COMPARISON AND COMPLEMENTARITY OF THE METHODS

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Content:

1. Introduction

In the previous chapters, the methods based on optical microscopy (OM), polymerase chain reaction (PCR), near-infrared spectroscopy (NIRS) and near-infrared microscopy (NIRM) were presented and discussed. Before comparing these methods, looking at their complementarities and proposing a global strategy for detecting banned animal by-products in feed, it is important to describe methods developed outside the framework of the STRATFEED project. The following sections present outlines of the immunological, chromatographic, NIR imaging, mass spectrometry and electronic nose methods.

2. Immunological methods

Immunological tests proposed in the application of the legislation banning animal by-products in feed are based on specific recognition through the antibody-antigen affinity. This method has been successfully applied for a long time for the analysis of raw and moderately cooked meat in food. Kits have been compiled to detect meat-and-bone meal (MBM) in compound feed, but they have limited application because of MBM heat treatment **[19]**. Ansfield **[1]** was the first to propose a method using antibodies thermo-stable antigens on heavily rendered material to detect animal species. This method, which included an extraction and purification step followed by a double-sandwich ELISA test, was patented and validated in-house **[2,3]**. There are some limitations for its routine application, particularly the interference from fats in compound feed.

Other approaches using immunological tests include: (i) a method using antibodies raised against heat-treated albumin **[18]**; and (ii) the dip stick method based on a lateral flow assay using antibodies to target heat-stable muscle protein (troponin I) conjugated into gold colloidal particles so as to facilitate the interpretation of the results. Dip stick kits have been compiled for detecting MBM in feed ingredients (with a detection limit of 5%) and in compound feed (a detection limit of 1%).

An intercomparison study for the determination of meat and bone meal in feed **[20]** comparing the performance of different analytical methods when applied to same samples revealed that the sensitivity of the currently available methods have been significantly improved. In particular, the sterilisation temperature of the meat and bone meal (MBM) that was considered until now a very critical factor does not pose a problem anymore. Depending on the target analyte and the applied immunoassay the achievable detection limit is between 0.1 and 0.5 % MBM in feed.

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 requires 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 also indicated that the methods need to be further improved in terms of sensitivity and specificity.

3. NIR imaging method

Since 2001, CRA-W has been developing a method based on the use of an NIR camera **[6,7,22]** to detect the presence of MBM in compound feed. An NIR camera (also called an 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. The NIR imaging method is still being developed, but initial results show that it has 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 **[14]**. Combined with the new chemometric method (involving SVM Support Vector Machines) as a classification algorithm, the NIR imaging method is very promising **[12,13].**

4. Chromatographic method

Recently, a method using high performance liquid chromatography (HPLC) to detect carnosin and related dipeptides (anserin, b-alenin) in animal tissue was proposed **[26]**. Aristosy *et al.* **[5]** described a method in which the sample preparation and manipulation steps were simplified. They subsequently demonstrated that this method has a detection limit of 0.5% **[4]**. Based on these studies, the quantification of and the ratio between dipeptides have been put forward as methods to determine the origin of animal protein **[27]**. Results from an intercomparison study **[20]** indicated that the achievable detection limit 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.

5. Mass spectrometry methods

Recently Ocaña et al. **[23]** reported on the use of gelatine as marker for the presence of PAPs in feed and the use of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS) as detection tools. The results confirmed the sensitivity of this approach, though the final check with feed adulterated with MBM needs still to be carried out. Major drawback of this approach is the lack of species specificity

6. Electronic nose method

The electronic nose is already a widely used analytical method in the food industry. Recently, a University of Milan research team 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 indicate that the electronic nose could be an interesting approach for screening raw materials in feed industry, but further studies on larger set of samples are needed. **[10]**.

7. Comparison of the methods

Table 8.1. summarises the main features of the OM, PCR, NIRS, NIRM, immunoassay and NIR-camera methods: (1) the analytical features; (2) possible interfering ingredients and processes; (3) species identification issues; and (4) miscellaneous features. The grey boxes indicate the main contributions that STRATFEED has made to the development of the various methods. The features of these methods were described by **[19]** in a chapter of the book published by OIE on risk analysis of prion diseases in animals. A comparison of the performance of the methods is also given in another publication **[19]**.

7.1. Analytical features

The main analytical features (1) covered in the table are the number of samples analysed by one analyst per day (1.1), the time needed to perform a complete analysis of one sample (1.2), the quantity of samples needed for the analysis (i.e., sampling, 1.3), the use of organic solvent or other reagents (1.4), the need for skilled and trained analysts (i.e., expertise, 1.5), the detection limit (i.e., LOD, 1.6), the percentage of false negatives (1.7) or false positives (1.8), the repeatability (1.9) and transferability of the method (1.10), the risk of contamination at laboratory level (1.11), and whether the method depends on the feed matrix used (1.12).

A comparison of the analytical features indicates that:

- the method with the highest throughput is NIRS;
- the OM and PCR methods require experienced analysts;
- the OM, NIRM and NIR-camera methods have a LOD in line with the legislation, while the NIRS method has a high LOD;
- the contamination risk at laboratory level is crucial with the PCR method

On analytical features point of view, the STRATFEED project has contributed greatly to developing tools (harmonised protocol, image database, decision-support systems) that have enhanced the performance (repeatability and transferability) of the OM, PCR and NIRM methods. The ARIES decision-support system provides a tool supporting the harmonised protocol developed in the Stratfeed project and gathering the data and skills needed to detect and identify animal-origin ingredients in compound feed using OM. The project has also contributed to an improvement in the performance of the NIRM method by lowering the LOD to the OM level, showing that the NIRM method gives equivalent results to OM.

7.2. Interfering ingredients and processes

The interfering ingredients and processes (2) that should be taken into account in developing a method to detect animal by-products are: the permitted animal products, including, milk (2.1), blood (2.2) and fat (2.3), the high-temperature heat treatments applied to animal by-products (2.4) and the size of the particles making up the samples (2.5.).

The presence of permitted animal products, and the use of processes that produce heat-treated materials do not affect the detection of MBM by OM. However, false positive results can be obtained using PCR if permitted animal ingredients are included in the compound feed. Obviously, this will depend on the quantity of DNA included in this material. For instance, the content of DNA in refined fat is very low and its use in the formulation of compound feed (e.g., 2–3%) does not automatically mean that PCR will detect the samples as positive. Technical limitations (instrument, manipulation) do not allow to acquire spectra of particles smaller than 20–100 µm using the NIRM or NIR-camera methods.

On interfering ingredients and processes point of view, the most important contribution of the STRATFEED project has been to develop an adapted protocol using PCR for the detection materials that have undergone high-temperature heat treatment (141°C). For the first time, the impact of the sterilisation temperature over a broad range was systematically investigated showing that this problem can be successfully addressed by selecting small DNA targets.
During the project it was also shown that this rendering process does not affect the detection of MBM by OM or NIRM.

7.3. Species identification issues

Current and proposed legislation requires the development of methods that can identify the species of the animal products present in samples (3). The ban that permits the use of fish meal for farmed animals, apart from ruminants, requires a distinction between fish ingredients and terrestrial animal ingredients (bovine, pig, ovine, poultry) (3.1). The objective of the legislation to prevent cannibalism in the use of animal by-products, because of its associated TSE transmission risk, requires developing methods that can identify the species of terrestrial animal protein sources; i.e., discriminate between poultry and other mammals (3.2) and between pigs and other ruminants (3.3).

All the methods were tested for their ability to discriminate fish material from terrestrial animal material. The results showed that the detection of MBM in presence of fish meal can be achieved using the OM, PCR, NIRS, NIRM, NIR-camera and immunoassay methods. The detection limit of MBM in presence of fish meal is generally increased, but the level of 0.1% can be achieved only in well-defined conditions.
On species identification issues point of view, the most important contribution of the STRATFEED project has been to develop PCR methods on-line with the legislation able to detect DNA from poultry, mammal, pig, ruminant and bovine.

7.4. Miscellaneous

For all the methods studied, the key points to take into account from an economic point of view when selecting a method are: the cost of the analytical instrument (4.10), the cost of analysis (4.11) and the cost of maintaining the existing facilities (4.4). From a legislative point of view, the validation of the method (4.3) is an important point. From a technical perspective, the key points are: the technical capacity to analyse the raw fraction (4.1.) or the sediment fraction (4.2.), as a confirmation method (4.6.), as a forensic method (4.5.) or as a quantitative method (4.7.). A last criteria is the additional information provided by the method (4.8.) which can be used for other topics. In infrared technology, the spectra can be used to identify other feed ingredients. In OM, the constituents analysed can be used for a study on the differentiation between muscle fibers, bone fragments, feathers, milk products. All these criteria are discussed in the following sections.

8. Strategy for detecting banned animal by-products in feed

One of the objectives of the STRATFEED project was to define strategies to detect MBM in feed based on an assessment of the advantages and disadvantages of the methods studied – microscopy (OM), spectroscopy (NIRS, NIRM) and molecular biology (PCR). No method fulfils all the requirements. It is necessary to combine several methods to evaluate the presence of MBM and identify the species origin. Moreover, several combinations of screening and confirmation methods can be proposed.

Currently, the OM method is the only official method for the detection of MBM. It has the advantage of having a low level of detection, few false negatives, very reliable results and heat insensitivity. However, it is based mainly on the presence of bone particles and further development is needed to improve species identification. The ARIES decision-support system developed within the framework of the STRATFEED project

provides a tool to help microscopists identify species based on morphological characteristics of particles of animal origin **[28]**.

To analyse large amounts of samples drawn from the huge mass of feed ingredients traded globally, a screening method is needed in order to detect suspect materials quickly. NIRS and immunoassay can both be used easily as screening tools. The advantages of these methods are the high number of samples that can be analysed per day, the low cost of analysis, the low level of expertise needed and the existence of appropriate facilities in the feed industry. If sensitivity and specificity requirements are fulfilled, immunoassay has an advantage over the NIRS method, which produces quite a high level of false negatives. However, both methods have a higher LOD than OM.

The micro-NIRS methods (i.e., NIRM and NIR–camera) are new methods that are able to detect and quantify low levels of added MBM. They combine the analytical advantages of microscopy and spectroscopy (i.e., the low level of expertise needed, as in NIRS, and a detection limit lower than 0.1 %, a low level of false negatives, high repeatability, and independence regarding the matrix used, as in OM). The main advantage of these methods is that they take into account, without additional analysis work, all the constituents of MBM (bones, muscles, etc.).

The PCR method is also a more recent method. It is able to make reliable identifications not only at species level (bovine, poultry, pig), but also at a higher taxonomic level (ruminant, mammalian). It is particularly useful in preventing cannibalism (i.e., controlling banned intra-species recycling). Using fluorescent dyes and silent quenchers for the probes, it would also be possible to check for the presence of several species simultaneously using real-time PCR in the same vial. However, with PCR there is a higher risk of contamination and possible interference with other animal species DNA sources that may well occur in milk, blood and fat pooled from multiple species aggregation.

Researchers at CRA-W demonstrated a strategy combining NIRM and PCR for the detection and species identification of MBM particles. It consists of analysing feed particles first, using NIRM. As NIRM is a non-destructive method, the particles classified as animal origin can then be selected and analysed by PCR to confirm their animal origin and determine the animal species origin. The combination of the two techniques could improve the advantages and reduce the disadvantages of each method. **[15].**

Another strategy is to combine microscopy as screening method for its very low level of false negatives, followed by PCR or immunoassay for confirmation and further identification of exclusively the positive samples. OM is limited in the differentiation of vertebrate classes (mammal, poultry) and depends primarily on the presence of bone fragments. In some cases, the poultry meal can hide the presence of mammalian meal. In those cases, PCR can answer the need of species specific control method required by the ban of intra-species recycling.
An extended strategy is to use PCR analysis for the analysis of exclusively the sediment fraction as help to OM to identify the animal species present. The analysis of the sediment fraction composed mainly of animal materials tends to increase the sensitivity of PCR to 0.1%, but this needs to be checked with a larger set of samples **[16]**.

	Optical microscopy	PCR	NIRS	NIRM	Immunoassay	NIR-camera
1 Analytical features						
1.1 Samples/day	10–15	5–10	100–200	3–5	100–200	?
1.2. Analytical time/sample	45–60 min	2 days	10 min	2 hours	30 min	1 hour
1.3. Sampling	5–10 g	0.1–1 g	5–100 g	0.2–10 g	10 g	0.2–10 g
1.4. Reagent	yes	yes	no	yes	no	yes
1.5. Expertise	yes	yes	no	no	no	no
1.6. LOD	<= 0.1%	+- 0.5%	3–5%	<= 0.1%	+-0.5%	<= 0.1%
1.7. False negative	< 5%	< 5%	> 5 %	< 5%	< 5%	< 5%
1.8. False positive	< 1%	< 1%	< 5 %	< 1%	< 1%	< 1%
1.9. Repeatability	high	medium	medium	high	high	high
1.10. Transferability	high	medium	high	high	high	?
1.11. Contamination risk	low	high	low	low	low	low
1.12. Matrix dependent	no	yes	yes	no	no	no
2 Interfering ingredients or processes						
2.1. Milk	no	yes	?	no	yes	no
2.2. Blood	no	yes	?	no	no	no
2.3. Fat	no	yes	no	no	yes	no
2.4. Heat -treated material	no	no <141°C	no	no	no<141°C	no
2.5. Particle size	no	no	no	yes	no	no
3 Species identification issues						
3.1. Terrestrial animal *vs* fish	yes	yes	yes/no	yes	yes	yes
3.2. Mammal *vs* poultry	Partly yes/no	yes	no	no	yes	no
3.3. Ruminant *vs* pig	no	yes	no	no	yes	no

4 Miscellaneous						
4.1.Analysis of the raw fraction	yes	yes	yes	yes	yes	yes
4.2.Analysis of the sediment fraction	yes	yes	no	yes	no	yes
4.3.Validated method	yes	no	no	no	no	no
4.4.Existing facilities	yes	yes	yes	no	yes	no
4.5.Forensic value	yes	yes	no	?	no	?
4.6.Confirmation method	yes/no	yes	no	yes	yes/no	yes/no
4.7.Quantitative method	yes(estimation)	no	yes	yes	no	yes
4.8.Additional information	yes	no	yes	yes	no	yes
4.9.Cost instrument	€20,000	€70,000	€60,000	€80,000	0	€160,000
4.10.Cost/analysis	€50–100	€150–200	€2–5	€50–100	€20-40	€50–100

Table 8.1. Main features of the OM, PCR, NIRS, NIRM, immunoassay and NIR-camera methods in the detection of MBM in compound feed.

9. Conclusion

The comparison table 8.1 shows (grey boxes) the progress made by the STRATFEED project in assessing the four methods. It is clear that the methods are complementary and offer laboratories various possibilities, depending on their facilities, expertise, financial resources, the purpose of the analyses and the level of processing of the feed matrices.

However, all four methods need improvement and validation.
With PCR, further in-house validation is needed before starting collaborative studies.
With NIRS, the results suggest this method could be used in a major collaborative study for detecting MBM in compound feed once its validation protocol has been refined and agreed by laboratories with NIRS expertise in compound feed analysis **[24]**.
With NIRM, the method was successfully transferred to the JRC **[8]**. The constraint in developing this method is the current lack of NIRM systems in European laboratories.

The future re-introduction, under strict conditions, of various animal by-products into animal feed formulations has been anticipated by several research laboratories.
Studies are undertaken to develop an NIRS method to detect MBM in hydrolysed feather protein **[17]**. It is also studying glyceroltriheptanoate as a marker for category 1 and 2 animal by-products **[21].**
Study aims to adapt NIRM and NIR-camera methods to identify and quantify a wider range of animal feed ingredients (e.g., blood, milk by-products, feathers). It is also looking at the use of FT-IR and PCR to discriminate species by animal fats. The spectroscopic methods, especially the middle infrared, could be used to discriminate animal fats, with PCR used as a forensic method for confirmation. **[25]**.

A NIRS method is also developed for the traceability of animal and vegetable fats and oil. **[9]**

Beside the Stratfeed project results, those perspectives of research were showed through lectures, posters and exhibition sessions planned during the International symposium entitled “Food and feed safety in the context of prion diseases“ organised in Namur (Belgium) on the 16-18th June 2004 to close the STRATFEED project. All the slides presentation, posters and abstracts are available on http://STRATFEED.cra.wallonie.be.

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