et al., 2007). This work is continued within the framework of the SAFEED-PAP project using the PCR targets already optimised or new targets to be developed to confirm the animal origin at the species level of suspicious particles detected by NIRM. One objective is to optimise the number of PCRs made with the DNA of one particle (Fumière et al., 2008).

Electronic nose has been also used to develop screening methods for the species-specific detection of animal proteins. An University of Milan research team (Campagnoli, 2004) evaluated its potential application for detecting processed animal proteins (PAP) in feed. Samples from the STRATFEED project were analysed using odour sensors. The initial results indicated that the electronic nose could be an interesting approach for screening raw materials in the feed industry, but further studies on larger set of samples are needed.

The total feed ban prohibits the use of PAPs in feed for farmed animals, however some feed ingredients such as milk, blood, fat, hydrolysed proteins or egg products, are authorised. This is posing an important limitation to the PCR approach since is not possible to distinguish whether the DNA is coming from an authorised product or not. However regarding the NIR technique; NIRS, NIRM and NIR camera methods have been adapted to identify and quantify a wider range of animal feed ingredients. Some studies showed that MBM can be detected in hydrolysed feather protein by NIRS (Geesing, 2004) and in blood by NIRM (Fumière, 2005).
Additional control tools in feed industry are presented in the chapter 1 of this book.

3.5. THE CONCEPT OF "ZERO-TOLERANCE" OF PAPS

Since there have been no tolerance limits for traces of PAPs in non-targeted feedingstuffs established yet, the sole detection of PAPs in such feedingstuffs – regardless of its concentration – led to rejection of the material. This concept includes the principle of "Zero-tolerance", which has been upheld in an EC judgment of 1 April 2004 (EC, 2004). This article lays down that the presence, even

accidental, of unauthorised substances in fishmeal used in the feeding of animals other than ruminants is prohibited and that they allow traders no level of tolerance. However, in between, Union legislation allows for deviation from this principle. For instance, the Commission Regulation (EC) No 1292/2005 (EC 2005b) enables the feeding of tuber and root crops contaminated with insignificant amounts of bone spicules to farmed animals, depending of a favourable risk assessment. The risk assessment shall take into account at least the amount and possible source of contamination and the final destination of the consignment (EC, 2005b). The new Commission Regulation (EC) 163/2009 extends this permission to all ingredients of plant origin (EC, 2009b). In 2007, the European Food Safety Authority (EFSA) was requested by the European Parliament to evaluate the introduction of certain tolerance levels with regard to small quantities of MBM in animal feed and the parameters which could be used to define these tolerance levels and quantities. This assessment considers such a tolerance for animal proteins of any species in animal feed. In the event that a tolerance level was required to be set up in order to quantify animal proteins in animal feed, the BIOHAZ Panel of EFSA considered the Limit of Quantification (LOQ) of the method used to set such tolerance level as the parameter required. However the BIOHAZ Panel concluded that it is currently not possible to set a LOQ because of insufficient data on the performance of relevant methods for quantification. It is therefore recommended that studies be conducted to define the LOQ for different types of animal proteins in feed (EFSA, 2007). It is generally accepted that an analytical method should show sufficient sensitivity at a concentration of 0.1% with a rate of false positive value inferior to 5% (Gizzi et al., 2003b).

The use of tolerance limits would be a new analytical challenge requiring control tools that are also able to quantify accurately the level of PAPs. When microscopy is used for quantification of animal constituents in feedstuffs as notified in the Directive 2003/126/EC (EC, 2003b), this method is depending on the presence of bones in the product and the accuracy is very depending on the bone content in the animal ingredient to be identified in a compound feed. Furthermore, the differentiation of bones from mammalians and poultry is very difficult and considerable expertise is necessary to make this differentiation. Within the framework of the Community Reference Laboratory for animal proteins in feedingstuffs (CRL-AP), two inter laboratory studies focusing on the implementation and performance evaluation of the method, revealed that the quantification by microscopy is very difficult (Veys, 2007; 2008). Moreover the implementation of the Commission Regulation (EC) No 163/2009 (EC, 2009b) involves a better knowledge of the bones from small animals living in the fields.

The response of PCR and immunochemical tests is influenced by the tissue content and by the process rendering parameters (pressure, temperature). Those techniques are therefore not dedicated for quantification purpose (Fumière et al., 2009). However, the studies by NIRS, NIRM and NIR imaging technique showed the potential of quantification of PAP (Garrido et al., 1998; Dardenne et al., 2002). All those methods are still at their initial stage of development: description, perspectives but also encountered difficulties of implementation are discussed in chapter 6 of this book.

3.6. DISCUSSION AND PERSPECTIVES

Enforcement of Union legislation requires the detection of PAPs at low level, the identification at species level and quantification of PAPs in order to allow a relaxation of the total feed ban. Up to now, no method presented in this chapter can answer completely those requirements.

Classical microscopy, the only official method up to now, is mainly based on the detection of bones with a limit of detection up to 0.1%. The method is not affected by the heat treatment applied during rendering. However, the determination at the species level needs more development.

The NIRS technique meets all the criteria in terms of speed of response, reliability, cost-effectiveness and fitness for a radically new approach to assess raw materials and finished feed products. It can analyse a sample in a few seconds, even for multiple constituents, and, very important, it is non-destructive. Various studies have shown that NIRS can predict instantly the percentage of PAPs. However, the limit of detection is too high (1%) when compared against the official method and needs to be combined with confirmatory techniques. It is a practicable methodology for the industry, which can apply it for the routine control of the gross volume of feedstuffs marketed in Europe. NIRM and NIR imaging methods can achieve 0.1% LOD and are the only methods suitable for quantification. However, the discrimination at the species level in increasing improvement needs still more studies. The advantage of the NIR technique is also the potential to discriminate between prohibited PAPs and some allowed ABPs.

The main advantage of immunoassays is that they are rapid and easy to perform. Part of the test can be performed in 10 minutes and only require boiling samples prior to analysis. Dip stick immunoassays do not require an analytical laboratory at hand and can be carried out on-site. These methods can be considered as screening methods. The results of the study carried out in the framework of the SAFEED-PAP project, also indicated that the methods need to be further improved

in terms of sensitivity and specificity. PCR is a powerful but costly technology. It is not the most suitable as a screening method, but as a tool in combination with optical or NIR microscopy it could improve the detection of animal material and even identify it at species level. A major drawback of the PCR is the limited test portion (100-200 mg) used for DNA extraction. The adaptation of the extraction procedure to larger sub-samples is evaluated in the framework of the SAFEED-PAP project. The Commission Regulation (EC) No 152/2009 gives guidelines regarding the sampling for the official control of feed.

Probably, the solution should be found by combining several methods, a screening method (Microscopy, NIR) and a confirmatory method (Immunoassay, PCR). The electronic nose could be an interesting approach for screening raw materials in feed industry, but further studies are needed. The development of new methods using high performance liquid chromatography and mass spectrometry techniques indicate that the species specificity is too low to be used as confirmatory method.

In order to ensure a high level of quality, a uniformity of the results and to assist in the development and the validation of new analytical tools and methods to support the detection of animal proteins in feedingstuffs, Commission Regulation (EC) No 776/2006, nominated the Walloon Agricultural Research Centre as Community Reference Laboratory for the detection of Animal Proteins in feedingstuffs (CRL-AP) for the 2006-2011 period (CRL-AP, 2009). The main missions of the CRL-AP are the following:

- to provide scientific advice and support to the European Commission
- to coordinate the activities of the network of National Reference Laboratories (NRLs),
- to organise interlaboratory studies and quality assistance for the NRLs,
- to develop and/or validate new analytical methods and tools and to improve existing methods,
- to organise training courses, formations and workshops.

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