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Official Feed Control Linked to the Detection of Animal Byproducts: Past, Present, and Future

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ABSTRACT: In the context of the expansion of the human population, availability of food, and in extension of animal feed, is a big issue. Favoring a circular economy by the valorization of byproducts is a sustainable way to be more efficient. Animal byproducts are an interesting source of feed materials due to their richness in proteins of high nutritional value. Prevention and control efforts have allowed a gradual lifting of the feed ban regarding the use of animal byproducts. Nevertheless, the challenge remains the development of analytical methods enabling a distinction between authorized and unauthorized feed materials. This Review focuses on the historical and epidemiological context of the official control, the evaluation of current and foreseen legislation, and the available methods of analysis for the detection of constituents of animal origin in feedingstuffs. It also underlines the analytical limitations of the approach and discusses some prospects of novel methods to ensure food and feed safety.

KEYWORDS: *BSE, processed animal protein, feed fraud, feed adulteration, light microscopy, PCR, spectroscopy, immunoassays, mass spectroscopy, PMCA, RT-QuIC, insect*

1. INTRODUCTION

Since the mid-1980s and the emergence of the epidemic, several thousand cases of classical bovine spongiform encephalopathy (BSE) have been reported in Europe. Measures of surveillance, feed ban, and feed control have been rapidly put in place. Fortunately, these extensive actions had a drastic effect on the number of BSE cases. To date, occasional cases of classical BSE in animals born following the total feed ban (BARB cases) still occur. In total, 61 BARB cases are currently recorded. Improper implementations of the feed ban or spontaneous incidents are some of the likely causes.[1] Even though the number of recent cases is very low, this should not be neglected. It is even more important to be careful because this disease is not completely understood. The current impossibility to establish an antemortem confirmation diagnosis provides a crucial role to the specified risk material (SRM) removal and the feed ban, given the zoonotic nature of BSE.

By now, there is an additional challenge to be faced by the animal feed industry: the feed availability. Solutions can be found by increasing the efficiency of feed production, finding new feed sources, and/or reusing byproducts. Animal byproducts are an interesting source of feed materials. Indeed, up to 50% of the slaughtered animal weight is not intended for human consumption. These materials are rich in proteins of high nutritional value and also have an economic interest because neglecting their use or underuse logically results in a loss of potential gains.[2] Since the first version of the feed ban in 1994, the regulations linked to the use of animal byproducts have been revised many times mostly for additional restrictions or, more recently, for partial lifting.[3] With each revision, the analytical scheme intended to check proper use of processed animal proteins (PAPs) had to be adapted and became more complex.

The aim of this Review is to summarize how the analytical framework is constantly being adapted to the changes in the legislation in order to ensure the control of the proper use of animal proteins in feed. The foreseen relaxations of the ban are reviewed together with the operational schemes that articulate the use of official methods depending on the feed destination. However, there are still analytical gaps that are highlighted. Alternative analytical methods developed to address them are considered. Finally, future challenges and some prospects to ensure food and feed safety are proposed.

2. BOVINE SPONGIFORM ENCEPHALOPATHY: ORIGIN, FEED-BORNE TRANSMISSION, RISK ASSESSMENTS, AND CURRENT EPIDEMIOLOGICAL SITUATION

BSE is a chronic disease causing a degenerative disorder in bovine neural tissue. The disease is due to a conformational conversion of a membrane glycoprotein, known as the cellular isoform of the Prion Protein (PrPc), naturally present in the nervous system and other extra-neural tissues, into an

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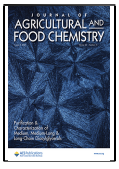

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extremely resistant form of the protein, the scrapie isoform of the Prion Protein (PrPsc).[4]

BSE emerged in cattle in the 1980s. The origin of the first classical BSE (C-BSE) cases remains unknown. The main hypotheses are the spontaneous occurrence and the scrapie transmission to bovine.[4] The cause of the BSE epidemic is clearer. Epidemiological studies related this outbreak to a feed-borne epidemic. A partial ban on the use of mammalian meat and bone meal (MBM) in ruminant feed was consequently put in place in 1994.[5] Although this measure resulted in a decrease in BSE cases, the epidemic was not stopped. One suggested explanation for this was that ruminant feed was being cross-contaminated with feed intended for other farmed animals for whom ruminant MBM was still authorized. In 2001, the feed ban was therefore extended to a prohibition of the use of PAPs of all species in feed for all farmed animals (i.e., a total feed ban).[6]

In parallel, other measures were put in place, including the removal of SRM from the food chain.[7] These measures were clearly justified by the zoonotic character of the disease, its long incubation time and the impossibility of direct detection of prions in feed.[8]

These measures have proved to be key actions to stop the progression of the disease. While the total number of C-BSE cases reported in the EU was 2174 in 2001, this number has drastically and continuously decreased to 37 cases in 2010, 21 cases in 2011, 11 cases in 2012, 2 cases in 2013, 3 cases in 2014, 2 cases in 2015, and only 1 case in 2016.[9,10] Worldwide, 2017 was the first year for which no classical BSE case has been reported. However, in the meantime, the United Kingdom confirmed a new case of classical BSE in 2018. It is still unclear if the few cases encountered indicate an inadequate implementation of the feed ban or a spontaneous occurrence of C-BSE.[11] This statement concerns the last two cases in March 2016 and October 2018 affecting animals born in 2011 and 2013, respectively, well after the total feed ban of 2001.

3. ANIMAL BYPRODUCTS AND DERIVED PRODUCTS NOT INTENDED FOR HUMAN CONSUMPTION

Since the BSE crisis, the legal framework on the feed ban and utilization of animal proteins in feedingstuffs has been in continuous development. In order to understand the challenges linked to the development of analytical methods, it is important to have an overview of the regulations linked to them.

3.1. Animal Byproduct Regulations. In 2002, the so-called animal byproduct legislation, Regulation (EC) No 1774/2002,[12] repealed and replaced by Regulation (EC) No 1069/2009,[13] defined animal byproducts (ABPs) as "entire bodies or parts of animal origin or other products obtained from animals, which are not intended for human consumption, including oocytes, embryos and semen".

This regulation introduced the classification of ABPs into three risk categories that also determine their subsequent use. Category 1 materials show the highest risk and must be destroyed by incineration or converted into biofuel. In addition to incineration or conversion into biofuel, ABPs of Category 2 can also be used as organic fertilizers or soil improvers following specific processing. Only Category 3 material may be used for the manufacturing of feed for farmed animals, fur animals, or pet food in accordance with Regulation (EC) No 1069/2009. ABPs of Categories 1 and 2 must be permanently marked with glyceroltriheptanoate (GTH). The goal of this labeling is to monitor potential contamination of Category 3 by Category 1 or 2 materials. In order to distinguish them, the term "MBM" is reserved for animal proteins derived from Category 1 or Category 2 materials whereas the term "PAPs" can only be used for Category 3 materials. Moreover, Category 3 materials must undergo a specific rendering process according to their type.[14]

Another important point of the ABP Regulation is the prohibition of intraspecies recycling. This rule is based on the "Species Barrier Concept" which means that transmission beyond the species barrier is more difficult. This prohibition is of paramount importance in the process of lifting the feed ban on the use of nonruminant PAPs in nonruminant feed.[15] This last point underlines the importance of the availability of species-specific methods to identify feed material of animal origin and ensure feed safety.[16]

3.2. Use of Animal Proteins of Category 3 in Feedingstuffs: Current Legislation. The prohibition of the use of ABPs of Category 3 in animal feed depends on three factors (Table 1): byproduct type, species of origin, and final destination (pets, fur animals or other farmed animals). These rules are described in Regulation (EC) No 999/2001.[6] While the species of origin and the final destination are two easy-to-understand concepts, byproduct type is more complex as it depends on the constituents of animal origin considered in combination with the production process undergone.[14]

Table 1. Summary of the Animal-Derived Products Currently Authorized in Feedingstuffs (Inspired by TSE Roadmap II[17])[a]

	animals for which the feed material is intended			
	farmed animals			
category 3 byproduct type	ruminants	nonruminants (except fish)	fish	pets and fur animals
ruminant PAPs including blood meal	NA	NA	NA	A
ruminant blood products	NA	NA	NA	A
gelatin from ruminants	NA	NA	NA	A
nonruminant PAPs other than blood meal and fish meal[c]	NA	NA	A	A
nonruminant blood meal	NA	NA	A	A
fishmeal	NA[b]	A	A	A
nonruminant blood products	NA	A	A	A
insect PAPs[d]	NA	NA	A	A
nonruminant gelatin	A	A	A	A
egg, egg products, milk, milk products, colostrum	A	A	A	A
hydrolyzed proteins from nonruminants or from ruminant hides and skins	A	A	A	A
hydrolyzed proteins other than those derived from nonruminants or from ruminant hides and skins	NA	NA	NA	A
di- and tricalcium phosphate of animal origin	NA	A	A	A
animal proteins other than the above-mentioned ones	NA	A	A	A

[a]A, authorized; NA, unauthorized. [b]Milk replacers containing fishmeal and intended only for unweaned ruminants are authorized. [c]Authorized since June 2013. [d]Authorized since July 2017.

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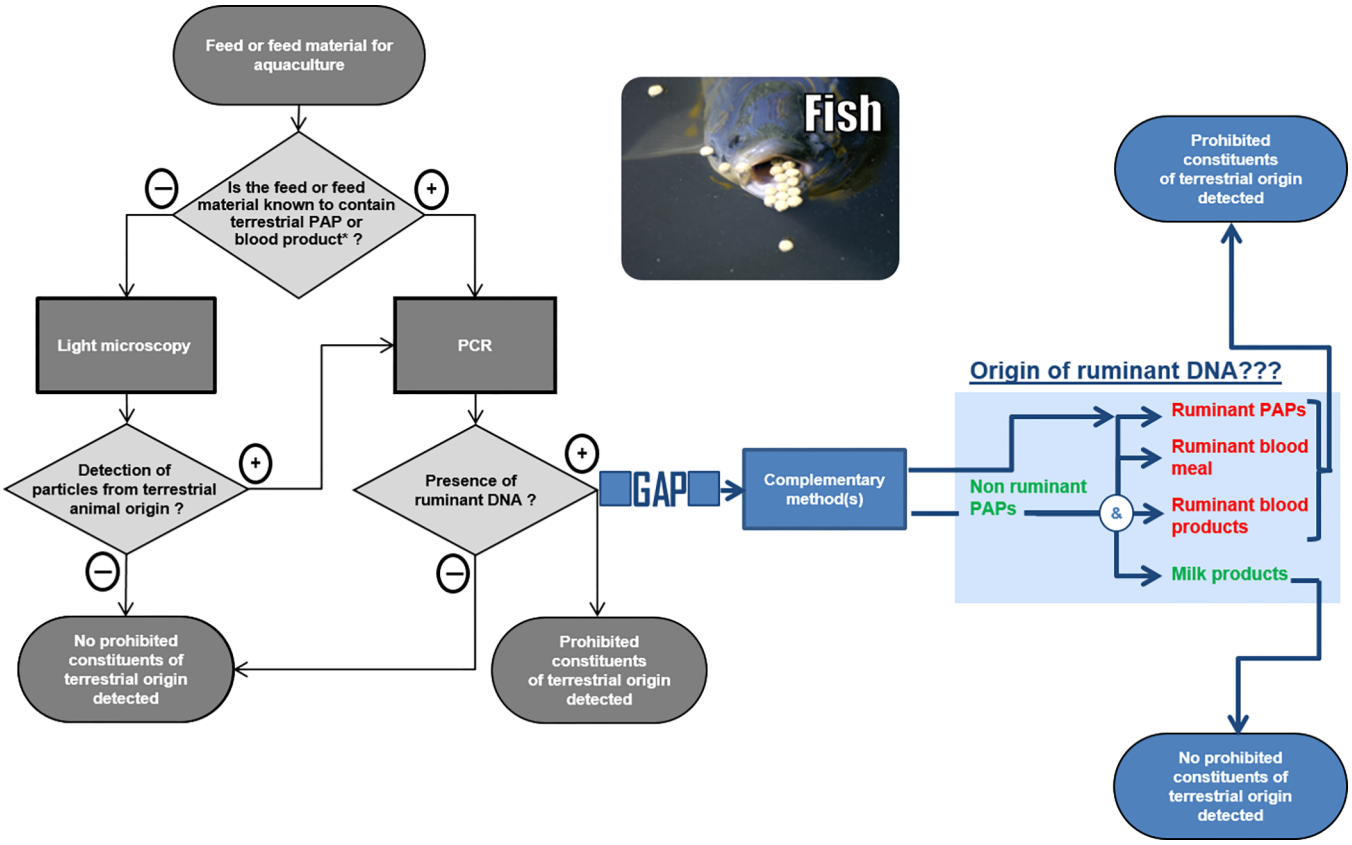

Figure 1. Operational protocol for the analysis of feed or feed material for aquaculture animals and current analytical gap.

Table 1 summarizes the current situation about the legal status regarding the use of animal-derived products in feedingstuffs. To date, ruminant PAPs and ruminant blood products are still forbidden in any type of feed other than for fur animals or as pet food. Following the lifting of the ban in June 2013,[3] nonruminant PAPs were reauthorized for aquafeed and now supplement nonruminant blood meal and fishmeal, which were already permitted. Nonruminant blood products and fishmeal are also authorized in feed for nonruminants other than fish. Fishmeal can also be used in milk replacers for unweaned calves or lambs. Besides that, nonruminant gelatin, egg, egg products, milk, milk products, colostrum, and hydrolyzed proteins derived from nonruminants or from ruminant hides and skins are authorized in all types of feed. Finally, since July 2017, a closed list of seven insect species (*Hermetia illucens*, *Tenebrio molitor*, *Musca domestica*, *Alphitobius diaperinus*, *Acheta domesticus*, *Gryllodes sigillatus*, and *Gryllus assimilis*) has been authorized for use in aquafeed.[18] Interestingly, only reared insects are authorized.[19] Therefore, according to EU regulation,[19] these insects are also on their turn considered as nonruminant farmed animals and are consequently also submitted to the same animal regulation rules.

When taking into consideration all the regulations cited above, one understands the complexity regarding the development of analytical methods enabling the correct application of these regulations.

4. METHODS OF ANALYSIS FOR THE DETERMINATION OF CONSTITUENTS OF ANIMAL ORIGIN FOR THE OFFICIAL CONTROL OF FEED

In order to control the presence of unauthorized products of animal origin in feed intended for farmed animals, analytical methods have been developed.[20,21] These methods are described in Commission Regulation (EC) No 152/2009.[22] Until 2013, official control was performed entirely by light microscopy (LM).[23] With the reintroduction of nonruminant PAPs in aquafeed, it was necessary to be able to identify the species of origin of the PAPs. For this purpose, polymerase chain reaction (PCR) for the detection of ruminant DNA was added as an official analytical method by amending Annex VI of the Regulation.[24] In what follows, the two methods are described as well as their advantages and limitations. The operational schemes currently in application are also discussed.

4.1. Light Microscopy. The light microscopic method (LM) is based on the identification of particles such as muscle fibers, cartilages, bones, horns, hairs, bristles, feathers, eggshells, and scales on the basis of typical and morphologically identifiable characteristics.[25] Before the microscopic observations, samples are prepared according to Annex VI of Commission Regulation (EC) No 152/2009, as amended by Commission Regulation (EC) No 51/2013.[24]

The LM technique is rapid, low-cost, and very sensitive with a limit of detection as low as 0.0025% (w/w), depending on the matrix and the type of PAPs.[26] However, LM requires experienced analysts and is unable to determine the species of origin of the detected particles. In the case of bone particles, microscopy is able to distinguish terrestrial bones from fish bones but is unable to determine lower taxa (e.g., cattle, pig,

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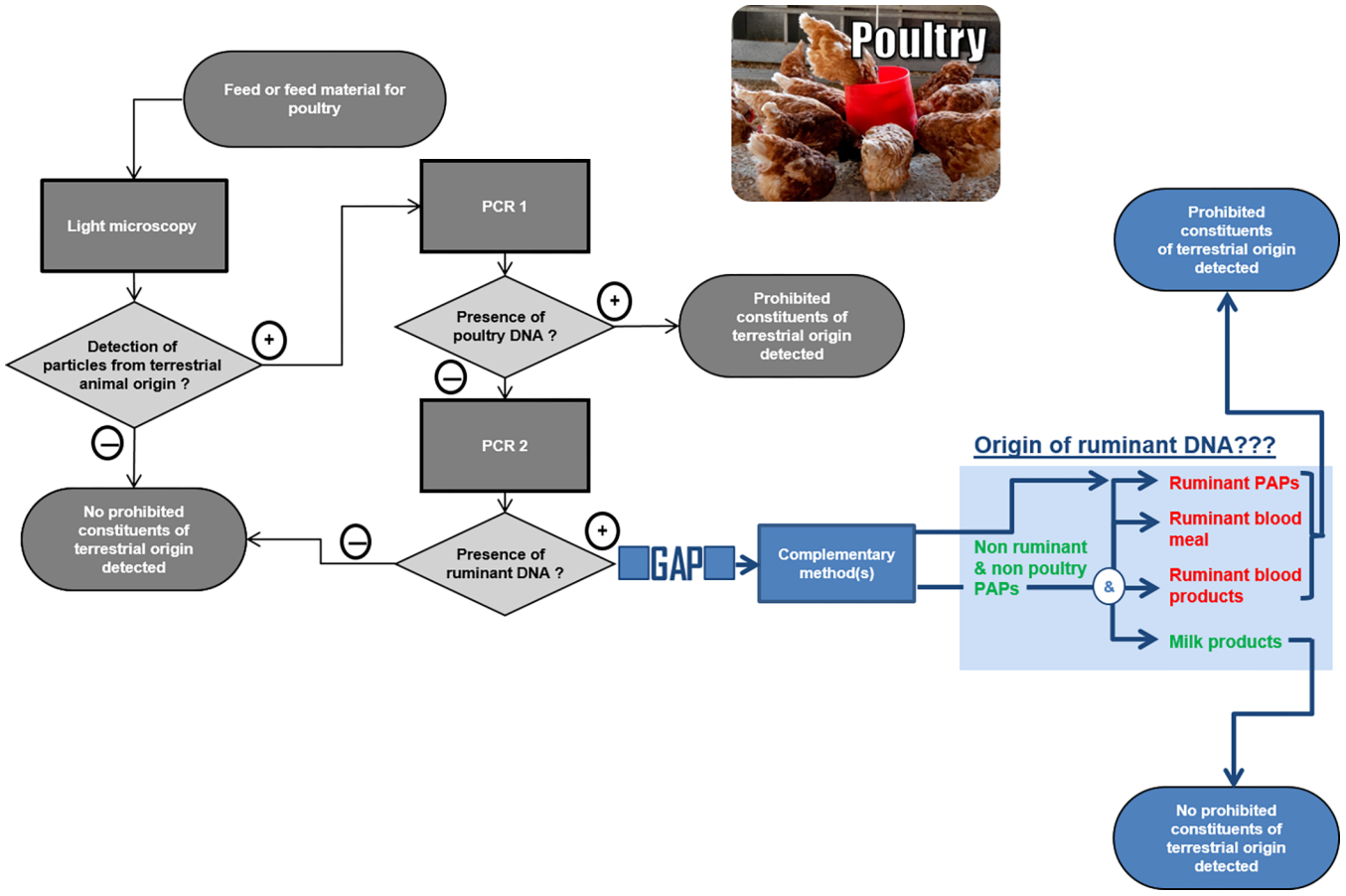

Figure 2. Analytical gaps in the analysis of feed or feed material for poultry in the context of a future lifting of the feed ban.

and poultry). Muscle fibers cannot be assigned to a species or a species group. Additional types of particle such as hairs, feather, eggshells, or fish scale can also be observed. The identification of feather or eggshell particles will indicate the presence of byproducts of poultry origin and fish scales that of fish. Hairs may confirm the presence of byproducts of mammal origin and the observation of their structure may even allow the species of origin to be determined. However, even when such particles are present, the simultaneous observation of terrestrial bone particles does not exclude the presence of PAPs of other origin.

4.2. Real-Time Polymerase Chain Reaction. Due to the limitations of LM regarding species determination, and in the context of the partial relaxation of the feed ban concerning nonruminant PAPs in aquafeed, it was crucial, before any legislation change, to have analytical methods able to distinguish ruminant PAPs from nonruminant PAPs.

An ad hoc real-time PCR assay was therefore developed and introduced in the legislation. PCR is based on the amplification of a particular DNA target specific to a species or taxon (e.g., ruminant). DNA extraction and amplification have to be performed according to the Standard Operating Procedure (SOP) established by the EURL-AP[27,28] as it has to be done in a harmonized way. Up to now, only the ruminant PCR test is part of the official method linked to Annex VI of Commission Regulation No 152/2009 but two other PCR assays were already validated and are ready to be introduced in the legislation (data not published). They target pig DNA and simultaneously chicken and turkey DNA, respectively.

Although PCR has limitations in the case of DNA degradation, the method developed allows ruminant DNA to be detected even in highly processed feed materials, thanks to the shortness of the DNA target (85 bp) as well as its multicopy character in a cell.[29] Potentially, PCR enables a clear identification to be made of various species or group of species.[30] It is also a very sensitive method and reaches the same limit of detection as light microscopy. However, although PCR provides information on the genetic origin of the DNA present in a feed, it cannot distinguish the cellular origin of the signal (e.g., leucocyte, osteocyte or myocyte). Therefore, this method is unable to discriminate between authorized and prohibited feed material from the same species of origin (e.g., milk is an authorized product that will react positively to the ruminant PCR test).

5. CURRENT OPERATIONAL SCHEMES AND RELATED ANALYTICAL GAPS

Depending on the type of feed being analyzed, the two official methods have to be applied differently. The operational protocols that have to be followed are described in the SOP for the combination of LM and PCR.[31] The final destination of the compound feed or feed materials determines the operational protocol that has to be followed.

For the analysis of aquafeed, the two methods are combined depending on the labeling and/or the LM results (Figure 1). If no terrestrial particle is detected by LM, no further analysis is necessary and the feed is declared free of prohibited constituents of terrestrial origin. However, if terrestrial particles

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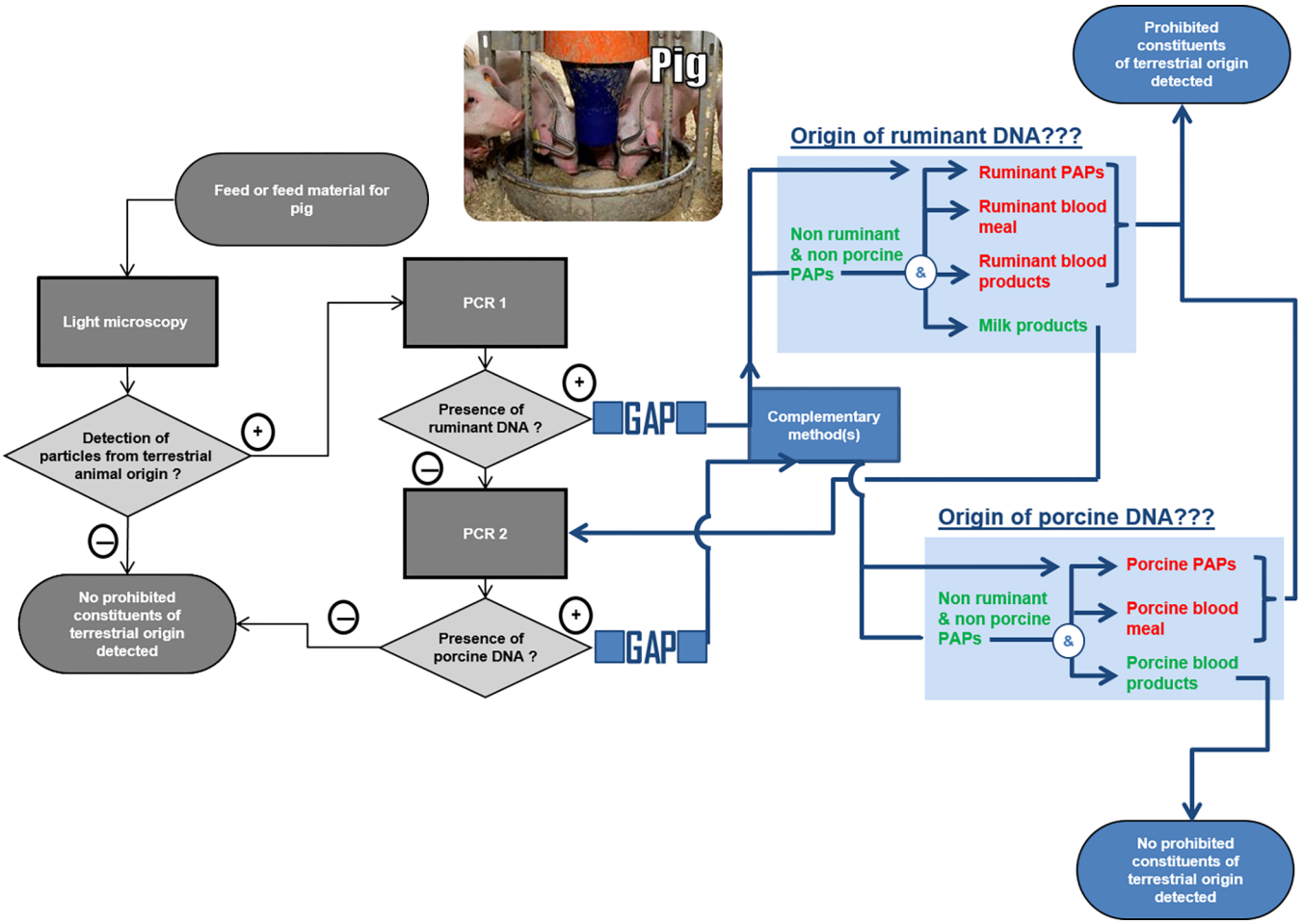

Figure 3. Analytical gaps in the analysis of feed or feed material for pigs in the context of a future lifting of the feed ban.

are identified or if the feed is known to contain terrestrial PAPs or blood products, ruminant PCR has to be performed. Following this, the detection of ruminant DNA in the feed leads to a single conclusion: the presence of prohibited constituents of animal origin.

When compound feeds are considered, a first analytical gap becomes clearly apparent. If a positive reaction is obtained by PCR using the official ruminant probe, the presence of ruminant DNA is considered as an indirect evidence of the presence of prohibited constituents of terrestrial origin.[31] This will be correct if the feed contains PAPs of ruminant origin (prohibited in aquaculture), but in the case of a feed containing milk products, as this product is authorized in aquaculture, the conclusion will be wrong. In such cases, additional analyses are needed to determine both the species and source of the animal products.[32] Fortunately, such cases have been evaluated as relatively uncommon as dairy products are rarely used as feed material in aquafeed. However, some producers have also argued that casein powder may sometimes be used in aquafeed as a carrier of feed additives. The case of an aquafeed declared as containing nonruminant PAPs, nonruminant blood products, and casein is a good illustration. All these ingredients are authorized in aquafeed. Terrestrial PAPs will be detected by LM, and a PCR analysis will be performed to detect the possible presence of ruminant DNA. The PCR result will logically be positive and can be explained by the presence of casein (according to the declaration) obtained from milk and still containing ruminant DNA. However, the additional presence of ruminant PAPs or ruminant blood products cannot strictly be excluded without complementary analyses.

Currently, for the analysis of feed or feed material intended for farmed animals other than aquaculture animals and fur animals, LM is sufficient to detect the presence of prohibited constituents of animal origin, as no PAP of terrestrial origin is authorized for use in such cases.

However, if the ban on the use of nonruminant PAPs in nonruminant feed is relaxed in the future, then the detection of terrestrial particles will not be sufficient to determine if prohibited feed materials are present or not with respect to prohibition of intraspecies recycling. It is very likely that PCR assays targeting poultry and porcine products specifically will be added to the analytical operational scheme, as the targets are already validated for this purpose (unpublished data). Figures 2 and 3 outline possible scenarios for analytical operational schemes in this context and the expected associated gaps.

As for aquafeed, with regard to poultry feed (Figure 2) or pig feed (Figure 3), if no terrestrial particle is detected by LM, no other analysis is necessary and the feed will be declared free of prohibited constituents of terrestrial origin. However, if terrestrial particles are present, PCR analysis will have to be performed.

For poultry feed (Figure 2), if poultry DNA is detected, the feed will be declared as containing prohibited animal material due to the intraspecies recycling prohibition. If no poultry

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DNA is detected, the presence of ruminant DNA will have to be controlled. If ruminant DNA is present, the current analytical methods cannot sort out if this response is linked to an authorized or unauthorized material (or a mix of both). In such case, additional analytical solutions will be needed in order to determine the tissue or cellular origin of the DNA and confirm the absence of prohibited constituents of ruminant origin.

For pig feed (Figure 3), ruminant DNA would be controlled first with the same pathway as for poultry feed. If no ruminant prohibited materials are identified, the feed will have to be controlled for the presence of porcine DNA due to the intraspecies recycling prohibition. If no porcine DNA is detected, no other analysis is necessary and the feed will be declared free of prohibited constituents of terrestrial origin. However, if porcine DNA is detected, additional methods will again be needed: they will be required to determine whether the porcine DNA is due to the presence of porcine PAPs or porcine blood meal, both of which are unauthorized, or due to porcine blood products, which are authorized in feed for pigs. It is important to underline that, by contrast with the situation in aquafeed, whey powder and porcine plasma powder are frequently used in piglet feeds,[33] making additional analysis crucial in this case.

As described, the combination of LM and PCR methods allowed the reintroduction of nonruminant PAPs in fish feed while ensuring feed safety thanks to LM's capacity to discriminate tissue coupled with PCR's capacity to identify species. However, if the use of nonruminant PAPs in nonruminant feed is authorized again in the future, even with the addition of pig and poultry PCR tests, these two methods will be unable to differentiate between authorized products and unauthorized products. This means that, in some cases, it will be impossible to confirm that prohibited animal products are absent. Therefore, to meet these requirements, complementary methods need to be developed.

6. ALTERNATIVE METHODS ALREADY INVESTIGATED

Since the beginning of the feed ban relaxation, several methods have been investigated in order to address these analytical gaps. Apart from LM and PCR, most of the research focused on spectroscopic or protein-based methods. The advantages and disadvantages of the different approaches and combinations of them have been discussed in several articles or reviews.[16,20,23,25,30,34−36]

Spectroscopy techniques were among the first to be investigated, as they are nondestructive and widely used for in situ analysis in the agri-food sector. Among them, near-infrared (NIR) spectroscopy methods were the ones mostly considered in the context of PAP detection.[37,38] The principle of the technique is the measurement of the absorbance of NIR light by the sample. The obtained spectrum gives a spectral overview of the molecular composition of the sample. This technique has the advantages of being rapid, easy to use and without long sample preparation steps. The resulting disadvantage is that the spectral information from a given specific particle is diluted by the information on neighboring particles. This explains the excessively high limit of detection (LOD) of NIR spectroscopy methods, about 1% (w/w), which makes them impracticable in the context of the prohibition of ABPs.

NIR microscopy (NIRM),[39−44] NIR hyperspectral imaging,[45−47] and Raman imaging[48] were then studied. These techniques combine the advantages of microscopy and spectroscopy techniques and are based on the NIR spectral absorbance or Raman scattering signatures of individual particles. The spectral signatures are then compared to a library database using chemometric analysis. In contrast to microscopy, the result is therefore independent of the operator's interpretation. When these techniques are applied to the sediment part of the sample, a LOD of less than 0.1% (w/w) can be obtained. Even though these techniques can identify and discriminate terrestrial particles from fish ones, this distinction is not sufficient to control the correct application of the feed ban in the context of its future relaxation.

More recently, synchronous fluorescence spectroscopy (SFS) was used for the detection of hemoglobin in various animal feeds through the identification of a hemoglobin signature.[49] SFS is an interesting method to characterize proteins as it takes advantage of intrinsic characteristics of their amino acid composition: their fluorescence. The limit of detection of hemoglobin powder or blood meal ranged between 0.5% and 1% (w/w) depending on the feed material in which they are. Even if this approach could be useful as a screening method for the detection of hemoglobin in feed, the method, as it is currently proposed, is not applicable in the control of the feed ban because it cannot tell what the species of origin is.

Proteomics is the second strategy investigated. Proteomics is defined as the study of an organism's proteome, just as genomics studies its genome. The proteome is the set of all expressed proteins in a cell, tissue or organism.[50] The study of the proteome will reflect both the genome and the cells' environment as the gene's expression, and the post-translational modifications (PTM) of the proteins is influenced by various conditions such as the type of cells, the stage in the life cycle, or different environmental conditions. The two main techniques currently used in proteomics are based on immunoassays or mass spectrometry.

Immunoassays have been widely studied in the context of PAP identification.[51−56] These techniques are based on the specific detection of an antigen by the use of antibodies. As antigens are in this case proteins or peptides, they can be selected in order to obtain a tissue- and species-specific method, making these techniques theoretically well adapted to the specific detection of animal proteins. Moreover, immunoassays are rapid, easy and cheap methods and do not require a highly trained operator. However, the main disadvantage of immunoassay techniques is the sensitivity of proteins to denaturation by high-temperature processing. Under high temperatures, most of the original tertiary structure of the proteins is modified. Many epitopes recognized by antibodies on the native molecule are therefore lost. Hence, in the context of PAP detection, thermostable antigens capable of withstanding severe rendering conditions must be chosen. Unfortunately, to date, immunoassays developed for PAP detection have not been able to reach the LOD of 0.1% (w/w) while keeping a good degree of specificity. For the detection of blood-derived products in particular, specific studies have been conducted on the development of immunoassays targeting bovine thermostable blood proteins by Rao and Hsieh,[57] Ofori and Hsieh,[58] and Hsieh et al.,[59] but, as yet, no robust method is available.

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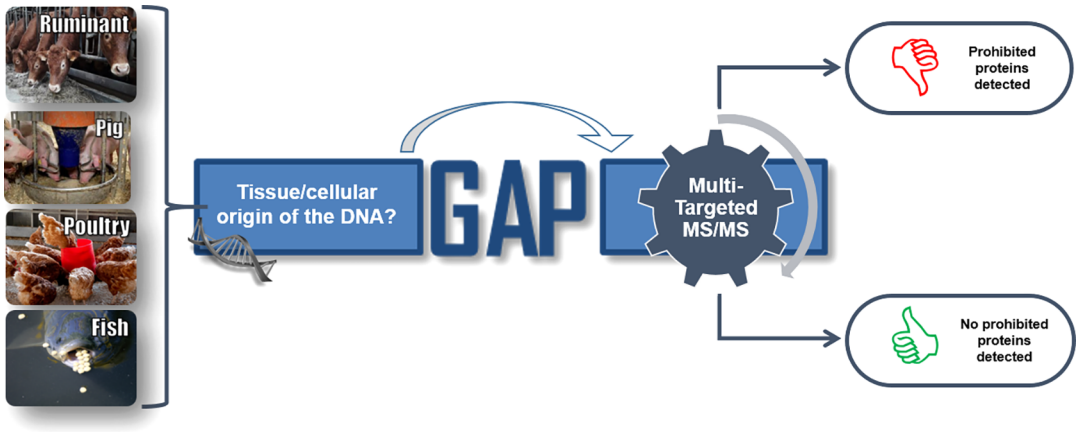

Figure 4. Resolving the analytical gaps by the use of a multitargeted MS/MS strategy for the determination of the tissue/cellular origin of the DNA.

Mass spectrometry (MS)-based proteomics is another protein-based method. Keeping the advantage of immunoassays regarding tissue and species specificity, this method bypasses the problem related to loss of conformation by focusing its detection on the mass-to-charge ratio (m/z) of its primary structure, the amino acid sequence. In the context of PAP detection, studies have initially focused on the identification of specific peptide biomarkers derived from the main PAP proteins:[60−63] myosin, troponin I, osteocalcin, collagen, and its hydrolyzed form, gelatin. In the last 2 years, the development of mass-spectrometry-based methods applied to PAPs identification has benefited from increased interest. Investigations were conducted for the development of targeted methods based on the detection of peptide biomarkers[64−71] or untargeted approaches using direct spectral library comparisons.[72] Generally, the 0.1% (w/w) level of detection was reached for the targeted MS approaches. The use of triple quadrupole mass spectrometers seems to be particularly adapted for use in routine analysis as this instrument is widely available in feed testing laboratories[73] and allows excellent analytic sensitivity for selected biomarkers.

7. INTRODUCING NEW FEED INGREDIENTS GENERATES NEW GAPS

Regarding the quest for protein source in feed, alternative sources have been considered for years by the industry and the authorities for sustainable and economic purposes. However, the introduction of new proteinaceous feed materials may also generate gaps in the current established analytical combination of methods, possibly even leading to more complex analytical schemes. The recent authorization of insect PAPs in aquafeed[18] illustrates perfectly this concern. Effectively, this introduction was supported by European authorities without beforehand having reliable methods for legal enforcement.[36,74] Therefore, this apparently minor change caused multiple problems of analyses and legal interpretation. For several reasons, the current combination of LM and PCR does not support the official controls that should be put into place for proper identification of insect derived proteins. First, classical tetrachloroethylene (TCE) sedimentation does not allow insect fragments to be concentrated because of their lighter density. To overcome this issue, a dedicated double sedimentation was recently developed[18] and validated.[75] Second, the validation study revealed that precise identification of insect PAP fragments requires new expertise to be gained by microscopists before enabling any legal implementation.[76] Third, as already mentioned, LM only authorizes the categorization of animal remains into "terrestrial animals" and "fish". The proper existence of only two categories will generate conflicting situations and lead to erroneous alerts from control authorities because it lacks taxonomic precision. In order to fix this, a third category, "terrestrial invertebrates," will need to be introduced into the legislation.[18] The introduction of such a third category will undoubtedly affect the current observation protocols and increase the workload. Therefore, conditions on when the presence of insects PAPs should be investigated must also be stated in the legal texts or the related SOP.[31] Fourth, since only a closed list of seven insect species is authorized so far, controls need to ensure the authenticity of species incorporated as feed ingredients.[18,74,77] In this respect, PCR methods offer complementary information for species determination and need to be applied. Although to date five insect species out of the seven authorized would be identifiable by specific DNA targets,[78−81] further developments and validations are still expected. The type of PCR technique used may also be questioned due to the multiplicity of targets that would be necessary, and so far real-time PCR has been commonly used but multiplex PCR for simultaneous detection is proposed,[81] provided thermal parameters of annealing for all primers can be encountered, which is an additional challenge to solve. However, even if the seven authorized species could be characterized by DNA-based techniques, the absence of unauthorized species remains to be proved. Whereas checking for the absence of ruminant DNA with a single target was eased by the low taxonomic level required (suborder), enforcement of control for the presence of unauthorized insect species will be challenging because of the high taxonomic level (class) and because of the omnipresence of insects in all environments and as a source of contamination. Therefore, alternative methods are developed for insect detection to complement the existing ones. NIR spectral imaging[82] could be used as a screening method based on the fatty acid profiles of insects against other PAPs from mammals, fish, or crustaceans. Mass-spectrometry-based proteomics, tested on several authorized species, successfully allowed specific discrimination,[83] although, for the future, dedicated spectral libraries still need to be created or completed for efficient data mining. As to reading, the single authorization of insect PAPs in aquaculture has created new

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analytical gaps, which, once filled by effective methods, will change the paradigm of official controls.

8. FUTURE PROSPECTS

This Review went through the present-day situation and the future challenges to ensure feed safety regarding the use of ABPs. In the context of a future relaxation, apart from the combination of the two official methods, at least a third method has proved necessary to discriminate the presence of authorized or prohibited feed material from the same origin. Several developments of analytical methods have been made recently for their detection. Currently, MS-based proteomics seems to be the most promising approach to solve the identified gaps. The use of a multitargeted MS/MS strategy (Figure 4) including multiple peptide biomarkers would allow application of it to the control of several animal ingredients or materials by the determination of the tissue/cellular origin of the DNA. Only the interpretation of the results would be adapted depending on the feed destination with respect to the regulation. The peptide biomarkers used could be selected by taking into account each regulation modification, resulting in an interesting flexibility of this analytical approach.

Looking to the example of aquafeed proposed in section 5, the presence vs the absence of prohibited materials and the origin of the ruminant DNA detected by PCR could be explained by a MS analysis using biomarkers specific of forbidden ingredients like blood products and PAPs.

Another reflection arising from this Review is that ABP regulations do not consider the analytical limitations. On the one hand, this is a good thing as it forces the analytical resource to constantly go beyond the limits, but, on the other hand, it also opens the possibility for fraud due to the lack of methodology. An adaptation of the legislation, while maintaining the maximum safety, but taking into account the analytical difficulties, could avoid many frauds. For example, a ban on the use of dairy products for fish, while the use of this kind of feed material is of no interest in this case, would simplify the analytical scheme for aquafeed. The argument of not being able to ban something nondangerous could be circumvented by the precautionary principle in order to avoid the presence of risk material. Restrictions regarding the use of porcine blood products in porcine feed would also make feed security easier. While maintaining the use of the porcine plasma powder in piglet feed, the prohibition of porcine hemoglobin powder would bridge the gaps. Indeed, hemoglobin peptides could be used in MS analysis to detect the presence of porcine PAPs or porcine blood meal while distinguishing them from the use of porcine plasma powder in pig feed.

Finally, another analytical way to guarantee the food and feed safety could be the direct prion detection. Novel approaches based on the amplification of prions have evolved considerably in recent years. These techniques exploit the ability of PrPsc to induce a conformational change of PrPc, so that small amounts of PrPsc could be amplified to a detectable concentration[84] by protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC). These methods are currently developed on a large range of tissues (e.g., brain, spleen), biological fluids (e.g., blood, urine, cerebrospinal fluid), and environmental materials (e.g., soil, grass, water)[85] and reach sufficient sensitivity for prion detection in blood in the asymptomatic phases.[86] Future research could lead to expanding the scope of these techniques to include feed analysis. These developments would be of particular interest in the context of controlling the removal of Category 1 materials (including SRM) from the food chain. As the detection of these dangerous materials is based on their marking with glyceroltriheptanoate (GTH), fraud consisting of the absence of marking makes them undetectable. The direct detection of prions would overcome this problem.

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Notes

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