# A ring trial for the detection of animal tissues in feeds in the presence of fish meal

L.W.D. van Raamsdonk and H. van der Voet

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CONTENTS page

## SUMMARY

A ring test was carried out based on a set of feed samples spiked at 0.1% with two types of animal meals (pure ruminant MBM and mixed species MBM) and with two types of fish meals at 5% (trimmings and whole fish), combined in every possible combination including blanks. This design resulted in nine treatments each analysed in duplicate by each participant; the total set comprised 18 results for each participant. Nine different laboratories from eight European countries participated in the trial. All participants reported complete sets of observations, including sediment percentages and presence/absence of terrestrial animal material (MBM) and of fish meal for each of the 18 samples.

Homogeneity was tested in three replicates of two samples each. The homogeneity appeared to be sufficient. The basic feed and the basic animal meals were checked and approved. The ruminant meal was produced specifically for research purposes in a pilot plant and showed some influences of high temperature treatment. The mixed species MBM was produced in a plant at industrial scale.

In four out of 108 observations of MBM containing samples the absence of terrestrial animals was erroneously reported. All these four false negatives occurred in the presence of fish meal and three of them applied to spiking with the ruminant MBM. The unusual appearance may in part account for three of the false negatives reported. Also a deviating individual lab protocol for the production of the sediment might has influenced the report of some false negatives. There were no false negatives for the presence of fish meal. There were four erroneous reports for presence of MBM. Two were found in the complete blanks, i.e. without fish meal present, and two were reported from samples spiked with fish meal. The latter two occasions can be due to the fact that certain fish bone particles might be confused with presumed terrestrial animal bone fragments. Good documentation and identification systems and training can improve this situation. The finding of false positives in complete blanks can be due to production procedures or lab practices. However, all these sources of contamination are ruled out and a further evaluation has to be carried out.

MBM of terrestrial animals can properly be detected at a level of 0.1% in the presence of fish meal, even in the case that the MBM consists of a low fraction of bones (13%). The unambiguous detection of MBM depends partly on the appearance of the MBM particles and on the type of the fish meal. Development of documentation systems and training is advised. A further harmonisation of the protocol compared to the guidelines of Directive 98/88/EC is recommended.

# 1 INTRODUCTION

For more than ten years the production of meat suffers by the presence of Bovine Spongiforme Encefalopathies (BSE), which is able to cause a variant of Creutzfeld-Jacob disease in human beings. The BSE occurrence is most likely caused by the feeding, mainly to ruminants, of slaughter by-products. This situation forced the European Commission finally to raise a ban on the feeding of all animal proteins to farmed animals (see for a more detailed review of the legislation Gizzi et al., 2003). A large amount of by-products needs currently to be destroyed or is used as fuel in electricity power plants. For reasons of environmental pollution and nutritional value of the material other applications are recommended. The feeding of fish meal to ruminants is temporarily prohibited as well. However, the use of pure fish material in the animal production chain poses no risk. It is accepted that fish do not carry Transmissible Spongiform Encephalopathy (TSE). The European Commission has stated that it requires a method to check for the presence of ruminant tissue in fish meal or in compound feed containing fish meal before it will allow fish meal into ruminant feeds.
Feed microscopy is currently the only method officially accepted by the Commission (Commission Directive 98/88/EC; EU, 1998) to test for the presence of animal proteins in feeds. This method claims a sensitivity of detecting 0.1% of animal products in feeding stuffs. However, the method is not widely accepted as a satisfactory test for monitoring fish meals because of possible difficulties distinguishing fish and mammalian bones. This study is intended to demonstrate the possibility to detect mammalian material in a feed in the presence of fish meal by at least eight laboratories.

## 1.1 Aim and framework of this study

The purpose of this study is to check the ability of microscopic detection of animal proteins to distinguish between material of terrestrial animals and of fish. A set of spiked samples with different treatments will be investigated blind by a range of laboratories to estimate the performance of microscopy detection of terrestrial animal meals in the presence of fish meals. The study is initiated by the International Fish meal and Fish oil Organisation (IFFO) and independently coordinated by RIKILT Institute of food safety.

# 2 METHODOLOGY AND ORGANISATION

## 2.1 Material and methods

All the samples are based on a typical ruminant feed that was specifically produced for the ring trial. The feed contained (in decreasing order of relative amount) maize, wheat, rape, soya and a premix containing minerals. The feed was checked on the presence of any cross contamination or non-intended animal material (e.g. rodents). The feed was approved to be free of animal tissues[1]. Corn and rape are present in the formula since these ingredients might introduce particles that might show a certain resemblance with particles of animal origin.

Two different animal meals (meat and bone meals; MBM) were chosen for the production of the feeds. A first one consists of exclusively ruminant material and has been produced in the United Kingdom, specifically for test purposes in a small scale pilot plant (fraction of bones: 54%). A second animal meal originates from a Dutch rendering plant and contains predominantly ruminant and pig material. Traces of poultry material might be present in this mixed species MBM (fraction of bones: 13%). Two different fish meals were included in the feeds. One consist of exclusively herring by-products (trimmings) and a second type of fish meal contains a mixture of different species (whole fish).

The fish meals were added at a concentration of 5%, whereas the feeds were contaminated at 0.1 % with animal meals. The design consists of nine different treatments with two replicates of each, making a total of 18 samples. The treatments are:

| Feed material code | | | (- t) | (r t) | (m t) | (- w) | (r w) | (m w) | (- -) | (r -) | (m -) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Feed material number (=preparation order) | | | 2 | 4 | 6 | 3 | 5 | 7 | 1 | 8* | 9* |
| Type of animal tissue: | | sediment % | | | | | | | | | |
| Trimmings 5% | t | 6 | + | + | + | - | - | - | - | - | - |
| Whole fish meal 5% | w | 16 | - | - | - | + | + | + | - | - | - |
| Ruminant MBM 0,1% | r | 54 | - | + | - | - | + | - | - | + | - |
| Mixed species MBM 0,1% | m | 13 | - | - | + | - | - | + | - | - | + |
| No. in first replicate | | | 6 | 9 | 2 | 8 | 1 | 5 | 3 | 7 | 4 |
| No. in second replicate | | | 14 | 11 | 18 | 12 | 16 | 10 | 17 | 13 | 15 |

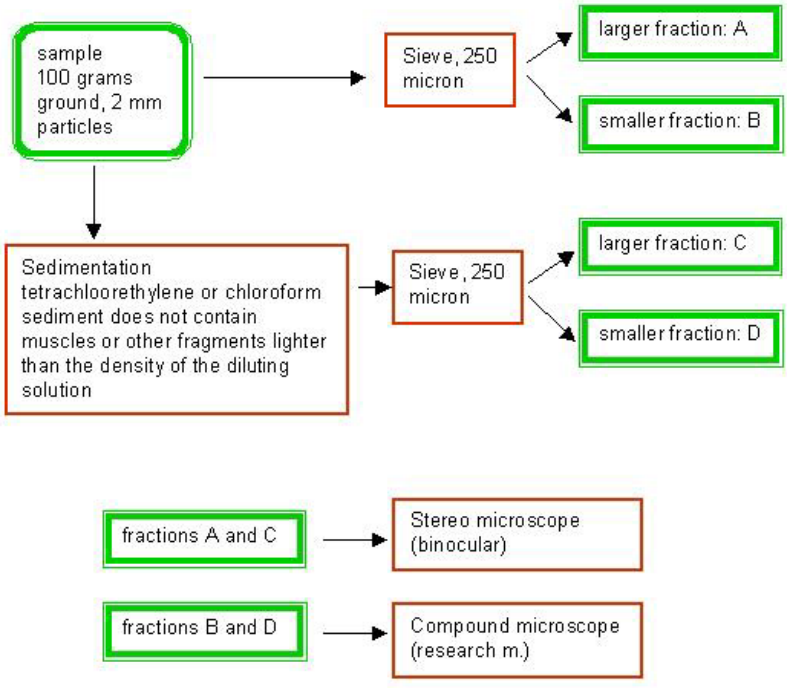
*: after an extra cleaning step

The preparation of the materials followed the applicable considerations in the ISO 5725 guidelines (ISO 1994a, 1994b). All material has been ground with a mesh size of 2 mm. This procedure results in a particle size of less then 0.8 mm. The particle size distribution is then suited for the normal microscopy procedure that includes sieving with a mesh size of 0.25 mm. Both resulting fractions are reasonable available.

The feed materials are prepared in a well defined order for avoiding any possible cross contamination. Two sets of glassware were used for trimmings and whole fish separately. The blanks (code (- -)) were prepared

[1] In legislation the term “animal proteins” is used. Since microscopy effectively detects the presence animal tissues or in practice the addition of meat meal, meat-and-bone meal, bone meal etc. this documents will use the term “animal tissues” in general and “MBM” in paragraphs referring specifically to the terrestrial animal material that was added to the feed samples. Fish meal indicates the dried and ground fish material.

at first and then the treatments with codes (- t) and (- w). After finishing the production of these feed materials the bags with the MBMs were opened. For both the fish meals and the MBMs the pure meals (i.e. trimmings and ruminant meal) were processed first in order to avoid contamination with ingredients of other animals as included in the mixtures (i.e. whole fish meal and mixed MBM). Finally the feeds with MBM and without fish meal have been produced after an extra cleaning step of the equipment.
Each feed has been prepared by using the process of stepwise dilution. That means that e.g. 140 grams of fish meal will be diluted in 140 grams of feed. This mixture with a concentration of 50 % will be diluted in 280 grams of feed (final concentration 25%). 180 grams of this mixture has been used for the final preparation in two steps of 900 grams of feed with 5 % of fish meal which in turn will be used for the production of feed contaminated with MBM. The entire procedure is presented in Appendix 2.
Each laboratory was asked to analyse two replicates of each feed material. It was decided to number the samples[2] in such a way that each material occurred once in the set 1-9 and once in the set 10-18. The order of the samples within each set was randomised (see table above). The samples numbered 1 to 18 have been offered blind to the participants in an amount of 25 grams each.
Two feed materials containing only MBM (feeds 8 and 9) have been investigated in three replicates each for testing the homogeneity by RIKILT. Every replicate of 25 grams has been taken randomly from the total batch of feed.
The samples have been prepared and investigated by the participants according to EU guideline 98/88/EC (see flow scheme). All participants have been asked to include some more detailed procedures in this general protocol. The main adjustment is the sedimentation in a closed sedimentation funnel allowing shaking instead of just stirring. In all cases 10 grams of each sample was proposed to use for sedimentation. Up to three slides were proposed to use for the detection of animal tissues. After having no material found in these three slides, the sample was reported negative. Details on the procedure as followed by the individual participants are given in Appendix 6.




Flow diagram of the microscopic method as described in Directive EU/98/88.

[2] "Feeds" refer to the nine treatments that were included in the experimental design. Every feed was included twice in the entire set, making a total of 18 "samples".

The results have been reported to the coordinator as presence or absence of MBM and of fishmeal. This qualitative representation of data does not allow the utilisation of the statistics as laid down in ISO 5725, since all these statistics are designed for quantitative results (ISO, 1994a, 1994b).
In order to test whether the number of errors from any single laboratory could be considered as an outlier a randomization test for qualitative results was implemented along the same lines as Grubb's outlier test for quantitative data, which is used in ISO 5725-2 (ISO, 1994b). In a randomization test (Edgington, 1995, Manly, 1991) a test statistic of interest (T) is calculated from the actual data ($T_{data}$) and also from a large number (e.g. 10,000) of randomized versions of the data set ($T_{rand}$). In this case we took the maximum number of errors of a certain kind per laboratory as our test statistic T. We then applied permutations of the error indicators over all samples from the same feed material. The 10,000 randomizations generated a null distribution of values $T_{rand}$. For a test at the usual 95% or 99% confidence level, we then compared the observed $T_{data}$ with the 95th or 99th percentile of the generated distribution. In analytical chemistry it is customary to denote a significant result at the 99 % confidence level as a statistical outlier, and a result which is significant at the 95 % but not the 99 % confidence level as a straggler (ISO, 1994b). More explanation and some discussion of this randomization test for qualitative outliers is given in Appendix 1.
For binary results (yes/no, positive/negative, etc.) standard statistics are accuracy, sensitivity and specificity. The accuracy is the fraction of correct results, either positive or negative. The sensitivity is the ability of the method to detect the contaminant when it is present, whereas the specificity is the ability to not detect the contaminant when it is absent. The following equations have been used to calculate the statistics:

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

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

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

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 *p* or as percentages after multiplication by 100. Accuracy (specificity or sensitivity) has been calculated for each feed material ignoring the laboratory structure (2 samples for each laboratory).
In simple situations, where only one type of variability is assumed, exact 95 % confidence intervals can be calculated from the binomial distribution. The lower limit is the smallest value of the binomial parameter *P* which generates the observed outcome *p* or higher values with at least 2.5 % probability. The upper limit is the highest value of *P* which generates the observed outcome *p* or lower values with at least 2.5 % probability.
In ring trials based on quantitative data usually within lab repeatability and between labs reproducibility are calculated. Recently comparable statistics have been developed for qualitative data (ISO, 2000; Langton et al., 2002). Accordance is the chance of finding identical results in pairs of replicates of the same treatment in the same laboratory under repeatability conditions. This is equivalent to repeatability for quantitative results. Several strategies have been applied in calculating the accordance. The two values to form a pair

can be sampled from the available values without replacement (Langton et al., 2002). Alternatively, the values for the replicates per treatment can be considered as representatives of a larger population of replicates and the two draws to form a pair can be sampled from that larger population, i.e. sampling with replacement is applied (ISO, 2000). The difference between the two calculation strategies decreases with an increasing number of replicates per treatment. In the current study there are only two replicates per treatment, which allows only one possible pair after sampling without replacement. Therefore, the strategy with replacement (ISO, 2000) is applied in this study. It is calculated by summing up the probabilities of matching one replicate to another of the same lab with the same result:

$$ACC = \frac{\sum_k \left\{ p_k^2 \ + (1 - p_k)^2 \right\}}{K}$$

where $K$ = number of labs and $p_k$ the fraction of replicates with result 1 for lab $k$. Due to the number of replicates in this study (2) $p_k$ can have the values 0.5 or 1. The closer the value of the accordance to one the better labs are performing.

Concordance is the chance of finding the same result for the same treatment in two different laboratories. ISO (2002) as well as Langton et al. (2002) presented calculation models based on sampling without replacement. Nevertheless, in comparison to accordance a calculation based on the same principle, i.e. sampling with replacement, will be proposed in this study. Then concordance is calculated from the chance of finding a pair of replicates from any laboratory with identical results, either 1 ( $p$ ) or 0 ( $1 - p$ ):

$$CON = \overline{p}^2 + (1 - \overline{p})^2$$

where $\overline{p}$ is the average fraction of replicates with result 1 for all labs. The closer the value of concordance to one the better the between lab reproducibility. The strategy followed in this study can be indicated as application of a random model, whereas the strategy of Langton et al. (2002) can be indicated as applying a fixed model.

## 2.2 Participating laboratories

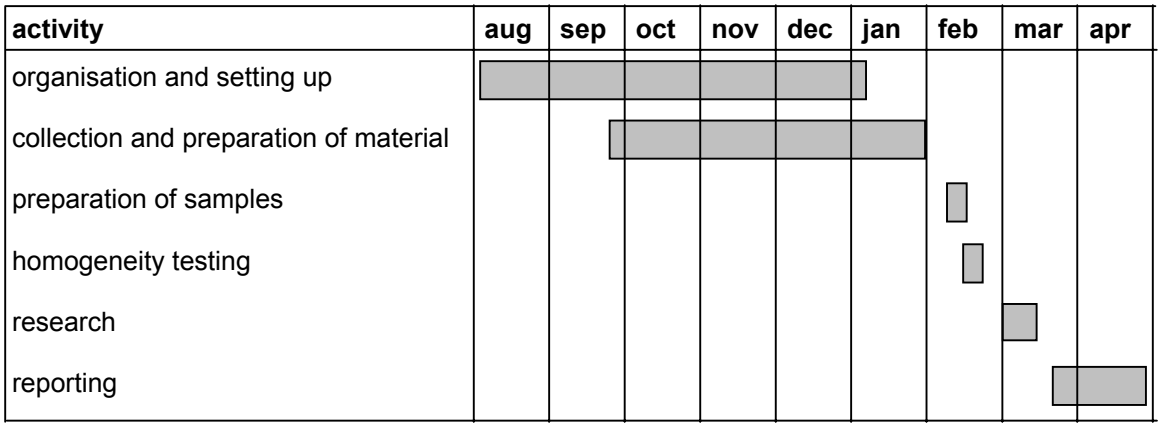
The following laboratories were asked and agreed to investigate the 18 feed samples:

Austria: Landwirtschaftliche Untersuchungen und Forschung Wien, Wien
Belgium: ROLT, Tervuren
Denmark: Danish Plant Directorate, Lyngby
Germany: Landwirtschaftliche Untersuchungs und Forschungs Anstalt Nord-West, Hameln
Netherlands: Maasweide Lab., NUTRECO, Boxmeer
Netherlands: RIKILT Institute for Food Safety, Wageningen
Spain: Laboratory of the Government of Catalonia, Barcelona
Switzerland: Swiss Federal Research Station for Animal Production, Posieux
United Kingdom: Veterinary Laboratory Agency, DEFRA, Luddington

## 2.3 Organisation and time frame

The organisation of the ring trial started during Summer 2002. There was a close collaboration between the stake holder IFFO and scientific coordinator RIKILT. This cooperation resulted in the mutual production of the sample sets. From that point RIKILT took the exclusive responsibility for the sending of the material, the communication regarding the scientific matters and the collection of the results. All participants have been asked to perform the actual analysis in week 9 of 2003 in order to comply to the related requirements with respect to repeatability conditions in ring trials (ISO, 1994b). The results are presented to the stake holder anonymously in the same form as to the public community.
The time schedule followed is presented in the following chart.

October 2002 – April 2003



# 3. RESULTS

## 3.1 Homogeneity testing and pure materials

The results for the homogeneity testing are presented in Appendix 3. The three replicates investigated for the two samples showed a sufficient resemblance within each sample. Since the replicates for this homogeneity test have been taken randomly from the total amount of material for each sample, it can be concluded that the production procedure ensures a sufficient level of homogeneity. The pure feed as well as the pure MBMs have been tested. The pure feed is free of any contamination. Based on these results the material is suited for the purpose of a ring trial.

## 3.2 Ring trial

The results of the sediment percentages (Appendix 5) show a high correlation with the contents of the samples. The pure feed shows a sediment percentage of approx. 1%. For pure feeds the sediment consists predominantly of minerals. The percentage for the samples without fish meal is only slightly higher, since the MBM concentration of 0.1% hardly adds to the sediment. The addition of 5% fish meal results in a considerably higher amount of sediment: 1.89% with fish compared to 1.19% without fish. The whole fish meal contains a higher amount of bones (16%), which is expressed in the higher amount of resulting sediment (Appendix 5). The standard deviations among partners per samples are predominantly low. This situation indicates a consistent application of the methods and therefore supports the comparability of the results. Participants C and I produced somewhat higher but consistent sediment percentages. The highest percentage of sediments was found after applying chloroform for sedimentation (participant I). This solvent has a lower density than the regular TCE, which results in relatively more particles that will sink during the sedimentation procedure, obviously producing a higher sediment fraction. This situation is not expected to influence the evaluation of the MBM detection.

The research by the nine participating laboratories included the detection of animal proteins of terrestrial origin (MBM) and of fish, as well as the detection of the percentage of the sediment relative to the whole sample. All participants reported complete results; there were no missing values.

The table in Appendix 4 shows the results of the detection of MBM and fish in the feed samples. In general there is good match between the real contents of the samples with the findings of the participants. The presence of fish meal was in all cases properly detected (no false negatives) and in only one case fish meal presence was presumed out of a total of 72 observations of samples lacking fish material (one false positive). This one false positive relates to a sample containing mixed MBM (participant H).

The results for the detection of MBM appear to be a bit more complicated. There is a total of four false negatives at a total of 108 observations. In this respect it is necessary to clarify the report of traces. Participant A reported the presence of “traces” of bones in samples 4, 9, 10 and 11. In their normal lab reporting procedures “traces” mean up to 1% of the target substance in the slides. The statement “one or two particles” would have been used in case of very few particles. Since this indication of “trace” differs considerable from the finding of participant C in both blanks, the report for the four mentioned samples from participant A is considered full positive. All these false negatives occur in the presence of fish meal and all of them except one apply to samples containing the pure ruminant MBM (table 1; treatment 5). The combination of ruminant MBM and whole fish meal showed the lowest accuracy (table 1). Concordance

| Feed material | number of errors (out of 18 results) | accuracy[1] | 95 % confidence interval | accordance | concordance |
|---|---|---|---|---|---|
| 1 (- - ) | 2 | 0.89 (*0.88*) | 0.65 – 0.99 | 1.00 | 0.80 (*0.78*) |
| 2 (- : trim) | 0 | 1.00 | 0.82 – 1.00 | 1.00 | 1.00 |
| 3 (- : whole) | 2 | 0.89 (*0.88*) | 0.65 – 0.99 | 0.89 (*0.88*) | 0.80 (*0.78*) |
| 8 (rum : -) | 0 | 1.00 | 0.82 – 1.00 | 1.00 | 1.00 |
| 9 (mixed : -) | 0 | 1.00 | 0.82 – 1.00 | 1.00 | 1.00 |
| 4 (rum : trim) | 0 | 1.00 | 0.82 – 1.00 | 1.00 | 1.00 |
| 6 (mixed : trim) | 0 | 1.00 | 0.82 – 1.00 | 1.00 | 1.00 |
| 5 (rum : whole) | 3 | 0.83 (*0.94*) | 0.59 – 0.96 | 0.94 | 0.72 (*0.88*) |
| 7 (mixed : whole) | 1 | 0.94 (*1.00*) | 0.73 – 1.00 | 0.94 (*1.00*) | 0.90 (*1.00*) |

Table 1. Accuracy, accordance and concordance of microscopic detection of animal tissues for nine contaminated feed materials from nine labs. Results without participant G (which was found to be a straggler in the outlier test) are given in parentheses when different.

[1] Equals specificity for the true negative feeds (1-3) and sensitivity for the true positive feeds (4-9).

| fish meal: | none | trimmings | whole fish | |
|---|---|---|---|---|
| **MBM** | | | | average |
| **ruminant** | 1.00 (*1.00*) | 1.00 (*1.00*) | 0.83 (*0.94*) | 0.94 (*0.98*) |
| **mixed** | 1.00 (*1.00*) | 1.00 (*1.00*) | 0.94 (*1.00*) | 0.98 (*1.00*) |
| average | 1.00 (*1.00*) | 1.00 (*1.00*) | 0.89 (*0.97*) | |

Table 2. Accuracy (equals sensitivity) for the results of the treatments involving the fish meals / MBM combinations. The values are extracted from table 1; column and row averages are given for easy comparison of ingredients. Results without participant G are given in parentheses.

(between lab reproducibility) is sub optimal for the detection of ruminant MBM in the presence of fish meal in general (table 2). Otherwise it is also clear that especially the whole fish meal caused a relatively low concordance for the detection of MBM, since all four false negatives are found in the presence of whole fish meal. The randomisation test for outliers was applied to the false negative and false positive results of the ring trial (see summary in table 3). Laboratory G reported three of the four false negative results, and this participant is therefore a possible outlier for false negatives. In four cases false positives were encountered. Two of them concern the blanks (feed material 1) as investigated by participant C, i.e. only one or two bone fragments of terrestrial animals were detected in the absence of fish meal. Participant C reported that for the two samples of feed 1 a second sediment has been produced in which the finding of one or two bone fragments in each was confirmed. Thus the results of laboratory C are retained as false positives and this participant is a possible outlier for false positive results.

| Laboratory: | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| feed codes of false negative results | 5 | - | - | - | - | - | 5,5,7 | - | - |
| feed codes of false positive results | - | - | 1,1 | - | - | - | - | 3 | 3 |

Table 3. Summary of erroneous results from the ring trial to detect MBM in 6 true positive (coded 4-9) and 3 true negative (coded 1-3) feed materials. Each laboratory analysed two (blinded) samples per feed.

In table 4 the results of the randomization tests are reported. Three false negatives in one laboratory occurred in less than 5 % of the randomizations (but not in less than 1 % of the randomizations), and therefore participant G with 3 false negative results can be called a straggler.

| | $T_{data}$ (potential outlier) | $T_{rand.\ 95\%}$ | $T_{rand.\ 99\%}$ | conclusion |
|---|---|---|---|---|
| false negatives | 3 (lab G) | 2 | 3 | Result G is a straggler |
| false positives | 2 (lab C) | 2 | 3 | not significant |

Table 4. Randomization test for one outlying high result in ring trial (10,000 iterations). *T* is the highest number of erroneous results per laboratory.

The result of participant C could not be considered as a statistical outlier or straggler (table 4). The other two false positives were found in the presence of whole fish meal (participant H and I) and both were found in the same sample (8).

# 4. DISCUSSION AND CONCLUSIONS

## 4.1 Discussion

The results of the homogeneity testing as well as the sediment percentages indicate that the results suits the purpose of a ring trial and that a proper evaluation can be carried out.

In most cases the presence of animal tissues is properly detected, even in the presence of a considerably higher amount of fish material. The overall accuracy is good or acceptable. Considering the situation that participant G can be indicated as an straggler, the concordance (between lab reproducibility) and accordance (within lab repeatability) are good. The used mammalian material includes two different sources, i.e. ruminant and mixed species, but more important these two materials represent two different ways of processing. The ruminant MBM was specifically processed in a small scale pilot plant (batch system) and showed some influences of high temperature treatment. The mixed species MBM was taken from a regular industrial plant in the Netherlands. The latter one, representing the normal rendering procedures, did not cause any difficulty for detection. The deviating appearance of the ruminant MBM (see images) might have caused somewhat more identification problems, despite the fact that the ruminant MBM contains significantly more bones than the mixed species MBM (54% vs. 13%). On the other hand, all false negatives and two of the false positives concern samples with whole fish meal included (table 1). This type of fish meal might contain particles that can more easily be confused with terrestrial animal tissues than found in the trimmings. A remarkable detail is the comment “difficult to recognise” that participant H added for sample 9 (treatment 4) containing the ruminant MBM, i.e. in the presence of trimmings instead of the whole fish meal.

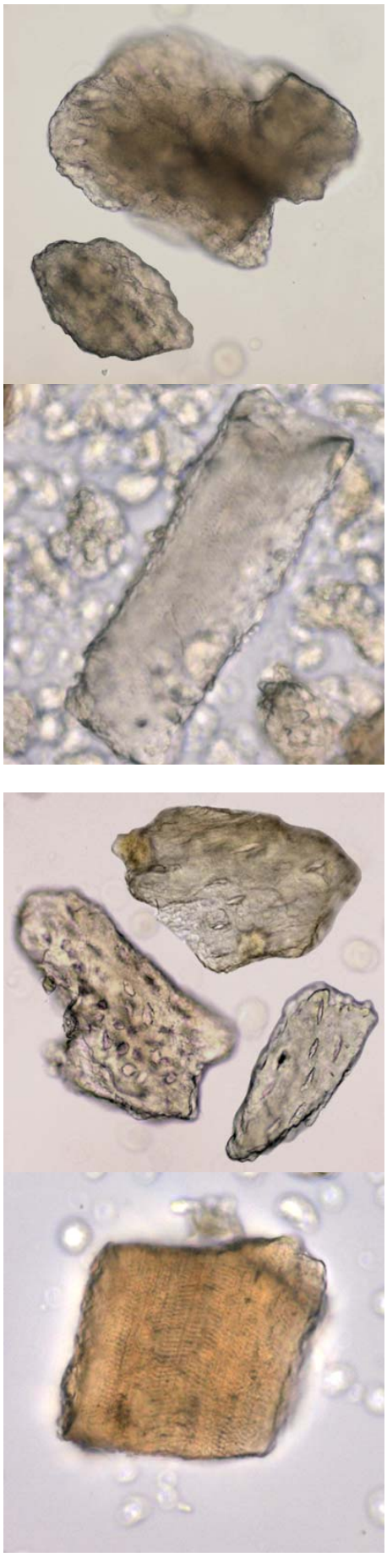
The results of the current study indicate the possibility of reliable detection and identification of terrestrial animal tissues against fish material. Different types of fish meals might influence the detection of terrestrial animal tissues. Documentation and information for support of the identification process can enhance the detection of animal tissues with less known appearances and the distinction from specific fish material. Identification support systems for this end are currently in development (Frick at al., 2002; Gizzi et al., 2003).

The specificity is good or in cases of a score lower than 100% errors can be considered as random based on the confidence intervals. The presence of some false positives relates to two different types of problems. In the three cases regarding presumed detection of MBM in the presence of fish meal (two occasions) and presumed detection of fish meal in the presence of MBM (one occasion) confusing of one or a few particles might cause the erroneous conclusion. It is known that especially cartilage particles might be confused. In all three cases the identical replicate was correctly identified, which points to random errors occurring occasionally. Also in these cases a proper documentation and identification system might help to avoid virtually all of such cases. The presence of two presumed detections of terrestrial animal material in the two blanks might theoretically be caused by either contamination of the pure feed by rodents (mice), cross contamination during sample preparation, cross contamination during lab research, or by confusion with plant material or with minerals. The feed was found to be free of animal proteins. During sample preparations the blanks have been produced prior to opening the bags with the MBM material. The absence of animal tissues in the blanks was approved by the results of eight labs. Lab procedures are usually carefully applied and cross contamination during research in the lab of participant C would have caused much more false positives than the current number of two. Therefore, cross contamination is considered

unlikely. The samples should only have been reported positive in the case of applying the zero tolerance rule. Some EU member states apply a certain lower level of detection in practice, in which case these samples should have been considered free of animal proteins. The particles are documented, which allows a further discussion and a proper evaluation in the framework of proposed documentation systems is recommended.

The protocol as followed by the participants appeared to be not completely harmonised (Appendix 6). Two participants reported a deviating sample size for sedimentation. A different type of separation funnel, a short sedimentation time and low number of slides observed was each reported once. The smaller or shorter the mentioned factors are the more likely traces can be missed. Participants B and I, without reporting any false negative, were apparently able to cope with the deviating circumstances. The short sedimentation time as applied by participant G might have influenced the number of false negatives for detecting MBM (3), in combination with the amount of 2 grams of material that has been used for sedimentation. Participant G did not report sediment percentages. Notwithstanding the results, all parameters as applied and reported by any participant in this study fit into the requirements of EU Directive 98/88/EC (EU, 1998).

Microscopic detection of animal tissue has proven to be effective in a ring trial carried out in 1997 and 1998 (Engling et al., 2000). The sample set in that ring trial consisted of three batches with two feed samples each, contaminated with a mixed species MBM at levels of 3%, 0.5%, 0.5%, 0.1%, 0.02% and 0%. Nineteen laboratories participated in the ring study. No false positives were found and only one false negative was found at the 0.02% contamination level. Fish meal was excluded from this ring trial (Engling et al., 2000). Taking this into consideration, the results are comparable with the current study except for the presence of some false positives as already discussed.



Images of bones and muscles of pure ruminant MBM (top) and of mixed species MBM (bottom). The ruminant MBM shows a more severely processed appearance; lacunae (holes containing bone cells) and muscle cross striation are less visible compared to the mixed species MBM.

## 4.2 Conclusions

The main conclusions of this study are:

- Terrestrial animal tissues can properly be detected in the presence of fish meal, even in the case that the MBM consists of a low fraction of bones (13%).
- The unambiguous detection of animal tissues depends partly on the appearance of the MBM particles together with a specific type of fish meal as well as on optimised lab protocols. Development of documentation systems and training is advised, as well as further harmonisation of the protocol as described in Directive 98/88/EC.
- The lower detection limit seems to be reached at contamination levels around 0.1 % when fish meal is present, since in several occasions only traces are found.
- When accepting a limit of detection higher than zero instead of the "zero tolerance" rule for the false positives would have been resulted in absence indications in most or all cases.

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# Appendix 1. Outliers in ring tests of analytical methods with qualitative results

For measurement methods with quantitative results the Grubbs' and Cochran's outlier tests are routinely used for the detection of deviating laboratories in ring tests (ISO, 1994b). In this note an outlier test is described for the case of a ring test of analytical methods where only qualitative results are reported.
As an example consider the data in table 1-1. Here the suspicion (formulated *a posteriori*) is that laboratory D performs worse than the other laboratories with regard to false negatives. Is such a conclusion statistically significant?

| Laboratory: | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| sample numbers of false negative results | 3 | - | 3 | 3,4,5,7 | - | - | - | 3,5 | - |

Table 1-1. Example 1. Artificial results from a ring test with 10 true positive samples.

Such hypotheses can be tested in a simple way using randomization tests. In a randomization test a null hypothesis about treatment effects is tested by generating the distribution of a test statistic based on permutations of the data (Edgington, 1995, Manly, 1991). In the case of an outlier test for one outlying laboratory producing more false results in a ring trial it is natural to consider the highest number of errors per laboratory as a test statistic $T$. So in the first example we have $T_{data} = 4$ from the actual data, for the 4 false negative results of laboratory D. Now under the null hypothesis that all laboratories have an equal performance, the distribution of the eight errors over the laboratories could have been different. In a randomization test a large number (*e.g.* 10,000) permutations of the error indicators are generated. For each permutation the same test statistic as before is calculated, thus generating a null distribution of values $T_{rand}$. For a test at the usual 95% or 99% confidence level, we can then compare the observed $T_{data}$ with the 95$^{th}$ or 99$^{th}$ percentile of the generated distribution of values $T_{rand}$. In analytical chemistry it is customary to denote a significant result at the 99 % confidence level as a statistical *outlier*, and a result which is significant at the 95 % but not the 99 % confidence level as a *straggler* (ISO, 1994b). The result for example 1 in table 1-2 shows that here laboratory D can indeed be considered a straggler.

| | $T_{data}$ (potential outlier) | $T_{rand.\ 95\%}$ | $T_{rand.\ 99\%}$ | conclusion |
|---|---|---|---|---|
| false negatives | 4 (lab D) | 3 | 4 | Result D is a straggler |

Table 1-2. Randomisation test for one outlying high result in example 1 (10,000 iterations). $T$ is the highest number of erroneous results per laboratory.

It is interesting to consider a second example (table 1-3 and 1-4). Here the numbers of errors are the same as in the first example, but the errors are distributed over more samples (seven, compared to only four in the first example). Now in a randomization test concerning a hypothesis on the laboratories we make no assumption that all samples are equally easy or difficult to analyse. Consequently, in the first example we want to respect the fact that there were four errors on sample 3 (perhaps this sample was more difficult than other samples), and two errors on sample 5.

| Laboratory: | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| sample numbers of false negative results | 6 | - | 3 | 3,4,5,7 | - | - | - | 2,9 | - |

Table 1-3. Example 2. Artificial results from a ring test in 10 true positive samples.

| | $T_{data}$ (potential outlier) | $T_{rand.\ 95\%}$ | $T_{rand.\ 99\%}$ | conclusion |
|---|---|---|---|---|
| false negatives | 4 (lab D) | 4 | 4 | not significant |

Table 1-4. Randomisation test for one outlying high result in example 2 (10,000 iterations). *T* is the highest number of erroneous results per laboratory.

In general, the errors should be randomized *within* samples. If we apply this procedure to examples 1 and 2 then we find 95$^{th}$ percentiles in the randomization distribution of $T_{rand}$ equal to 3 and 4, respectively. This means that the result of laboratory D ($T_{data}$ = 4) is considered a straggler in example 1, whereas the same result is not significant at this confidence level in example 2. This is a reasonable conclusion because the relatively high number of errors in laboratory D stands out against the baseline error rate more clearly in the more structured sample-by-error matrix of example 1.
Note that the test described so far is a one-sided test, which is reasonable in this case: we do not expect laboratories with significantly less errors than the rest (and in any case we would not be able to detect such laboratories, unless the typical situation would be that almost all laboratories would make errors on several samples in the ring trial).
The randomization test for qualitative data described here is the equivalent of Grubb's test for quantitative data as it tests for outlying levels. For qualitative data there is no equivalent for Cochran's test (which tests for laboratories with unusually high variation in the results) because for binomial data the variation is directly related to the level, but is zero both when no errors are made and when all samples are misclassified.
In analogy with the Grubbs' test for two upper outliers (ISO, 1994b) we may also test if the two largest numbers of false results are outliers. Using the same randomizations as for the single-outlier test, we just take the test statistic *T* now as the sum of the the two highest numbers of errors, and compare this with the same statistic calculated from the data ($T_{data}$ = 4+2 = 6 for the false negative results in both examples given here, see table 1-5).

| | $T_{data}$ (potential outliers) | $T_{rand.\ 95\%}$ | $T_{rand.\ 99\%}$ | conclusion |
|---|---|---|---|---|
| example 1 | 6 (labs D,H) | 5 | 6 | Pair (D,H) is a straggler |
| example 2 | 6 (labs D,H) | 6 | 6 | not significant |

Table 1-5. Randomisation tests for two outlying high result in examples 1 and 2 (10,000 iterations). *T* is sum of the two highest numbers of erroneous results per laboratory.

When laboratories analyze multiple replications of the samples then care should be taken not to consider these as separate samples. If each of *L* laboratories analyze *s* subsamples, then the total number of subsamples is $n = Ls$ and the randomization of error indicators should be among all *n* subsamples of each sample.

# Appendix 2. Preparation of the samples

### Stepwise dilution

Initially feed material is contaminated with fish meal (right column) in order to produce stock material for all treatments that are based on feed containing fish at 5%. 140 grams of fish meal are mixed with 140 grams of feed, this mixture is mixed with 280 grams of feed. The resulting mix of 560 grams contaminated feed has a concentration of 25 % fish. From this mixture 180 grams is taken to produce 900 grams of feed with a final contamination level of 5% of fish.

Likewise 2 grams of MBM was taken to mix with 4 grams of feed (left column), either with or without fish. The resulting 6 grams were mixed with 10 grams of feed w/o fish. After a third mixing step 32 grams had been produced with a contamination level of 6.25% of MBM. From this mixture 14 grams was taken to mix with 28 grams of feed w/o fish to produce 42 grams of mixture with a contamination level of 2.08%. After two further mixing steps a final amount of 856 grams of feed contaminated with MBM at 0.1% was obtained.

| **MBM** | | feed | | | mix time |
|---|---|---|---|---|---|
| | | | % | | min |
| **2** | + 4 = | 6 | 33,33 | | 3 |
| 6 | + 10 = | 16 | 12,50 | | 3 |
| 16 | + 16 = | 32 | 6,25 | | 3 |
| 14 | + 28 = | 42 | 2,08 | | 3 |
| 42 | + 84 = | 126 | 0,69 | | 3 |
| 126 | + 730 = | 856 | 0,10 | | 3 |
| 856 | | | | | |

| **fish** | | feed | | | mix time |
|---|---|---|---|---|---|
| | | | % | | min |
| **140** | + 140 = | 280 | 50 | | 3 |
| 280 | + 280 = | 560 | 25 | | 3 |
| 180 | + 180 = | 360 | 12,5 | | 3 |
| 360 | + 540 = | 900 | 5 | | 3 |
| 900 | | | | | |

# Appendix 3. Homogeneity testing

| treatment | Basis | Sediment % | Animal proteins |
|---|---|---|---|
| 8 (r -) | Mix of cereals and rape | 0.8 | present |
| 8 (r -) | Mix of cereals and rape | 0.8 | present |
| 8 (r -) | Mix of cereals and rape | 0.8 | present |
| 9 (m -) | Mix of cereals and rape | 1.0 | present |
| 9 (m -) | Mix of cereals and rape | 1.0 | present |
| 9 (m -) | Mix of cereals and rape | 1.0 | present |

## Appendix 4. Basic results

Results of nine labs (A to I) for 18 spiked feed samples. The real contents with respect to MBM and Fish are given in columns "real" (1 = present, blank = absent). The type of MBM is indicated. Samples are numbered according to the processing scheme in Appendix 1. False negatives are indicated by "-", false positives are indicated in bold and italic; traces in brackets.

| | | real | | A | | B | | C | | D | | E | | F | | G | | H | | I | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | type | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F | MBM | F |
| 3 | - | | | | | | | *(1)* | | | | | | | | | | | | | |
| 17 | - | | | | | | | *(1)* | | | | | | | | | | | | | |
| 7 | ruminant | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | |
| 13 | ruminant | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | |
| 4 | mbm | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | |
| 15 | mbm | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | *(1)* | 1 | |
| 9 | ruminant | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 11 | ruminant | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 1 | ruminant | 1 | 1 | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 1 | 1 | (1) | 1 |
| 16 | ruminant | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 1 | 1 | 1 | 1 |
| 2 | mbm | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 18 | mbm | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | mbm | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 1 | 1 | 1 | 1 |
| 10 | mbm | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | - | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 |
| 14 | - | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 |
| 8 | - | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | ***1*** | 1 | ***1*** | 1 |
| 12 | - | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 | | 1 |
| | PA | | | 11 | 12 | 12 | 12 | 14 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 9 | 12 | 13 | 13 | 13 | 12 |
| | NA | | | 6 | 6 | 6 | 6 | 4 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 5 | 5 | 5 | 6 |
| | ND | | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| | PD | | | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| | Accuracy | | | 0.94 | 1.00 | 1.00 | 1.00 | 0.89 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.83 | 1.00 | 0.94 | 0.94 | 0.94 | 1.00 |

## Appendix 5. Sedimentation

Results of the sedimentation procedure, in percentages of the total feed. The real contents with respect to MBM and Fish are given in columns "real" (1 = present, blank = absent). The type of MBM is indicated. Samples are numbered according to the processing scheme in Appendix 1.

| | | real | | | | A | B | C | D | E | F | I |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type | | MBM | F | average | SD | | | | | | | |
| 3- | | | | 1,28 | 0,28 | 1,1 | 0,93 | 1,5 | 1,3 | 1,1 | 1,3 | 1,75 |
| 17- | | | | 1,33 | 0,18 | 1,4 | 1,32 | 1,5 | 1,1 | 1,2 | 1,2 | 1,62 |
| 7 | ruminant | 1 | | 1,24 | 0,43 | 1,1 | 0,92 | 1,3 | 1,3 | 1,0 | 0,9 | 2,13 |
| 13 | ruminant | 1 | | 1,12 | 0,32 | 1,0 | 1,00 | 1,4 | 1,0 | 0,8 | 0,9 | 1,72 |
| 4 | mbm | 1 | | 1,09 | 0,22 | 1,1 | 1,06 | 1,4 | 0,9 | 1,0 | 0,8 | 1,34 |
| 15 | mbm | 1 | | 1,11 | 0,21 | 1,2 | 1,08 | 1,1 | 0,9 | 0,9 | 1,1 | 1,52 |
| 6- | trimmings | | 1 | 1,61 | 0,18 | 1,7 | 1,59 | 1,6 | 1,7 | 1,5 | 1,3 | 1,89 |
| 14- | trimmings | | 1 | 1,71 | 0,26 | 1,5 | 1,64 | 2,0 | 1,7 | 1,5 | 1,5 | 2,14 |
| 9 | ruminant trimmings | 1 | 1 | 1,63 | 0,28 | 1,5 | 1,69 | 2,0 | 1,5 | 1,4 | 1,3 | 2,00 |
| 11 | ruminant trimmings | 1 | 1 | 1,55 | 0,28 | 1,1 | 1,38 | 2,0 | 1,7 | 1,5 | 1,6 | 1,57 |
| 2 | mbm trimmings | 1 | 1 | 1,79 | 0,35 | 1,5 | 1,53 | 2,0 | 1,7 | 1,7 | 1,6 | 2,48 |
| 18 | mbm trimmings | 1 | 1 | 1,63 | 0,24 | 1,5 | 1,51 | 1,7 | 1,6 | 1,6 | 1,4 | 2,13 |
| 8- | whole fish | | 1 | 2,10 | 0,21 | 2,0 | 2,22 | 2,3 | 2,4 | 1,8 | 2,0 | 1,98 |
| 12- | whole fish | | 1 | 2,35 | 0,47 | 2,9 | 2,22 | 3,0 | 1,8 | 2,5 | 1,9 | 2,10 |
| 1 | ruminant whole fish | 1 | 1 | 2,03 | 0,42 | 2,0 | 2,25 | 2,5 | 1,6 | 1,6 | 1,7 | 2,58 |
| 16 | ruminant whole fish | 1 | 1 | 2,15 | 0,20 | 2,2 | 1,87 | 2,3 | 2,1 | 2,1 | 2,0 | 2,50 |
| 5 | mbm whole fish | 1 | 1 | 2,12 | 0,26 | 1,9 | 2,36 | 2,2 | 2,1 | 1,7 | 2,1 | 2,46 |
| 10 | mbm whole fish | 1 | 1 | 2,04 | 0,29 | 1,9 | 1,93 | 2,5 | 2,1 | 2,0 | 1,6 | 2,26 |
| | | | | | | | | | | | | |
| average without fish | | | | 1,19 | | 1,15 | 1,05 | 1,37 | 1,08 | 1,00 | 1,03 | 1,68 |
| average with fish | | | | 1,89 | | 1,81 | 1,85 | 2,18 | 1,83 | 1,75 | 1,67 | 2,17 |
| average total | | | | 1,66 | | 1,59 | 1,58 | 1,91 | 1,58 | 1,50 | 1,46 | 2,01 |
| solvent | | | | | | TCE | TCE | TCE | TCE | TCE | TCE | $CHCl_3$ |

# Appendix 6. Procedure

Details on the procedure followed by the participants. The sample size is the amount of material used for producing the sediment. The abbreviations for the solvent are explained in the foot note. The time reported is the period that the material was allowed to sink. Two fraction can be observed: the flotation and/or the sediment. The stereo microscope can be used to view the larger particles.

| participant | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| sample size (g) | 10 | 10 | 10 | 10 | 10 | 10 | 2 | 10 | 2 |
| type of solvent | TCE | TCE | TCE | TCE | TCE | TCE | TCE | TCE/ CHCl3 | CHCl3 |
| separation funnel | closed | closed | closed | open | closed | closed | test tube | open | open |
| time (min) | 5 | 5 | 6 | 20 | 3-5 | 5 | 1 | 2-5 | 2 |
| observed fractions | both | both | both | both | sediment | sediment | sediment | both | both |
| # of slides | 3 | 1 | 3 | 2 | 4 | 2-4 | 3 | 1-5 | 3 - >3 |
| stereo microscope | yes | no | yes | yes | yes | no | yes | yes | yes |

Densities:

| Abbr. | Name of the solvent | density |
|---|---|---|
| CHCl3: | chloroform | 1.48 |
| TCE: | 1,1,2,2-tetrachloro ethane | 1.60 |