THE VALIDATION OF THE MICROSCOPIC METHOD SELECTED IN THE STRATFEED PROJECT FOR DETECTING PROCESSED ANIMAL PROTEINS (WP7)

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1 Introduction

Before using an analytical method developed in a research project for routine purposes, it is necessary to show that it is fit for its intended purpose through a validation process. In a validation study, experiments are conducted to assess the performance characteristics of the method. There are various ways of performing a validation study, such as a single-laboratory validation or an intercomparison study involving many laboratories. Based on the results of these trials, it is decided whether the method is suitable or needs to be further refined before being subjected to a new validation study. In the STRATFEED project both situations had to be dealt with.

The objective of this paper is to describe the basic aspects of validation studies, focusing mainly on intercomparison studies. The paper will therefore present the results of the recent intercomparison study of the microscopic method, developed in the project, which complies with the official EU method [**6**]. The results from other intercomparison studies for the detection of meat and bone meal (MBM) in feed will also be summarised [**10, 15**]. The objective of the study [**10, 12**] conducted on behalf of the European Commission's DG Health and Consumer Protection (DG-SANCO) was to evaluate the proficiency of European control

laboratories and compare the various analytical methods applied to the analysis of feed samples. This study was not part of the STRATFEED project but its results were crucial for the design of the recent validation study for the refined microscopic method. A third study [**15**] was focused on the determination of MBM at 0.1% in the presence of fish meal, and the results of this exercise were also essential for evaluating the outcome of the STRATFEED validation study.

When organising intercomparison studies, the preparation of well-characterised test material is pivotal. The test material used in validation studies should reflect typical characteristics of real world samples and needs to be homogenous enough for the intended use, ensuring that each laboratory performs the experiments on samples containing a similar amount of MBM. This paper will therefore also describe how samples meeting these criteria were prepared in this project.

2 Intercomparison Comparative studies

This section discusses the most important aspects of organising intercomparison studies and provides general information on the objectives of intercomparison studies, as well as on the specific target of the validation of the microscopic method presented here.

2.1 The objective of the method validation

Before conducting method validation studies, it is important to address some issues, as outlined in **Figure 1**.

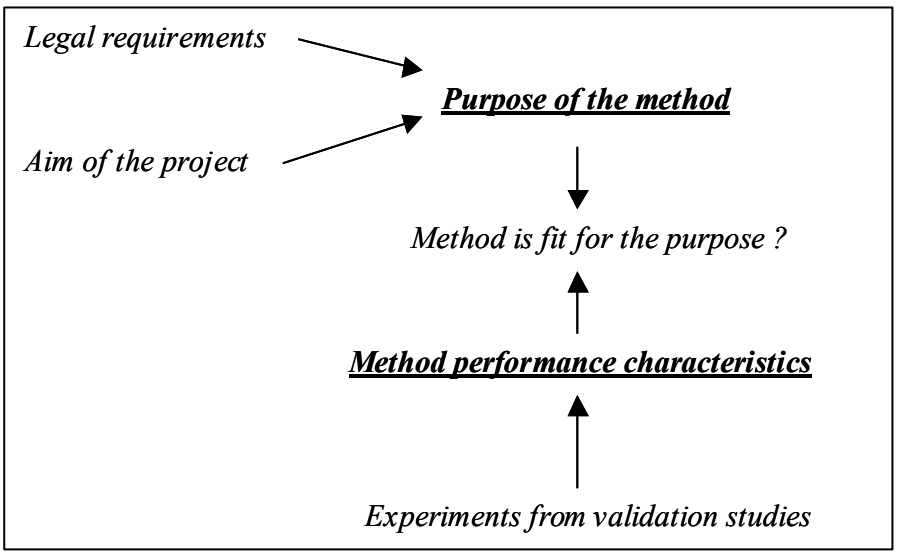

Figure 1: Structure of a validation study

Since the objective of method validation is to establish whether a method is fit for its intended purpose, this purpose needs to be defined. The primary aim of the analytical methods in the present case is evident in the title of the STRATFEED project, the aim of which is to develop methods for detecting mammalian tissue. However, it is also important to take into account the requirements of European legislation introduced in 2000[1] imposing a total ban on feeding ruminants with processed animal proteins (PAPs) whatever the origin of those PAPs. The legal situation is different for non-ruminants. Future

[1] The total MBM ban has been introduced after the approval of the STRATFEED project and could therefore not be taken into account in the design of the project.

legislation depends on the capacity of analytical methods to determine the animal species in PAPs. This aspect will be discussed in chapter 3.

Irrespective of whether the method is used to detect PAPs from terrestrial animals or exclusively from mammals, the ban imposed under European legislation does not envisage a legal limit. Thus, the presence of bone particles indicates an infringement of European Union (EU) legislation, whatever the actual concentration of MBM in the feed is. The purpose of the method is therefore to establish the presence of PAPs; a quantification of PAPs is not required. When validating qualitative methods, the most important performance characteristics are sensitivity, indicating the achievable detection limit, and specificity, giving information on false positive results. These statistics will be discussed in paragraph 5.3.

In this study, 0.1% of MBM in feed as a target concentration was selected as required by European legislation establishing microscopy as official control method [**6**]. In addition, a concentration level of 0.5% was included in the STRATFEED validation study in line with a recommendation by the DG-SANCO's Scientific Steering Committee that cross-contamination with mammalian MBM (MBMB) should be condemned at a level above 0.5% [**14**]. In order to establish whether the microscopic method [**6**] is fit for this purpose, laboratories were asked to analyse samples and report the presence or absence of MBM to the organiser of the intercomparison study. The fraction of positive results at the target concentration was calculated, as well as the fraction of negative results in the case of the blank samples. In both cases, the fraction should be above 95%. Based on the calculated method performance characteristics it can be decided whether the method is fit for its intended purpose.
In particular, the aim of the validation study was to establish the performance characteristics of the microscopic method as modified in the STRATFEED project when used for the following purposes:

- Determination of terrestrial (specifically mammalian) MBM (MMBM) at a concentration level of 0.1% in compound feed, and in compound feed with fish meal
- Determination of MMBM in compound feed containing poultry meal and compound feed containing fish meal and poultry meal

An additional objective was to test whether the decision-support system ARIES, which was developed under the STRATFEED project and contains explanations on the microscopic method and many figures of particles specific for PAPs, would help laboratories identify MBM in feed.

2.2 Validation study versus proficiency test

In an intercomparison study, laboratories analyse the same batch of blind samples and report the results to the study organiser. The results are then subjected to statistical analysis.

The intercomparison studies conducted in this field had two purposes: the STRATFEED study sought to assess *method* performance characteristics, whereas the DG-SANCO study focused mainly on the *proficiency* of European control laboratories. In the former case, the laboratories had to apply a specific protocol; in the latter, they could use whatever method protocol they wanted to. The major differences of these approaches are summarised in **Table 1**.

Table 1: Comparison of validation study and proficiency test.

	Validation study	**Proficiency test**
Purpose	Assessing method performance characteristics	Assessing the proficiency of the laboratories
Analytical method	All laboratories apply the same method	The laboratories are free to use a method protocol of their choice
Extreme values	Extreme values need to be eliminated if justified	No elimination of extreme values
Principle of statistical analysis	Pooling results from all laboratories for each sample type	Pooling results from all sample types for each laboratory

Since the objective of the current study was to assess method performance characteristics it was necessary to be sure that the participating laboratories were experienced enough in microscopic analysis and that they knew the modified microscopic method. Therefore, a training period was included in the study. During this stage, the laboratories had to analyse samples and report the results to the study organiser. Thus, it was established whether each laboratory was meeting the basic criteria in order to include them in the validation study. More details of the training period are given in section 5.5. After the training phase, the samples for the validation study were sent out.

3 The meat and bone meal ban under European legislation

In the EU the use of PAPs is governed by two regulations. The first is the "animal by-product" (ABP) Regulation (EC) 1774/2002 [**7**], which prohibits feeding animals with proteins from the same species and establishes three categories of ABPs according to their safety risk to the food chain. Only material from category 3 can be used for feeding farmed animals, since this class poses the lowest risk. The second is Regulation (EC) 999/2001 [**8**], which explicitly prohibits the feeding mammalian PAPs to ruminants. However, various problems such as the lack of appropriate methods to detect mammalian PAPs in the presence of PAPs from other animals led to the introduction of a temporary MBM ban for all farmed animals (total MBM ban) in 2001. The temporary MBM ban recently became a permanent one under amending [**9**] Regulation (EC) 999/2001. It is important to note that the revised regulation clearly indicated the need for a reappraisal of the total MBM ban once new and more specific control methods were available. For instance, reliable methods for detecting MMBM in feed in the presence of fish meal could be the justification for lifting the ban on fish meal as an ingredient in feed for ruminants. The only official EU method is microscopy as described in Commission Directive 2003/126/EC [**6**].

4 Characterisation of the test material

An important aspect of organising an intercomparison study is the preparation of suitable test material. This material has to meet specific criteria in order to ensure that the right conclusions can be drawn from the results of the study. In the current study, it was necessary to select appropriate feed to which the MBM was added and to establish the type of MBM used for the test material. Given the low target concentration level of MBM in feed, it was also important to use a preparation procedure that allowed for a homogeneous distribution of the MBM particles in the test material.

4.1 The feed

The test material prepared for the intercomparison study was typical compound feed, both with and without traces of MBM. Compound feed is a mixture of various ingredients such as grain, food industry by-products, minerals and feed additives. Since these compounds can interfere with sample analysis, it is important to select typical material produced under real world conditions. For example, the microscopic analysis focused on the sediment of the test material, which contained higher density particles, such as bones. Minerals and other sediment constituents that do not indicate the presence of PAPs can interfere with the identification of bones and are therefore a challenge for the microscopic method. If they are not present in the compound feed, the analysis might be too straightforward and therefore not mirror a typical situation in a routine laboratory. In the STRATFEED study, the test material was compound feed with about 1.6% sediment and was prepared in the dedicated pilot plant at NUTRECO, a feed company and one of the partners in the STRATFEED project. The composition of the compound feed is shown in **Table 2**.

Table 2: Composition of the compound feed

Raw material	Mass fraction (%)
Wheat	8.0
Corn	2.0
Corngluten feed	22.0
Soyabean meal	1.2
Rapeseed meal	8.0
Palm kernel meal	16.0
Coconut meal	9.0
Beet pulp	13.0
Citrus pulp	10.0
Molasses	10.0
Vegetable fat	0.2
Magnesium oxyde	0.4
Vitamins/mineral mixture	0.2

4.2 Meat and bone meal (MBM)

The two MMBM samples (MMBM 134°C and MMBM 127°C) used in the DG-SANCO study were produced in the same commercial rendering plant, using the batch type system described elsewhere [**16**]. They contained equal portions of porcine and bovine material and the proportion of bones was about 12.7% and 10.5%, respectively. The MMBM used in the STRATFEED validation study was also a mix of bovine and porcine processed material, with a bone content about 12%. The MMBM feed ingredients were analysed by polymerase chain reaction (PCR) in order to check the species present in the sample, confirming that these samples contained mainly bovine and porcine materials, as well as low amounts of poultry material.

The fish meal was obtained directly from a fish meal producer and its proportion of bones was about 12%. The poultry meal was obtained from a pilot plant and was produced from poultry by-products; its proportion of bones was about 3.3%. The fish meal and the poultry meal were analysed by PCR to check the species composition of the samples. The results indicated that the fish meal did not contain bovine, porcine or poultry materials and that the poultry meal did not contain bovine and porcine materials.

4.3 Preparation of the test material

4.3.1 Some theoretical considerations

Prior to selecting a suitable sample preparation technique, the intended use of the test material, especially in terms of required homogeneity of the samples, needs to be specified. In the STRATFEED study the samples were used exclusively for validating the microscopic method in line with European legislation. Major aspects to consider were that the achievable detection limit was at least 0.1% MBM in feed and that *qualitative* analysis was more important than *quantitative* analysis, in the context of the EC imposing a total ban of MBM, without setting a legal limit (as noted in section 2.1). Therefore, the test material could be considered as homogenous enough for the purpose of the study if each sample item contained enough particles at the target concentration level to be detected by microscopy.

It was necessary to take into account the following aspects when considering how to prepare the samples and the degree of homogeneity to be achieved:

1. MBM is a mixture of particles of various sizes, with a considerable portion larger than 100 μm.
2. Microscopy can detect MBM particles above 50 μm, but there is a risk that smaller particles will remain undetected, especially when the MBM is present in feed at a very low concentration level. In addition, microscopy focuses primarily on the sediment of the samples, which contains the bones. In the STRATFEED study MBM with a bone fraction of about 12% was used. The presence of muscles in the 'sieve' fractions helps detect the presence of MMBM.
3. Grinding the feed samples needs to be carried out by the laboratories and should not be done by the study organiser when preparing the samples.
4. The sample amount was set at 10 g. The laboratories were advised that the homogeneity was only ensured *between* the samples and not *within* the individual samples, and they therefore had to subject the whole sample to analysis. It should be noted that the official method requires using at least 5 g for the sedimentation and 5 g for the producing the sieve fractions.

A closer look at the composition of the test materials shows that the samples containing 0.1% MBM could be considered the most critical in terms of the equal distribution of the MBM particles between samples. This concentration corresponded to an absolute amount of 10 mg of MBM in 10 g of the samples, and we needed to establish the number of MBM particles present in the samples. In principle, a high number of particles (e.g., above 1.000) would make a homogeneous distribution of the MBM in the test material more likely, whereas a low number of particles (e.g., below 100) would make it more difficult. Particle size analysis of the MBM revealed that a typical particle was about 200 μm to 500 μm long. It was assumed that the MBM contained only this particle range, although we were aware that real world MBM contains particles that are smaller and others that are larger than this range. Counting the number of particles of this range revealed that 10 mg contained about 350 particles. The number of bones particles, however, was only about 45, since the bone fraction of the MBM used was 12%. The major challenge in preparing the test material was therefore to distribute the MBM particles among the sample items, ensuring that each item contained enough particles for microscopic analysis. One option was to fortify each sample containing pure compound feed with 10 mg of MBM, but this strategy was not feasible because of the huge amount of work required to weigh 10 mg of MBM for each individual sample. The test materials

were therefore prepared by mixing the compound feed with MBM to produce homogeneous bulk material. In a second step, subsamples of 10 g were randomly taken from the bulk material and put into sachets. This procedure is illustrated by MAT 6 (**Table 4**), which contained 0.1% MBM. By taking into account the required number of samples, 1000 g of compound feed with 1 g of MBM, corresponding to about 4.500 bone particles, had to be prepared so that each sub-sample of 10 g contained about 45 particles.

In assessing the achievable between-sample homogeneity, two major sources of error were considered, stemming from the mixing procedure (step 1) and the sub-sampling of the 10 g samples (step 2). To assess the influence of the sampling error relating to step 2, it was assumed that the prepared test material had a completely random distribution of the MBM particles in the feed. A random distribution means that the chance of finding 45 MBM particles in a sample would be the same irrespective of the place in the bulk material from which the sub-sample was taken. According to Cochran [**2**], the Poisson distribution can be used to calculate the confidence limits, since the fraction of MBM particles in the feed is extremely small. Applying the Poisson distribution showed that the actual number of particles in the samples varied from 32 to 60 at a confidence level of 95%. It is important to note that this range corresponded to perfect mixing of the bulk material, whereas under real world conditions the range of the number of particles in the individual samples would probably be higher. Error arising from imperfect homogeneity adds to sampling error. Thoroughly prepared test material is therefore extremely important if the samples are to contain a similar number of MBM particles.

4.3.2 Composition of the test material

Two batches of test material were prepared. Batch A (see **Table 3**) contained samples for the training period, during which the participating laboratories could also analyse known samples and thus became familiar with the modified method protocol and learned how to use the decision-support system ARIES.

Table 3: Composition of the samples for the training period (Batch A)

Code	Label	Description*	Samples per laboratory
A	Known	MMBM 0.5%	1
B	Known	MMBM 0.5% + fish meal 5%	1
C	Blind	Fish meal 5%	1
D	Blind	Blank	1
E	Blind	MMBM 0.5%	1
F	Blind	MMBM 0.5% + fish meal 5%	1
G	Blind	MMBM 0.5% + poultry 5%	1

**MMBM = mammalian meat-and-bone meal*

For the actual validation study, a different sample design was selected to validate the method according to the objectives described in section 2.1. The composition of the samples in this batch (Batch B) is shown in **Table 4**. All the samples were sent out as blind replicates.

Table 4: Composition of the samples for the validation study (Batch B)

Code*	Description	Samples per laboratory
MAT 1	Blank compound feed	4
MAT 2	Mat 1 with poultry meal 5%	3
MAT 3	Mat 1 with fish meal 5%	4
MAT 4	Mat 1 with MMBM 0.1% + 5% fish meal	4
MAT 5	Mat 1 with MMBM 0.5% + 5% poultry meal	3
MAT 6	Mat 1 with MMBM 0.1%	3
MAT 7	Mat 1 with MMBM 0.5% + 2.5% poultry meal + 2.5% fish meal	3

** MAT = Material*

4.3.3 The mixing procedure

In order to obtain enough homogeneity, the materials were prepared by a stepwise dilution of the MBM with the blank feed, ensuring that in each dilution step the mass ratio of the two materials to be mixed, did not exceed a factor of 3. Depending on the target level of the PAP concentration, the procedure included between 4 and 10 steps. For example, MAT 6 (0.1% MMBM) was prepared by mixing 1 g of pure MMBM with 3 g of compound feed (MAT 1), obtaining 4 g of the MBM/feed mixture. In the second step 4 g of MAT 1 was added, followed by extensive mixing. Since the amount from the second step was 8 g, the same amount of MAT 1 feed was added, obtaining 16 g of the mixture. This procedure was repeated until 480 g of the material had been prepared, and this was then mixed with 520 g of MAT 1, to produce, finally, 1000 g of MAT 6 containing exactly 0.1% of MMBM. At each dilution level the materials were mixed for 30 or 60 minutes. Aliquots of 12 g of the test material were filled immediately after the preparation of the mixtures in small plastic-aluminium bags.

Sufficient homogeneity of each material was established by analysing 10 randomly selected bags using near-infrared microscopy (NIRM) as described by Baeten *et al*. [**1**] This method is based on analysing several hundreds of particles of the sediment fraction using an NIR spectrometer linked to an adapted microscope. A sedimentation step was performed to remove the major part of the unspecific feed matrix, thereby allowing the NIRM analysis to focus on particles more likely to indicate the presence of MBM. MBM particles were then unequivocally identified by their specific IR-spectrum. By analysing 10 g of the test material from several sachets each containing 12 g of the test material, only between-sample rather than within-sample homogeneity was evaluated. The laboratories participating in the study were therefore advised to use the whole sample for analysis or to apply appropriate sub-sampling when requiring less test material for conducting the experiment. The results from the homogeneity study confirmed that there were enough MBM particles in all the samples. This also applied to MAT 6 which contained only 0.1 % MMBM.

5 The STRATFEED validation study

5.1 The organising team

The study was conducted jointly by the Institute for Reference Materials and Measurements (IRMM) of the EC Joint Research Centre (JRC), the Walloon

Agricultural Research Centre (CRA-W) and the Institute for Food Safety (RIKILT). It was coordinated by IRMM.

5.2 Outline of the study

Before conducting a validation study of the microscopic method, it was important to establish that the proposed method was ready enough to be validated by an intercomparison study. Generally, this is done by subjecting the method to in-house validation, including a test for robustness. The method had already been tested by several laboratories both in and outside the STRATFEED project, and the protocol had been shown to be robust. Therefore, an in-house validation was not necessary.

Since the laboratories had to apply a specific microscopy protocol, a training period to familiarise them with the new protocol was organised. To ensure coherence with European legislation [**6**] the following modifications to the STRATFEED protocol were made [2]:

- In addition to an electric mill with specific requirements, a simple mortar could be used, especially for samples with a high fat content.
- Sedimentation in an open beaker was allowed in addition to a sedimentation funnel.
- The use of immersion oil as well as an embedding agent was acceptable, resulting in a range of media with more diverse viscosity

A letter of invitation was sent on the 1st July 2003 to 37 laboratories in 17 countries asking if they were interested in participate in the study. On 21st October 2003 CRA-W sent Batch A (a set of 7 training samples) to 31 laboratories. The samples contained typical compound feed (**Table 2**) fortified with PAPs from various species at different concentration levels, as shown in **Table 3**.

The laboratories were asked to report on the two main parameters to be used for validating the method, and to assess the method performance characteristics. The two parameters were (1) PAPs derived from mammals and/or poultry (terrestrial animals) and (2) PAPs derived from mammals. Two additional parameters were considered in the evaluation of the participants' results: (3) PAPs derived from poultry (avian) and (4) PAPs derived from fish.

As the microscopic method produces only qualitative data, the laboratories had three options for reporting their results: (1) present (2) not present and (3) no results. Option 3 corresponded to inconsistent results, or was used to indicate that a laboratory was unable to assess a particular parameter (e.g. PAPs from mammals). For instance, laboratories that could not confirm the presence of PAPs derived from mammals when they confirmed the presence of bones from mammals or poultry.

In order to study the applied protocol more carefully, the laboratories were also asked to indicate the sediment percentages of each sample.

Part of the study was a test of the ARIES system. This computer program supports the process of sample treatment and evaluation, and the identification of collected particles. It helps to discriminate between animal particles and confusing plant material, between terrestrial animal material and fish material and between mammalian and poultry material. A special web connection with the server in Wageningen (RIKILT) was established for the participants using ARIES.

In order to handle the great amount of information (more than 3.100 data: 33 laboratories x 24 samples x 4 parameters), a special Excel worksheet was designed to process the results submitted by the participants. The Excel sheet also allowed for an automatic identification of the

[2] The recent modification of the EU protocol for the microscopic identification was mainly based on the results of the STRATFEED project. However, after the consultation of all national representatives, deviations to the STRATFEED protocol have been introduced as they are applied in different European countries.

sample codes and an automatic calculation of the performance characteristics. When compiling the Excel sheet, a table summarising the results was automatically established. This summary table, from each laboratory, was used for the analysis of results. To obtain information on how strictly the laboratories followed the protocol, a list of questions mainly about sample preparation and the use (or not) of ARIES was included in the Excel file.

The participant laboratories received, with the samples, a detailed protocol on the classical microscopy method developed by the STRATFEED project, a copy of the manual for using ARIES and the Excel file for reporting results.

All the results were submitted by 10th December 2003, using the Excel file and ARIES. In addition, the laboratories gave specific information about their experience when using the STRATFEED protocol to conduct the analyses.

Based on these results, the final selection of laboratories to participate in the study was made. More detailed information on the results of the training is given in paragraph 5.5.

On 7th January 2004, CRA-W sent the 24 Batch B samples (see **Table 4**) to the 33 laboratories taking part in the validation study. All laboratories received the samples before 15th January and had 3 weeks to analyse them before submitting the results to the JRC following the procedure used in the preceding round. The results reached the JRC between 22nd January and 16th February 2004.

5.3 Statistical analysis of the results

For binary results (yes/no, positive/negative, etc.), standard statistics relate to accuracy, sensitivity and specificity. Accuracy is the fraction of correct results, positive or negative, calculated as

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

where *PA* is the number of correct positive identifications (positive agreements), *NA* the number of correct negative identifications (negative agreements), *PD* the number of false positives (positive deviations) and *ND* the number of false negatives (negative deviations). The statistics can be presented as fractions or as percentages after multiplication by 100.

Sensitivity is the ability of the method to detect PAP when it is present in the samples. Specificity is the ability to show, correctly, the absence of PAP in the blank samples. The following equations were used:

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

Accuracy is numerically identical to either specificity or sensitivity, depending on the test material and the specific PAP sought, since either the number of false negatives (*ND*) when the feed is not spiked with PAPs, or the number of false positives (*PD*) when PAPs are deliberately added, is equal to zero. For evaluating the various treatments, the statistics can generally be referred to as 'accuracy'.

In the raw results, at the level of distinguishing the terrestrial animal classes (mammals and poultry) there were a lot of 'No results' reported. For calculating accuracy, this would greatly influence the calculated statistics. To get comparable results, a modified equation – *adjusted* accuracy – was used:

$$\text{adjusted Accuracy } AC' = \frac{PA + NA}{PA + ND + PD + NA + mv}$$

where *mv* is the number of 'No results'. The denominator is equal to the total number of data points for a specific treatment.

The 95% percent confidence limits were calculated, based on a Bernoulli distribution (equals a Binomial distribution with n=1). The values for *PA* or *NA* and for *n* were used to calculate, using the cumulative sampling distribution, the limits for *pi* = 0.025 and *pi* = 0.975, which are limits of the 95% confidence interval.

The validation study included 7 materials (**Table 4**) and 4 parameters (PAPs at the various levels), resulting in 28 test material/parameters combinations. The accuracy was calculated for each combination.

5.4 Overview of the results

The overview of the results presented in this section does not take into account the individual capability of the participating laboratories. The assessment of the method performance characteristics requires that the results from laboratories which were shown not to be experienced enough to conduct the microscopic analysis were excluded from the statistical evaluation. A detailed description of the valid results produced by the competent laboratories and the final calculation of the method performance characteristics are given in paragraph 5.5.

On the report sheet, the participants were asked whether they followed the protocol strictly and used the decision-support system ARIES during identification of the samples. With regard to the protocol, 26 of the 33 laboratories had followed it; five laboratories gave various reasons for not applying it and two did not give any information on it.

With regard to the use of ARIES, 14 had used it during the identification process, while 17 reported that they had not, for various reasons, and two laboratories did not provide any information on using ARIES. We will elaborate on the participants' use of the protocol and of ARIES in section 5.5.

Table 5 and **Table 6** summarise the results submitted by 33 laboratories for seven materials according to the two parameters – terrestrial animals and mammals.

The detection of PAP of different origin and at different concentrations is sufficient to enforce the total ban of PAP in ruminant feed. The results for the terrestrial animals parameter (**Table 5**) showed that the ability of the microscopic method to detect the presence of PAP in animal feed was generally good. For MAT 1 and MAT 3, and for MAT 4, there was a notable number of false positives or false negatives, respectively, partly because some participants did not follow the method protocol. The number of reported 'No results' was very low. In almost all cases the participants seemed confident enough to draw a conclusion.

Table 5. Results for the parameter PAPs from terrestrial animals. Total observations (n); present (P), not present (NP) and no result (NR). Correct result in bold.

PAPs from terrestrial animals					
MAT	**Composition**	**P**	**NP**	**NR**	**n**
1	Blank	20	**109**	3	132
2	Poultry meal 5%	**94**	5	0	99
3	Fish meal 5%	24	**106**	2	132
4	MMBM 0.10% + 5% fish meal	**91**	40	1	132
5	MMBM 0.5% + 5% poultry meal	**97**	2	0	99
6	MMBM 0.10%	**94**	5	0	99
7	MMBM 0.5% + 2.5% poultry + 2.5% fish meal	**97**	2	0	99

For the parameter PAPs from mammalians as shown in **Table 6**, the high number of false positives when reporting on MAT 2 (poultry) and the high number of 'No results' demonstrated the limitations of the microscopic method to discriminate between mammalian and poultry materials.

Table 6. Results for the parameter PAPs from mammals. Total observations (n) present (P), not present (NP) and no result (NR). Correct result in bold.

PAPs from mammals					
MAT	**Composition**	**P**	**NP**	**NR**	**n**
1	Blank	11	**113**	8	132
2	Poultry meal 5%	34	**33**	29	96
3	Fish meal 5%	13	**109**	10	132
4	MMBM 0.10 % + 5% fish meal	**55**	49	27	131
5	MMBM 0.5% + 5% poultry meal	**61**	9	26	96
6	MMBM 0.10%	**57**	12	29	98
7	MMBM 0.5% + 2.5% poultry + 2.5%fish meal	**58**	11	27	96

As noted earlier, a second objective of the study was to test the decision-support system ARIES. The participants had to indicate whether or not they used the program as a support during the identification process, particularly in distinguishing between mammalian and avian material. The results for PAPs from mammalian sources are presented in **Table 7**, separated into 'users' (14 laboratories) and 'non-users' of ARIES (17 laboratories).

Comparing the results reported by the user group with the non-user group, there was no significant difference in the performance of the two groups in detecting PAPs in general. It seems that the use of ARIES does not improve the performance of the method when detecting the presence of *terrestrial animals*. However, when discriminating between mammals and poultry, ARIES proved to be a very useful tool detecting MMBM in the presence of poultry and fish meal, as shown the substantial decrease in the number of 'No results' reported by ARIES users compared with 'non users' (**Table 7**).

Table 7. Comparison of results on the use/non-use of ARIES for the mammals parameter Present (P), not present (NP) and no result (NR).

PAPs from mammals							
		ARIES			**NO ARIES**		
MAT	**Composition**	**P**	**NP**	**NR**	**P**	**NP**	**NR**
1	Blank	5	60	*3*	6	45	*5*
2	Poultry meal 5%	22	21	*5*	9	12	*21*
3	Fish meal 5%	9	57	*2*	4	44	*8*
4	MBM 0.10%+ 5% fish meal	32	31	*4*	23	12	*21*
5	MBM 0.5%+ 5% poultry meal	36	7	*5*	22	2	*18*
6	MBM 0.10%	36	10	*4*	19	1	*22*
7	MBM 0.5% +2.5% Poultry+2.5%fish meal	36	8	*4*	19	3	*20*

The combined results from **Table 5** and **Table 6** showed that for the terrestrial animals parameter, all the laboratories reported results on all the materials (**Table**

5: MAT 1: 33 participants x 4 replicates = 132; etc.). However, for the parameter PAP from mammalians a few data points were missing (**Table 6**: MAT 2: 33 participants x 3 replicates = 99, 96 reported, 3 void; MAT 4: 33 participants x 4 replicates = 132, 131 reported, 1 void; etc.)

We can also conclude that, in general, the structure of the Excel reporting sheet, the type of questions asked and the explanations provided were clear to the users. In some cases, illogical results were reported (e.g., an absence of both mammalian and avian material and a presence of terrestrial animal material). The questions on details of the method performed were part of the report sheet. Most participants answered these questions; only two did not. This indicates that a good structure for reporting the results of a qualitative proficiency test had been developed.

The next section elaborates upon the analysis of the results of both the training period and the STRATFEED validation study, and presents the criteria used to select the final set of laboratories to participate in the study after the training. It also evaluates the comments from the laboratories on the methodology applied and on ARIES, and compares the STRATFEED results with the results of the DG-SANCO [**10, 12**] and IFFO [**15**] studies in terms of accuracy.

5.5 Detailed evaluation

A training phase involving 31 laboratories was organised prior to the validation study. For organisational reasons, two STRATFEED partners were allowed to take part in the final validation study without going through the training phase. The purpose of the training was twofold: to familiarise the participants with the type of materials, and to test their performance. Out of 31 laboratories, 25 submitted good results for the five blind training samples (Batch A). The remaining six failed for at least two of the five samples; they were still allowed to take part in the final validation study, and it was decided to accept their results for the final set of 24 samples (Batch B) provided that there had been a considerable improvement.

*Table 8. Parameters in the protocol for preparing slides for microscopic evaluation. The requested and the deviating type of the indicated parameters according to the STRATFEED protocol are listed with, in brackets, the number of participants applying the parameters. Two participants did not provide details on the protocol. * Accepted in the validation*

Parameter	Requested (No. users)		Deviating (No. users)	
Sample size (g)	5	(23)	10	(8)
Grinding	mill	(13)	mortar	(16)
Solvent	TCE	(30)	CCl_4	(1)
Separation funnel	closed	(21)	open	(10)
Time (min)	=> 4	(28)	=< 3	(3)
Sieving	yes	(19)	no	(12)
Binocular	yes	(27)	no	(4)

In the validation study, 33 laboratories submitted their full results for the set of 24 samples. Two laboratories appeared to have not improved since the training. Two other laboratories produced an average amount of sediment that was considerably

higher than the declared amount of sediment in the feed used (8.6% and 7.8%, respectively, compared with 0.6 – 0.9% obtained by the other laboratories). This high amount indicated an incorrect application of the protocol. Four laboratories used other parameters than those that had been set, which would have substantially influenced the observations in a negative way: embedding the sieve fraction in ether petroleum combined with sedimentation in CCl_4, embedding the sieve fraction in several agents not included in the set protocol combined with a sedimentation time of 2-3 minutes, embedding the fine sediment fraction in water combined with a sedimentation time of only 1 minute, and embedding the sieve fraction in immersion oil combined with the high viscosity of the embedding agent of the fine sediment fraction.

All these laboratories (eight) were excluded from the final evaluation of the results of the validation study, for the given reasons. One laboratory showed a substantial improvement in their results for the final set of 24 samples compared with the training set and was accepted for the final evaluation.

As shown in **Table 8**, a substantial number of participants used a deviating state for several parameters. Many of them used the starting amount of 5 g of the sample that is allowed under the EC Directive 126/2003 [**6**]; the original STRATFEED protocol called for the use of 10 g of original material. More than half of the participants used the mortar because of the small size of the original samples. The use of the open sedimentation beaker was excluded in the original STRATFEED protocol, but was allowed in the procedure as currently accepted by the EU [**6**] (see chapter 3). The lack of sieving and of the use of binoculars for examining the coarse fractions was allowed in the final evaluation, although requested in the protocol.

After excluding the eight laboratories, the results of remaining 25 participants were used for the final evaluation. They are summarised in **Table 9**. The 'accuracy' measure means specificity in the case of false positives (e.g., terrestrial animal material in MAT 1, a blank; mammalian material in MAT 3) or sensitivity in the case of false negatives (e.g., mammalian material in MAT 6; avian material in MAT 2).

Table 9. Summary of results expressed as accuracy values (AC) for detecting animal proteins in seven contaminated feeds with, in brackets, the number of 'No results'. n: total number of observations.

	Material	**n**	**AC**			
			Terrestrial	**Mammalian**	**Avian**	**Fish**
MAT 1	blank	100	0.908 (2)	0.933 (7)	0.953 (11)	0.880 (0)
MAT 2	poultry 5%	75	0.960 (0)	0.587 (29)	0.769 (36)	0.907 (0)
MAT 3	fish 5%	100	0.857 (2)	0.919 (10)	0.963 (15)	0.990 (1)
MAT 4	MBM 0.1% + fish 5%	100	0.768 (1)	0.639 (27)	0.800 (39)	0.970 (0)
MAT 5	MBM 0.5% + poultry 5%	75	1.0 (0)	0.959 (26)	0.878 (34)	0.865 (1)
MAT 6	MBM 0.1 %	75	0.987 (0)	0.896 (27)	0.632 (37)	0.920 (0)
MAT 7	MBM 0.5% + poultry 2.5% + fish 2.5%	75	1.0 (0)	0.898 (26)	0.718 (36)	0.947 (0)

The detection of terrestrial animal material and fish material did not generally present significant problems. Exceptions were the reports of some false positives for terrestrial animal material in MAT 3 and for fish material in MAT 5. A special case was the detection of 0.1% terrestrial animal material in the presence of 5% fish meal (MAT 4), for which 23 false negatives in a total of 99 observations were reported (and 1 'No results'). The detection of specific mammalian or avian material appeared to be more difficult for some materials. This was illustrated not only by the lower values for accuracy, but also by the number of 'No results' (as noted in paragraph 5.4), which in some materials was more than half the number of data points for the presence of avian material. For a more detailed and balanced view of the results, an equation for adjusted accuracy was developed (see paragraph 5.3).

The adjusted accuracy values per material and target contaminant are presented in **Table 10.** The detection of classes of terrestrial animals was, as expected, more difficult than detecting terrestrial animals in general. There appeared to be generally more positive observations for the detection of mammalian material than avian material, except for MAT 4 where there were higher values for adjusted accuracy. Only for MAT 2 was the accuracy value for mammalian material lower, since a positive in this material was a false positive, whereas for MAT 5, 6 and 7 the positives were correct.

Table 10. Recalculated results expressed as adjusted accuracy values (AC') for the detection of animal proteins in seven contaminated feeds. n: total number of observations.

	Material	**n**	**AC'**			
			Terrestrial	**Mammalian**	**Avian**	**Fish**
MAT 1	blank	100	0.890	0.865	0.844	0.880
MAT 2	poultry 5%	75	0.960	0.253	0.400	0.907
MAT 3	fish 5%	100	0.840	0.823	0.813	0.980
MAT 4	MBM 0.1% + fish 5%	100	0.760	0.465	0.485	0.970
MAT 5	MBM 0.5% + poultry 5%	75	1.0	0.627	0.480	0.853
MAT 6	MBM 0.1 %	75	0.987	0.573	0.320	0.920
MAT 7	MBM 0.5% + poultry 2.5% + fish 2.5%	75	1.0	0.587	0.373	0.947

Participants were asked to indicate whether or not they used the decision-support system ARIES. No distinction was made between using ARIES for providing knowledge and/or for active support in the detection and identification process. The laboratories that used ARIES only for providing background knowledge were considered as ARIES users. Three STRATFEED partners that indicated that they did not use ARIES were classified in the group of non-users. The group of ARIES users consisted of 13 participants, while the non-users group consisted of 12 participants. There was no significant difference in the performance of the two groups in detecting terrestrial animal material and fish material.

These results accorded with the predominantly high level of accuracy values presented in **Table 9** and **Table 10**. Similarly, there was hardly any difference in the critical detection of terrestrial animal material in the presence of fish meal (MAT 4). The results for the detection of mammalian and avian material are presented in **Table 11**. Again, at this level there seemed generally to be no seriously

deviating results according to whether or not ARIES had been used, except for a few situations, such as in the detection of mammalian material in MAT 2 (0.353 vs. 0.583) and in MAT 4 (0.702 vs. 0.520) and in the detection of avian material in MAT 7 (0.759 vs. 0.6). However, the number of 'No results' was significantly higher for the group of participants that did not use ARIES. Therefore, the adjusted accuracy values were calculated for both groups of participants, as shown in **Table 12**. In all cases the values were higher when using ARIES. The report of correct negatives for mammalian material in feed that is exclusively adulterated with poultry material and the detection of avian material in feed contaminated only with mammalian material are special cases for further attention. Where ARIES was not used, there was no difference in adjusted accuracy (0.194; values in italics in **Table 12**). When ARIES was used, it seemed easier to correctly indicate the absence of avian material in MAT 6 (0.436) than the absence of mammalian material in MAT 2 (0.308).

Table 11. Summary of results expressed as accuracy values (AC) for the detection of two types of animal proteins in seven contaminated feeds, according to users and non-users of the ARIES system, with, in brackets, the number of 'No results'. n: total number of observations.

Material		AC with ARIES			AC without ARIES		
		n	Mammalian	Avian	n	Mammalian	Avian
MAT 1	blank	52	0.935 (2)	0.935 (2)	48	0.930 (5)	0.974 (9)
MAT 2	poultry 5%	39	0.353 (5)	0.800 (6)	36	0.583 (24)	0.667 (27)
MAT 3	fish 5%	52	0.891 (2)	0.932 (3)	48	0.950 (8)	1.0 (11)
MAT 4	MBM 0.1% + fish 5%	52	0.702 (4)	0.718 (9)	48	0.520 (23)	0.952 (27)
MAT 5	MBM 0.5% + poultry 5%	39	0.941 (5)	0.871 (5)	36	1.0 (21)	0.900 (26)
MAT 6	MBM 0.1 %	39	0.857 (4)	0.607 (8)	36	1.0 (23)	0.700 (26)
MAT 7	MBM 0.5% + poultry 2.5% + fish 2.5%	39	0.917 (3)	0.759 (7)	36	0.846 (23)	0.600 (26)

Table 12. Recalculated results expressed as adjusted accuracy values (AC') for the detection of animal proteins in seven contaminated feeds, according to users and non-users of the ARIES system. n: total number of observations. For values in italics, see text.

	Material	n	AC' with ARIES		n	AC' without ARIES	
			Mammalian	Avian		Mammalian	Avian
MAT 1	blank	52	0.896	0.896	48	0.833	0.792
MAT 2	poultry 5%	39	*0.308*	0.615	36	*0.194*	0.167
MAT 3	fish 5%	52	0.854	0.854	48	0.792	0.771
MAT 4	MBM 0.1% + fish 5%	52	0.647	0.549	48	0.271	0.417
MAT 5	MBM 0.5% + poultry 5%	39	0.821	0.692	36	0.417	0.250
MAT 6	MBM 0.1 %	39	0.769	*0.436*	36	0.361	*0.194*
MAT 7	MBM 0.5% + poultry 2.5% + fish 2.5%	39	0.846	0.564	36	0.306	0.167

Reclassifying the three STRATFEED partners as ARIES users rather than non-users made a substantial difference for some materials. In the detection of mammalian material in MAT 4 most correct positives came from these three partners; reclassification resulted in AC' values of 0.667 and 0.110 for the user (n=16) and non-users (n=9) groups, respectively. All the correct positives for avian material in MAT 2 were reported only by the three STRATFEED partners in the no-user group.

The pivotal material was the feed contaminated with 0.1% of MBM in combination with 5 % fish meal (MAT 4), where the AC value for all participants was 0.768. This indicated that some of the laboratories were not able of correctly distinguish between the two sources of animal proteins. It is important to emphasise, however, the expertise and proficiency that the laboratories needed to have in order to correctly analyse the samples. The STRATFEED study can be compared to the proficiency study carried out by JRC (commissioned by DG-SANCO; [**10; 12**]) and the validation study organised by RIKILT (commissioned by IFFO; [**14**]).

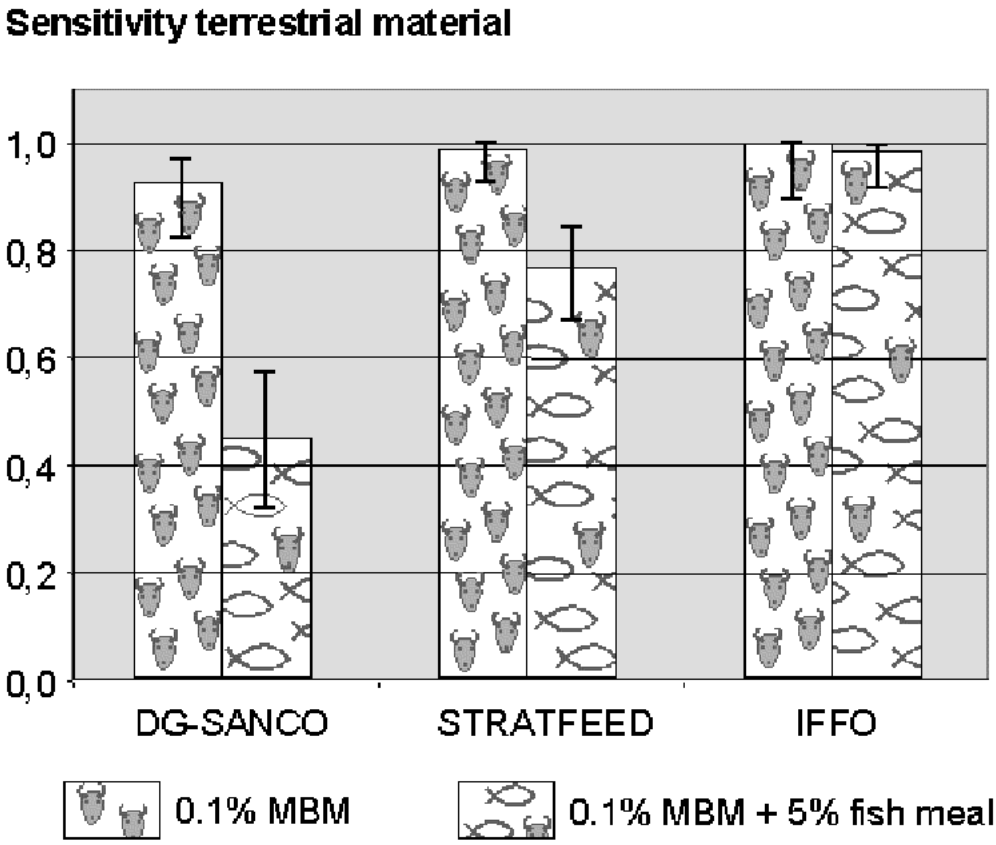

Figure 2. Sensitivity values showing the 95% confidence limits for the detection of two types of animal meal in feeds (terrestrial animal meal versus fish meal). The names in capitals on the horizontal axis refer to the stakeholders in the original studies.

The DG-SANCO study aimed at providing information on the proficiency of the laboratories and therefore all participants were allowed to use their routine procedures. The participants in the IFFO study applied only the STRATFEED procedure, which is stricter than the protocol currently accepted by the EU [**6**]. The DG-SANCO study did not report statistics on specificity and sensitivity for

individual materials, since this is not an aspect of proficiency testing. However, the reports from 18 laboratories in the DG-SANCO study classified as group A or B (see [**10**] for details) were used to calculate the required statistics for the comparison shown in **Figure 2**. The results for the material with 5% fish meal and 0.1% MBM (MAT 4 here) of the DG-SANCO study showed an accuracy of 0.444 (n=63 data points). The IFFO study reported an accuracy of 0.984 (n=64). The STRATFEED study reported a value of 0.768 (n=99), which is higher than the DG SANCO value but lower than the IFFO value. A second observation of interest is the detection of terrestrial animal material in feed adulterated with exclusively 0.1% MBM (MAT 6). The accuracy values were 0.921 (n=63), 1.0 (n=32) and 0.987 (n=75) for DG-SANCO, IFFO and STRATFEED, respectively. For both materials, the results for the detection of terrestrial animal material in the STRATFEED study were intermediate between the other two studies. There was a major improvement compared with the normal situation as described in the DG-SANCO proficiency test. Since the characteristics of the test materials in the three studies were not the same, the reason for the different outcomes of these studies could not be completely inferred from the results. However, the superiority of the IFFO study might have stemmed from differences in the methodology used, but this requires further investigation. Possible reasons for the lower AC values in the STRATFEED study compared with those in the IFFO study are the changes to the original strict STRATFEED protocol in line with current legislation [**6**]. The use of an open sedimentation beaker was not expected to have a major influence, since previous proficiency studies indicated a good performance. The use of 5 g instead of 10 g for sedimentation, however, might reduce the chance of detecting the few particles that are present in the sediment. Also, the small quantity of the samples provided to the participants in the STRATFEED study required the use of a mortar. In future studies (for validation or proficiency testing) a higher amount of material per sample is necessary. Focusing on the results of the STRATFEED validation study might show that microscopy is not suited to the intended purpose when analysing compound feed with 0.1% MBM in the presence of fish meal. However, the results from the well-performing laboratories in the DG-SANCO study and all the laboratories in the IFFO study clearly confirmed that microscopy is able to detect MBM at this level, provided that the laboratories have enough experience in applying the microscopic method properly. With respect to ARIES, it should be noted that the participants of the validation study showed, by means of investigating a training sample set prior to the proper study, to have a sufficient level of expertise for the type of material that was included in the validation study. This starting situation might explain why there is no significant difference between the group of users and of non-users for the detection of terrestrial animal proteins in general, and of fish meal. It is clear that using ARIES enhances the possibilities of detecting and identifying classes of terrestrial animal material. The correct indication of the absence of mammalian material in poultry-contaminated feed appeared to be more difficult than vice versa, indicating the absence of avian material in mammal-contaminated feed (AC’ = 0.31 vs. AC’ = 0.44, respectively). This might be because some poultry long bones are similar in appearance to mammalian bones [**13**]. These bones can be indicated as confusing elements, whereas in the vice versa situation they are almost absent. These results indicate that a further improvement of ARIES can be achieved. The first official release ARIES version 1.0 will include such improvements.

6 Conclusions

We can summarise the results of the study as follows:

- The overall performance of the microscopic detection of animal proteins in feeds is generally good.
- In the STRATFEED validation study the detection of terrestrial MBM 0.1 % in the presence of fish meal turned out to be not sufficient. Additional investigations need to be conducted in order to establish whether a further harmonisation of the analytical protocol improves the performance of the microscopic method as indicated by the results of the IFFO study.
- Comparing the current results with those of the previous study commissioned by DG-SANCO [**10; 12**], it can be concluded that the improved protocol [**6**] is significantly better than the one established by the former Directive 98/88/EC [**5**].
- When comparing the STRATFEED study with the IFFO study [**14**], the laboratories trained in applying the optimised STRATFEED protocol were able to obtain an acceptably low rate of false negative results for the detection of 0.1% terrestrial MBM in presence of fish meal (64 data points in the IFFO Study). Adequate experience of the control laboratories is an extremely important factor.
- The use of ARIES did not improve performance in determining terrestrial MBM, but ARIES did help in detecting mammalian MBM in the presence of poultry and fish meal. Three of the four laboratories (from a total of 12) that did not use ARIES but correctly classified MAT 4 (mammalian MBM in the presence of fish meal) were STRAFFEED partners. Since they all participated in developing ARIES, they had the main features of ARIES available without being physically connected to the Aries server. The other eight laboratories reported only false negatives.

7 Recommendations

Based on the results of the studies, we recommend the following:

- It is important to provide training in critical aspects, such as cleaning to avoid cross contamination, the correct application of the sedimentation procedure, the use of binoculars, and the proper identification of assumed animal particles.
- Proficiency tests should be organised regularly; the amount of material supplied for each sample should be enough to enable laboratories to use the right grinding equipment and perform all aspects of the protocol.
- The information in ARIES needs to be updated as a planned result of the validation study.
- The harmonisation of the interpretation of detection levels (i.e., whether one bone particle is proof of a positive sample, the status of a duplicate analysis) should be approved by the EC.

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