Chapter 4
Towards reference materials as a tool for validating results of MBM in animal feed

Jean Charoud-Got[1], Marie-France Tumba-Tshilumba[1], Vincent Baeten[2], Jeroen Van Cutsem[3] and Håkan Emteborg[1*]

(1) European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium;
Jean Charoud-Got jean.charoud-got@ec.europa.eu
Marie-France Tumba-Tshilumba marie-france.tumba-tshilumba@ec.europa.eu
Håkan Emteborg hakan.emteborg@ec.europa.eu

(2) Walloon Agricultural Research Centre (CRA-W), Gembloux, Belgium;
Vincent Baeten baeten@cra.wallonie.be

(3) Federaal Laboratorium voor Voedselveiligheid Tervuren (FLVVT), Tervuren, Belgium.
Jeroen Van Cutsem jeroen.vancutsem@favv.be

* To whom correspondence should be addressed: Tel. +32 14 571 708.

TOWARDS REFERENCE MATERIALS AS A TOOL FOR VALIDATING RESULTS OF MBM IN ANIMAL FEED

SUMMARY

In order to ensure the quality of data obtained from control laboratories measuring meat and bone meal (MBM) in feed, IRMM has developed concepts and starting materials for the possible production of reference materials in this field. The measurements to be verified are both qualitative and quantitative to their nature. Two main routes have been tested for the development of such materials, namely dry-mixing of bulk materials and direct gravimetric preparation of individual samples. The first approach consisted of addition of known amounts of meat and bone powders to a maize powder and subsequent 3-dimensional mixing. The resulting powder was then split up in different sample containers and checked for homogeneity. The second approach consisted of a production with exactly known masses of meat and bone meal powder added to a standard background matrix of maize powder prepared directly in each jar. The latter samples could only be used in single-shot analysis. The existing official method (as described in Annex VI of Commission Regulation EC 152/2009) based on sedimentation and light microscopy was used for evaluation of materials as well as NIR microscopy and PCR. This chapter describes the concept, raw materials, the two different approaches for preparation and analytical results. The results are promising in so far that the best set of results obtained for the samples prepared by direct gravimetric preparation were within 10 % of the known target with a precision of ±11 % RSD using the official EU-method. Possible routes for future material preparation and measurement developments are also discussed.

Key words: Meat and Bone meal, European feed legislation, Reference Materials, Validation, Light Microscopy.

4.1. INTRODUCTION

Meat and bone meal (MBM), processed animal protein (PAP) of terrestrial animals, is banned for use in animal feed in the European Union. The ban is implemented to eliminate the risk of TSE (Transmissible Spongiform Encephalopathy) transmission (mad cow disease) in livestock and subsequent transmission to humans. The Commission Regulation EC 152/2009 is describing how detection, identification and estimation of MBM in feed should be performed. A simplified description of this method reveals that it is mainly based on sedimentation of bones from at least 5 g of animal feed in tetrachloroethylene (density 1.62 g/ml) and subsequent counting of bone particles in the resulting sediment using light microscopy. Until now, validation of the analytical method by using reference materials has not been possible because of lack of such materials. A reference material is a material that is sufficiently *homogeneous* and *stable* with respect to one or more specified properties, which has been established to be *fit for* its *intended use* in a measurement process as defined in ISO Guide 30. The homogeneity of the key component in the sample taken for analysis (i.e. the bones) is therefore of vital importance for any candidate reference material in this field. Indeed the stability of the key components is important too, but fortunately it is safe to assume that bone particles are not easily destroyed in the dry matrices investigated here so this fundamental aspect has not been included in the feasibility studies. It should be noted that the degree of heterogeneity that can be detected for a certain parameter in a material is also interlinked with the repeatability of the measurement method. The relationship between material and method is illustrated in Figure 1 and how an iteration process comprising of measurements and improvements of the material preparation leads to a material with a better homogeneity that eventually could be used as a reference material. The aim of the study has been to check if the current methods (sedimentation, light microscopy, PCR and NIR microscopy) are reliable enough to be used for preparation of reference materials for MBM in animal feed. Two main approaches have been used to spike blank feed with bone and meat namely dry mixing and direct gravimetric preparation. The current research as been part of the SAFEED-PAP project work package 6, Task 15, entitled "Feasibility study of the production of reference materials". The research that has been undertaken is exploratory as there are currently no reference materials in this specific field.

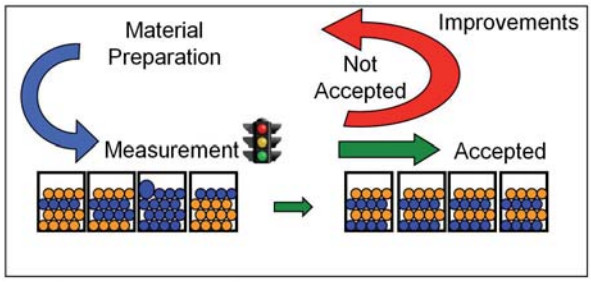

Figure 4.1. Schematic illustration of material preparation based on repeated measurements of the heterogeneity of a key parameter and improvements in the material processing. The improved homogeneity is illustrated among the samples to the right with no between bottle heterogeneity.

4.2. MATERIAL AND METHODS

4.2.1 Concept

Mixtures of pure bone meal, pure meat meal of bovine and porcine origin in maize powder has been prepared in two different ways. The underlying principle for the preparation of all these materials has been to control the mass ratio bone to total MBM (*f*-factor) as outlined in the Commission regulation 152/2009 by using a pure meat and a pure bone component dispersed in one single background matrix of maize. The assumption is that this simplified system would be easier to prepare and evaluate in comparison with real samples that have a multitude of components of different origin and no clear information on the *f*-factor. The problem of the complexity between these simplified matrices and real samples has not been addressed explicitly. The likelihood increases however that bias or problems connected with the official method can be detected and overcome if one is able to verify the trueness and precision of the official method on these "simplified samples". Otherwise small problems with the method are swamped by the huge impact very heterogeneous samples have on the results. This approach is normal in breaking new ground for reference material preparation. One normally starts with a simple system and then increases the complexity of the samples in order to tune the laboratories and methods to higher levels of skill and performance.

4.2.2 Dry mixing

In this approach a relatively large amount of bulk materials are carefully mixed and then split up over many sample containers. To be more specific, the materials were dry-mixed using a three-dimensional mixer (Dynamix CM-200, WAB, Basle, Switzerland) as depicted in Fig. 2 and the resulting bulk material was equally divided over individual sample containers using a vibrating feeder. It is possible to take subsamples from the same jar with this approach.

Figure 4.2. The WAB Dynamix CM-200 three-dimensional mixer.

4.2.3 Gravimetric preparation

A known quantity of bones or meat was precisely weighed and added to 0.7 g or 10 g maize in each jar with a precision of ± 1 mg per meat or bone component. The exact composition of the mixture in each jar was therefore known with a high degree of certainty. The content of each jar must be used in single-shot analysis. It is not possible to take subsamples from the same jar with this approach. It is also not necessary to shake the bottle before use. Transfer of all material must be ensured.

4.2.4 Raw materials

Refined and de-fatted samples of pure meat and bone were obtained from Rendac (Son. NL) and Sonac Vuren, NL through CCL Nutritional (Veghel, The Netherlands) and CRA-W (Gembloux, Belgium). All MBM products had previously been treated thermally at 135 °C to destroy possible pathogens. Virtually no meat particles in the bone material and no bone particles in the meat material were present in the raw materials. To achieve this purity, the porcine bones meal was also treated enzymatically at 65 °C for 30 minutes using alkalase and the bovine bones meal were obtained by sedimentation of a bovine bone/meat mixture and checked by light microscopy for purity. Prior to mixing the different starting materials were characterized using particle size distribution measurements by laser diffraction

(Sympatec Helos, Clausthal-Zellerfeld, Germany) and water content measurements by volumetric Karl-Fischer titration (Metrohm, Herisau, Switzerland). It can be stated, in general terms, that powder with similar particle size distribution mix better as reported by Johnson in 1974. But there are numerous other parameters that affect mixing behaviour like density, water content, fat content, particle shape etc. The maize was provided from an internal IRMM source and had been rinsed in water, milled and sieved (< 500 µm) in previous experiments. The meat was milled using a Fritsch Pulverisette mill with a 500 µm sieve insert prior to use (Idar-Oberstein, Germany). The bones were sieved on an industrial sieve (Russel Finex, London, UK) and the fraction smaller than 500 µm was used in further experiments.

Micrographs were taken to verify the findings of the laser diffraction measurements. An example of particle size distributions of the ingoing components of pork bone and pork meat and maize is displayed in Figure 3.

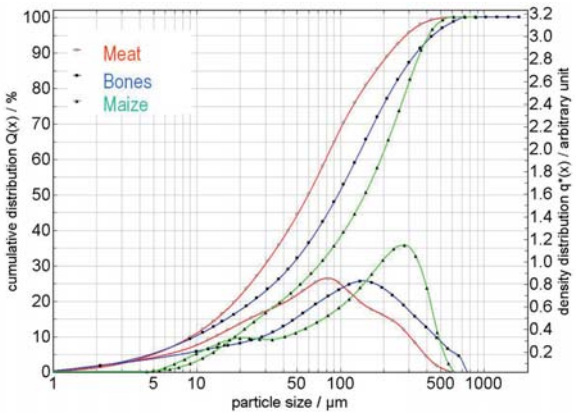

Figure 4.3. Overlay of particle size distributions of maize, porcine bones and porcine meat using the cuvette and lens R6 with isopropanol as dispersant. Open circle = meat, square = bones and triangle = maize.

The water content is an important parameter for stability as it controls the biological activity in a sample. The result in Table 1 is an average of two water measurements ± one standard deviation. Following the screening of the ingoing components it can be concluded based on previous experience that they are sufficiently dry not to jeopardise the quality of the materials to be prepared although only a rigorous stability test can prove stability in the case of a real production of a reference material.

Table 4.1. Summary of water content in the different components for n = 2.

Sample	Water content , % (m/m)
Bovine meat	4.2 ± 1.0
Bovine bones	6.2 ± 0.0
Porcine meat	1.0 ± 0.0
Porcine bones	1.7 ± 0.2
Maize	1.3 ± 0.2

Two collaborating laboratories measured one sample each of 10 g maize provided in a clean glass bottle in order to check that the blank maize itself does not contribute significantly to the mass of 'bone' measured by weighing of the sediment. The result is not completely unambiguous since some sediment was found by both laboratories although at rather low level as given in Table 2. These particles are not bone but possibly starch particles or other heavier particles, such as originating from the seed coat, present in the blank maize as confirmed by light microscopy. No correction has been done to subtract the blank value because the levels found are low and vary significantly. In addition there does not seem to be reports in the literature that describe the completeness or selectivity of the sedimentation method or rule out that 'lighter' particles of plant origin could still be found in the sediment. Indeed the blank maize was also checked with PCR and no DNA of animal origin (pig) was detected.

Table 4.2. Blank test on maize performed on approximately 10 g of maize.

Laboratory	Mass of maize (g)	Mass of sediment detected (g)	Mass fraction of sediment in maize % (m/m)
A	10.02	0.0184	0.18
B	10.00	0.0118	0.12

4.2.5 Analytical methods

The following four analytical methods have been used by the collaborating laboratories (CRA-W and FLVVT) for assessing the homogeneity of the materials with the target analyte in parenthesis.

1. Sedimentation with tetrachloroethylene (C_2Cl_4) and weighing of sediment (bones)
2. Sedimentation using C_2Cl_4 followed by light microscopy according to the Commission Regulation EC 152/2009 (bones).
3. Sedimentation using C_2Cl_4 followed by NIR microscopy (bones)
4. Real time polymerase chain reaction (PCR) of target DNA fragments, (meat + bone)

As can be seen the major emphasis lies in detecting bones, although the current Commission Regulation lists several other animal tissues that may be present in meat and bone meal (horn, hair, cartilage muscle etc.). The component that is easiest to discern using a light microscope in the resulting sediment is nevertheless bone particles. From a material preparation point of view it can be safely assumed that the risk of segregation (leading to heterogeneity) is larger for the bone particles than for the meat particles in the maize background matrix due to the higher density and compact shape. It is therefore advantageous to try to establish to which degree the material is homogeneous for the bone fraction in the resulting mixtures since the meat fraction can be expected to be less affected by these segregation effects. Nevertheless, the meat fraction has to be estimated by PCR although it is well known that this method has inherently a poor precision which is not ideal when trying to establish homogeneity for a material. In addition, the MBM component is heavily heat-treated resulting in degradation of DNA. Finally only 0.1 to 1.0 g of sample is normally used for the extraction step preceding PCR analysis which is much lower than the 10 g or 3 g applied in this work for the sedimentation method.

4.2.6 Sample preparation by dry-mixing for samples with high and low level

In order to achieve good and efficient mixing of the ingoing components a Dynamix CM-200, three-dimensional mixer (WAB, Basel, Switzerland) was used throughout (Fig. 2). The high mixing efficiency is achieved by its mode of operation. When running this mixer at 53 % of the maximum speed on both axes, and when applying the same direction of rotation of both axes throughout the programme, the mixing motion is highly similar to that of a T-200 Turbula mixer. The mixing in a Turbula mixer is based on the Paul Schatz principle which can be described as an inverted mixing-motion describing the figure eight (kinematic inversion). In essence this mixing movement is very efficient, rapid, and at the same time gentle and does not result in segregation or milling effects. The movement is gently pulsating and the particles experience forces in two directions at the same time. It has therefore a very different mode of action in comparison with a traditional V-mixer or roll-mixer. Over the past two decades a T-200 Turbula mixer has been used for numerous reference material preparations where homogeneity has been proven for a large number of different materials and analytes. All certification reports give evidence of homogeneity for the target analytes and hence successful homogenisation by using the T-200 Turbula mixer (http://irmm.jrc.ec.europa.eu/html/homepage.htm). For the first dry-mix experiment a sample was prepared from 12.5 g of pork bones, 12.5 g of pork meat and 475 g of maize powder, all previously characterised as described in section 4.2.2 to obtain a 5 % mixture (m/m) of MBM in maize (2.5 % for each component). The components were placed in a plastic drum with a screw-cap lid (Overtoom, Den Dolder, The Netherlands) maintaining a sufficient head-space volume for mixing. Thereafter the drum was placed centrally in a stainless steel drum mounted in the Dynamix CM-200 mixer and mixed for 4 hours. After mixing of the 5 % material 100 g of this material was withdrawn and diluted with 400 g of maize and again subjected to 4 hours of mixing in the Dynamix CM-200. The resulting mixture was consequently 1 % in MBM with an *f*-factor of 0.5 (m/m). Both materials were filled in 11 g portions in 60 ml amber glass bottles with a PE-insert. Finally 43 bottles of the 1 % material and 34 bottles of the 5 % material were produced.

4.2.7 Direct gravimetric preparation

Direct gravimetric preparations of control samples were performed under strict control on a calibrated analytical balance with four decimal digits to provide information on bias and precision of the analytical methods used. Such an evaluation can only be made if the whole content of the bottle is used. Obviously the dry-mix samples can not be used for such purposes because one can not be sure that the components are equally distributed over the samples produced. For direct gravimetric preparation, two sample preparations and subsequent evaluations have been made called Phase-1 and Phase-2 samples, respectively. In tables 3 and 4 the compositions are given for the Phase-1 and Phase-2 materials. An exact amount of pork bone of the same origin and treatment as the bone used to prepare the dry-mixture was carefully weighed into a 10 ml vial as given in Table 3. Thereafter about 0.7 g of maize was added to obtain the Phase-1 samples. The maize was added to facilitate

quantitative transfer of the bones when subsequently analysing these samples. During the gravimetric preparation, the masses placed in the sample container were checked by controlling the sum of the individual components added and the measured total mass of the container with the components. Only samples that passed the criterion of a deviation below ± 1 mg per component have been sent to the collaborating laboratories. Based on that pass criterionr the relative standard deviation (RSD) of the bone component in material D was, only 1.4 % at the 0.7 % contamination level (m/m). In the Phase-1 sample, where 0.7 g samples of maize were prepared (Table 3) the samples were evaluated by sedimentation only. In Phase 2, the 10 g samples of maize as given in Table 4) were evaluated by sedimentation followed by light microscopy. In addition to that the Phase-2 samples contained more than two components, namely porcine bone meal, porcine meat meal, bovine bone meal and bovine meat meal.

Table 4.3. Exact composition of the control samples in Phase-1.

Sample number	Mass of bones (g)	Mass of maize (g)
1	0.0238	0.7657
2	0.0255	0.7112
3	0.0240	0.6992
4	0.0269	0.7249
5	0.0221	0.7023
6	0.0243	0.7001
7	0.0229	0.7134
8	0.0236	0.7067
9	0.0222	0.7314
10	0.0212	0.7056
11	0.0221	0.7330
12	0.0216	0.7023
13	0.0220	0.7138
14	0.0211	0.7063
15	0.0237	0.7164
16	0.0230	0.7468
17	0.0222	0.7051
18	0.0252	0.7512
19	0.0229	0.7406
20	0.0243	0.7248

Table 4.4. Composition of the control samples in Phase-2, all entries in mg.

Component	Material			
	A	B	C	D
Pork bones	70	-	35	70
Pork meat	-	70	35	-
Bovine bones	30	-	30	-
Bovine meat	-	40	30	-
Maize	10000	10000	10000	10000

4.3. RESULTS AND DISCUSSION

4.3.1 Analysis of high and low level dry-mixtures of MBM in maize by sedimentation and NIRM – single shot analysis

In initial experiments, the whole content of one bottle of 11 g was used to follow the official sedimentation protocol based on the Commission Regulation EC 152/2009. In Table 5 the results are shown for these experiments based on ten samples per level for the high (H) and low (L) level materials. If the precision is calculated for the low and high level materials one obtains 0.52 ± 0.03 g /100 g (5.2 % RSD) and 2.25 ± 0.15 g /100 g (6.5 % RSD), respectively. Since no subsamples per individual bottle were taken it was not possible to obtain information on the within-bottle homogeneity and ANOVA could not be used to evaluate the homogeneity. Nevertheless the result was encouraging both with respect to precision and deviation from the actual (intended) amount of bone in the low (L) and high (H) level material. It must be also mentioned that NIR microscopy was used to check the sediment for presence of bones. Here on average only 54 % of the sediment-particles checked by NIRM were detected as bones in the low level material and 73 % of the particles in the high level material were detected as bones. The reason for this discrepancy is not known. It may depend on the relatively large fraction (approximately 20 %) of small pork bone particles (<20 μm) present in the sediment that do not generate good NIR spectra and hence remained undetected. Further use of NIR Microscopy was therefore abandoned in this study.

4.3.2 Homogeneity test 1: Analysis of dry-mixed samples of low level MBM in maize by sedimentation –triplicate analysis and homogeneity test by ANOVA

Three replicates per bottle were taken from eight samples of remaining low-level material from the dry mixing. Since the total content per bottle was 11 g, only 3 g per replicate has been taken for analysis in order for the contents to suffice for three replicates. In Table 6 the raw data for the ANOVA evaluation of the between bottle homogeneity study is displayed. The eight bottles (B1–B8) were measured in random order with respect to the sample identification number with three replicates per bottle. The data is normally distributed (prerequisite for ANOVA). In Table 6 the data are sorted by individual sample number and replicate. Theoretically the result should be 0.5 % bones since the sample is a 1 % mixture of MBM in maize with an *f*-factor of 0.5 which means that the result is rather close to the expected level. The outcome of this homogeneity study as evaluated by ANOVA and calculated using SoftCRM (v. 2.0.10, only available in-house at IRMM) resulted in a relative between-bottle variation of 5.6 %. The relative within-bottle variation was found to be 6.8 %. The between-bottle homogeneity found by ANOVA is very similar to the 5.2 % RSD found for the first ten samples for 11 g of the same material as given in Table 5. Thus, lowering of the sample intake did not lead to a deteriorated precision in this case.

Table 4.5. Initial experiments on the high and low level samples using the whole sample of approximately 11 g as found by sedimentation with tetrachloroethylene.

Sample number and type high (H) or low level (L)	Theoretical level, % bones, (m/m)	Found level, % bones, (m/m)
04 (L)	0.5	0.51
09 (L)	0.5	0.53
10 (L)	0.5	0.51
13 (L)	0.5	0.48
14 (L)	0.5	0.52
18 (L)	0.5	0.54
24 (L)	0.5	0.53
25 (L)	0.5	0.55
33 (L)	0.5	0.57
39 (L)	0.5	0.49
08 (H)	2.5	2.16
13 (H)	2.5	2.12
15 (H)	2.5	2.36
16 (H)	2.5	2.34
18 (H)	2.5	2.33
20 (H)	2.5	2.32
27 (H)	2.5	2.41
28 (H)	2.5	2.43
32 (H)	2.5	2.12
33 (H)	2.5	1.99

Table 4.6. Raw data for the ANOVA-evaluation of the between-bottle homogeneity is displayed. The result is sediment mass taken directly as % bones in the low level material (m/m).

	B1	B2	B3	B4	B5	B6	B7	B8
Replicate 1	0.55	0.52	0.51	0.46	0.58	0.52	0.54	0.50
Replicate 2	0.55	0.46	0.52	0.56	0.59	0.54	0.53	0.54
Replicate 3	0.51	0.52	0.51	0.53	0.68	0.56	0.62	0.54

4.3.3 Homogeneity test 2: Analysis of low level dry-mixtures of MBM in maize by PCR – triplicate analysis and homogeneity test by ANOVA

The outcome of the homogeneity evaluation by ANOVA was calculated using SoftCRM (v. 2.0.10). In order to have a more realistic view of the performance of PCR the evaluation has to be made for copy numbers instead of number of cycles. The raw data for the copy numbers can be found in Table 7 for three replicates and five samples. The data is normally distributed and was measured in random order to be able to separate possible drift from the sample number which is also the fill sequence. The data displayed for the PCR has a method repeatability of 53.6 %. The effect of poor repeatability can be eliminated by performing multiple replicates. However, in case that insufficient number of replicates have been performed, quantification of between-unit homogeneity is usually not possible. In this case, one can estimate the maximum heterogeneity that could be hidden by method repeatability (i.e. the "limit" of detection of the homogeneity study) by calculating u^*_{bb} as outlined by Linsinger et al.. In this case, u^*_{bb} was calculated to 21 %. This means, that between-bottle variation above 21 % can be excluded, but no stricter limits can be set on the homogeneity due to the poor method repeatability. The result is not convincing but it does not mean that the material is inhomogeneous with respect to the meat content but rather that PCR is unsuitable to be used for homogeneity assessments in this kind of samples. The data shown in Table 7 gives evidence that PCR can be used for qualitative detection of meat in maize: the number of cycles in the 1 % MBM material was 24 to 26 cycles i.e. well below the set threshold of 40 cycles at which a sample is considered as not containing the target DNA. There should be no difficulties in detecting 0.1 % MBM which is the limit of detection in the animal feed regulation, Commission Regulation EC 152/2009, Annex VI, Paragraph 2.

Table 4.7. Raw PCR data for the copy numbers used in the ANOVA-evaluation for the between-bottle homogeneity in the low level material which is 0.5 % in meat (m/m) and 0.5 % in bones (m/m).

	B1	B2	B3	B4	B5
Replicate 1	2.23E+9	1.44E+9	0.89E+9	3.60E+9	0.93E+9
Replicate 2	1.81E+9	0.74E+9	1.16E+9	1.41E+9	0.85E+9
Replicate 3	3.59E+9	2.41E+9	2.18E+9	0.91E+9	1.98E+9

4.3.4 Bias check using the direct gravimetric preparation samples, Phase-1 samples

The gravimetric samples were prepared in order to test the performance of the sedimentation method as described in section 4.2.7. The results as obtained by two different laboratories are reported in Table 8. The amount of bone in the Phase-1 control samples was ± 25 mg, which roughly corresponds to the 15 mg present in 3 g of the dry-mixed low level sample (0.5 % of bone (m/m) as reported in Table 6). The only difference being the 0.7 g maize present in the control sample in comparison with the 3 g maize present in the dry-mixed sample. If, for some reason, the sheer amount of maize present in the tetrachloroethylene during the sedimentation process affects the sedimentation yield of bones one may argue that the control samples are not identical to the dry-mixed samples for which sample intakes of 3 and 10 g have been employed. The differing amount of maize could be an explanation for the deviating results where the precision is indeed much better on the higher sample intakes although the amount of bone is almost the same. The results that have been obtained for these control samples suggest that there may be some inherent problems with the sedimentation technique since the result for the amount of bone in this material is 27.4 ± 9.7 % below target (all results in Table 8). Furthermore the two sets of results reported in Table 6 and 8 are contradictory because it is not to be expected of a method that has a relative precision of 35.5 % for the phase-1 sample listed in Table 3 to perform at 8.6 % relative precision % in a highly similar material with a much lower bias. This problem is discussed more in detail in the conclusions. The results from the Phase-1 study consequently called for more detailed studies on samples by direct gravimetric preparation also involving light microscopy whereby the Phase-2 samples were prepared.

Table 4.8. Results obtained by laboratory A and B on the gravimetric control samples, Phase-1 samples.

Sample number	Laboratory	Actual mass of bone (g)	Found mass of bone (g)	Bias in %
1	B	0.0238	0.0172	-27.7
2	B	0.0255	0.0157	-38.4
3	B	0.0240	0.0193	-19.6
4	B	0.0269	0.0185	-31.2
5	B	0.0221	0.0150	-32.1
6	B	0.0243	0.0118	-51.4
7	A	0.0229	0.0183	-20.1
8	A	0.0236	0.0172	-27.1
9	A	0.0222	0.0152	-31.5
10	B	0.0212	0.0141	-33.5
11	B	0.0221	No result	-
12	A	0.0216	0.0159	-26.4
13	A	0.0220	0.0170	-22.7
14	A	0.0211	0.0199	-5.7
15	A	0.0237	0.0159	-32.9
16	A	0.0230	0.0188	-18.3
17	B	0.0222	0.0188	-15.3
18	B	0.0252	0.0176	-30.2
19	A	0.0229	0.0155	-32.3
20	A	0.0243	0.0186	-23.5

4.3.5 Bias check using the direct gravimetric preparation samples, Phase-2 samples

As outlined in section 4.2.8 and Table 4, the Phase-2 samples for the gravimetric control samples were in fact four different kinds of samples. Material A contained bovine and porcine bone meal, Material B contained bovine and porcine meat meal, material C contained bovine meat meal and bone as well as porcine meat meal and bone meal, finally material D contained porcine bone meal alone. For all materials A-D the MBM was dispersed in 10 g maize and the levels of MBM varied from 0.7 to 1.3 % (m/m). Each sample was used in single shot analysis and the sediment was subsequently evaluated by light microscopy.

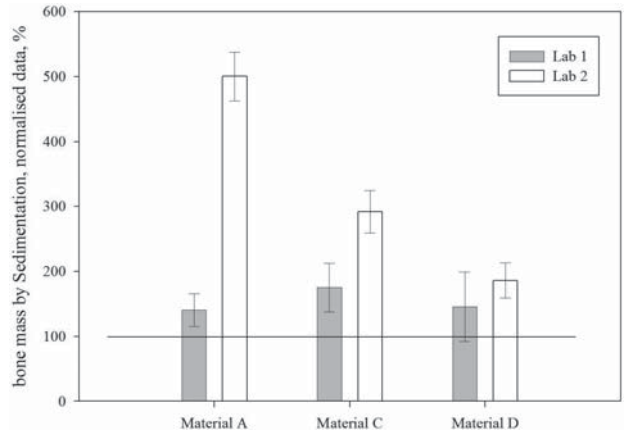

Figure 4.4. Results for Phase-2 samples using sedimentation alone. Material B could not be characterised using sedimentation as it contains no bone. The result on the Y-axis is normalised to the added bone mass, target is 100%. The error bars are relative standard deviation for n = 10.

The results in Fig. 4 are based on 10 single-shot measurements of ten unique samples. As can be seen in Figure 4 the results were not particularly good when using sedimentation alone to evaluate the amount of bone in the samples. The situation improved for lab 2 when light microscopy was used for evaluation of the ten collected sediments as depicted in Figure 5. However lab 1 seriously underestimated the actual content of bone in the sample. The explanation could be that the fine bones (very small particles) had not been assigned as bone when counting under the microscope by the analyst in Lab 1. It can also be seen in Fig 4 that Lab 2 has collected more sediment than Lab 1 possibly explaining part of this bias even if the laboratories had agreed on a similar sedimentation protocol. It should be mentioned that the best results were obtained for material D with an average of about 11 % above target with a relative standard deviation of 10 % for the ten replicates as shown in Figure 6.

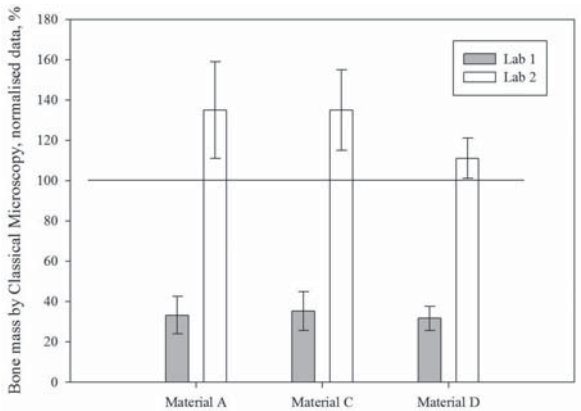

Figure 4.5. Results for Phase-2 samples using optical microscopy on the sediment. Material B could not be characterised using sedimentation as it contains no bone. The result on the Y-axis is normalised to the added bone mass, target is 100. The error bars are relative standard deviation for n = 10.

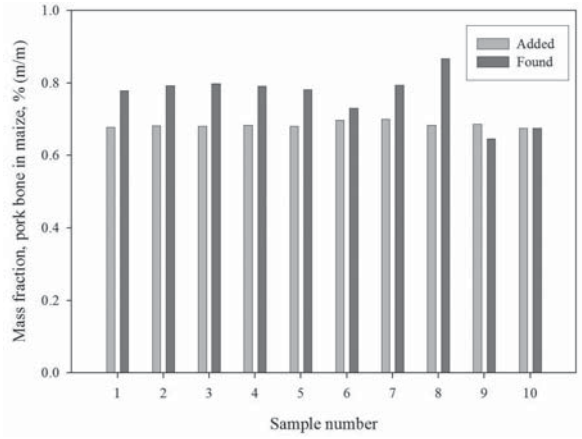

Figure 4.6. Results for 10 single shot analyses of material D of the Phase-2 samples using optical microscopy on the sediment. Results obtained by Lab 2.

Graphically the ten shingle-shot analyses for material D as analysed by Lab 2 are displayed shown in that figure. The exact numerical data used to generate Figures 4 and 5 is given in Table 9. Even if the official method for sedimentation and microscopy is applied, (Commission Regulation EC 152/2009, Annex VI) there is plenty of room for slight differences between the protocols (waiting time, means of agitation, design of separation funnels, beakers, uniform systematic approaches of counting the slides etc). Preceding these experiments the collaboration laboratories had discussed and agreed on a highly similar protocol trying to avoid as many sources of systematic differences as possible but, as can be seen, there is still a bias that must be overcome. Logically future material developments should involve materials containing a bone fraction with less fine particles more suitable for light microscopy.

Table 4.9. Results obtained by laboratory 1 and 2 on the Phase-2 gravimetric control samples containing bones. All data is normalised to the target 100 %. Ten samples of each matrix was measured by each laboratory, the spread is reported as relative standard deviation.

Method	**SAMPLE A**		**SAMPLE C**		**SAMPLE D**	
	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
Sedimentation	140	500	175	292	146	186
RSD, %	25	37	38	33	54	27
Microscopy	33	135	35	135	32	111
RSD, %	23	24	24	20	15	10

4.4. CONCLUSIONS

In general homogeneous subsamples of complicated matrices like compound feeds with a low MBM content are not easy to prepare by dry-mixing. As with all reference materials sufficient between-bottle homogeneity needs to be proven before laboratory inter-comparisons are organised. With the results obtained here it has been shown that dry-mixing works sufficiently well for the current level of method performance, although direct preparation of gravimetric control samples by precise weighing of components into each bottle results in materials that can be used for trueness check and is also a more reliable method for material preparation. If the latter approach is chosen one can prepare kits with samples containing a number of different components of different species mixed into any suitable background matrix. This approach is more labour intensive but it is not inconceivable with access to appropriate machines like precise filling machines for low masses (10-500 mg). One should also stay close to a sample amount of 10 g in the preparation of these kits which should be used up completely when subjected to analysis. The bulk maize could even be supplied separately from the meat and bone components just making sure that a complete transfer has taken place alongside with a protocol for achieving this in a uniform way between different laboratories.

The results reported here cast doubt on the sedimentation test method whether its performance is sufficient to allow meaningful value assignment. A good reason for choosing the kit-approach is that the composition and amount of meat or bone meal is known with certainty when the whole bottle content is used. If the aim is to be able to lift certain bans in the EU-legislation concerning animal feed and MBMs, the certified materials to be used when evaluating the test methods must be clearly without any suspicion. The best way to achieve this, in a first step, is to use sources of different 'clean' species and background matrices that can be mixed in a conceivable way with exact information of the composition in every bottle. Only then each method and laboratory can be clearly evaluated alongside with each other to reach consensus on further steps to be taken. Future experiments should be performed on new samples with an even lower complexity by narrowing the particle size distribution as it is suspected that the presence of many fine particles is disturbing sedimentation and counting of bone particles under the light microscope. In subsequent steps one should increase the complexity of the samples again to obtain samples that are more similar to real samples. This could for example involve adding more background components like wheat, barley and/or soya. To this end one should also characterize commercially available animal feeds with respect to particle size distribution.

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