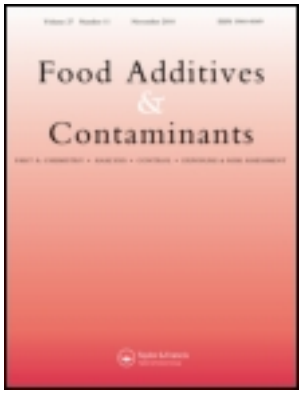

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Validation of a near infrared microscopy method for the detection of animal products in feedingstuffs: results of a collaborative study

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Validation of a near infrared microscopy method for the detection of animal products in feedingstuffs: results of a collaborative study

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The performance characteristics of a near infrared microscopy (NIRM) method, when applied to the detection of animal products in feedingstuffs, were determined via a collaborative study. The method delivers qualitative results in terms of the presence or absence of animal particles in feed and differentiates animal from vegetable feed ingredients on the basis of the evaluation of near infrared spectra obtained from individual particles present in the sample. The specificity ranged from 86% to 100%. The limit of detection obtained on the analysis of the sediment fraction, prepared as for the European official method, was 0.1% processed animal proteins (PAPs) in feed, since all laboratories correctly identified the positive samples. This limit has to be increased up to 2% for the analysis of samples which are not sedimented. The required sensitivity for the official control is therefore achieved in the analysis of the sediment fraction of the samples where the method can be applied for the detection of the presence of animal meal. Criteria for the classification of samples, when fewer than five spectra are found, as being of animal origin needs to be set up in order to harmonise the approach taken by the laboratories when applying NIRM for the detection of the presence of animal meal in feed.

Keywords: near-infrared microscopy; inter-laboratory validation; animal feed; processed animal proteins

Introduction

One of the important measures taken by the European Union against the spread of bovine spongiform encephalopathy (BSE) was the introduction of a total ban on the use of processed animal proteins (PAPs) including meat and bone meal (MBM) for any animal farmed for the production of food (European Union 2003). Already in the first TSE Roadmap (European Commission 2005), the European Commission specified that a restricted use of animal proteins for non-ruminants would be considered if analytical tests to differentiate between PAPs from the various animal species were available. In consequence, the development and validation of robust analytical methods for species-specific detection of MBM in compound feedingstuffs is crucial to enforce current and upcoming European legislation on the use of PAPs in animal nutrition (Fumière et al. 2009; European Commission 2010). Moreover, the use of PAPs under specific conditions will require a further reinforcement of the control of feed for ruminants, since the total ban of PAPs for this feed material will not be changed. For this task there is a strong need for reliable methods capable of detecting traces of PAPs regardless of their origin in compound feed. The only European official method to enforce the total feed ban is classical microscopy (European Union 2009), but quite different and alternative methods are currently proposed for the identification of PAP traces in feed. The motivation of this research is to address one drawback of classical microscopy related to the fact that its successful application depends on the rather specific experience of the scientist performing the visual inspection of the particles. Several studies have been dealing with the use of near infrared spectroscopy for the detection of MBM (Garrido-Varo and Fernández 1998; Baeten and Dardenne 2001; Murray et al. 2001, 2004, 2005; Garrido-Varo et al. 2005; de la Haba et al. 2007, 2009; Yang et al. 2007, 2008). Near-infrared microscopy (NIRM) follows the same principle than classical microscopy, but the visual evaluation of the appearance of the particles as required by the latter technique is substituted by the measurement and subsequent interpretation of NIR spectra from individual particles. This approach allows for an objective, rapid, sensitive and highly selective identification of animal particles in compound feed. Different studies performed during the last years have demonstrated the

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powerful characteristics of NIRM for the detection of MBM in feedingstuffs (Baeten et al. 2001, 2005; Murray et al. 2005; Zengling et al. 2011).

The specific NIRM method presented in this paper delivers qualitative results in terms of the presence or absence of animal particles in feed and differentiates animal from vegetable as well as mineral feed ingredients on the basis of the application of specific decision rules for the measured absorbances at specific wavelengths. The ruggedness of this concept has been demonstrated in a former study (von Holst et al. 2008), and subsequently the method was successfully transferred to two independent laboratories.

The purpose of the present study, conducted in the frame of the European project SAFEED-PAP, is to determine the method performance characteristics of this NIRM method by conducting a collaborative study, where the participating laboratories used their own NIRM instrumentation. Since the method is based on the identification of particulate matter, in this paper the term "animal particles" stands for the key components in terrestrial MBM and fishmeal, which are targeted by the NIRM method. The participating laboratories had to take spectra from compound feed samples as such, called the raw fraction, and from the sediment fraction, which have been previously prepared by the organiser's laboratory applying the European official control method, i.e. classical microscopy (European Union 2009). In the sedimentation step the compound feedingstuff sample is treated with a chlorinated solvent of high density thus allowing heavier particles such as bones to be concentrated in the sediment fraction. Another objective of sedimentation is to separate the major fraction of the sample which contains mainly not-target components of vegetable origin from the sedimented fraction. Spectroscopic analysis is then applied on the sediment fraction and identification of traces from animal particles is achieved due to the specific NIR absorption of bones particles. When taking spectra from the raw fraction, the matrix is more complex compared with the sediment fraction and the concentration of the sought animal particles is lower. On the other hand, specific components of MBM such as muscle tissues, which are present in the raw fraction but not in the sediment fraction, may contribute to the identification of animal particles.

The performance characteristics were expressed in terms of correct identification of (1) samples containing animal particles in feed at trace level and (2) blank compound feed samples free of animal particles. An important fit-for-purpose criterion was the successful detection of traces of animal particles in the sedimented fraction at a concentration of 0.1% expressed in terms of mass fraction MBM in the sample, since this level is achievable by classical microscopy, the European official control method.

Methods and materials

Study design

Seven laboratories from four European Union member states and one laboratory from China participated in the study. The number of laboratories working with the very specific instrumentation required for the participation in this study is not very large; however, according to the International Union of Pure and Applied Chemistry (IUPAC) harmonised protocol for the design of method-performance studies, the study may be conducted if an absolute minimum of five laboratories reporting results for each material is ensured. The organising laboratories also participated in the study. However, the scientists conducting the analyses were not involved in the organisation of the study, thus ensuring that they had just the same knowledge on the samples as the other laboratories.

The laboratories had to apply a specific NIRM method that was already applied for the detection of MBM in the sediment fraction of compound feeds (Baeten et al. 2005). For this reason and in order to allow the participating laboratories to become familiar with the method protocol, a step-by-step approach was selected, including an initial training period previous to the validation study. The training also allowed the participants to evaluate their effective performance on a limited number of samples and to check their rigorous application of the protocol. The participating laboratories were provided with a detailed protocol of the method describing the analytical procedure to be followed strictly. The protocol also specified the parameters to be fixed in the instrument as well as the experimental conditions on how to handle the samples (European Commission 2009). Only laboratories that successfully passed the training phase where invited to participate in the actual validation study. The results from the validation phase were finally used to assess method performance characteristics.

The training was divided into the following three steps. The first part was comprised of the analysis of a standardisation cell containing a set of 12 sealed and well-characterised materials, including PAPs from different species and feed ingredients, in order to evaluate the performance of the laboratories' instruments.

The same standardisation cell was sent to each of the participating laboratories that collected 10 spectra per material during 2 consecutive days. By measuring from the same standard cell we excluded possible effects of matrix variations on the spectra measured in the laboratories. More detailed information on the instrument standardisation will be published in a separate paper (Fernández Pierna et al. Submitted).

In a second step the participants had to assess spectra that had been previously measured and provided by the organiser of the study and which corresponded to materials from different origin.

Table 1. Description of the samples used in the training and the validation phase of the study.

	Material	Composition	Sediment
Training phase	Material 1	Pure MBM	No/yes
	Material 2	Pure fish meal	No/yes
	Material 3	Compound feed blank	No
	Material 4	Compound feed blank	Yes
		Terrestrial MBM at:	
Validation study	Material 5	0.5%	Yes
	Material 6	0.1%	Yes
	Material 7	0% (Blank)	Yes
	Material 8	2% (1% Terrestrial + 1% fish)	No
	Material 9	1%	No
	Material 10	0% (Blank)	No

Notes: The samples were sent as such (Sediment/No) and/or sedimented (Sediment/Yes) after sedimentation was performed in the laboratory by the organiser of the study and by applying the procedure specified for classical microscopy (European Union 2009). Materials 6 and 9 were sent in blind duplicates; materials 2 and 3 were sent as sediments and as such (Sediment/Yes or no).

The purpose of this exercise was to evaluate the participants' capability to classify correctly the spectra as from animal origin or not based on visual observation and applying the decision rules as specified in the method protocol.

Finally, the laboratories had to measure and to evaluate the spectra of a set of six blind samples consisting of pure MBM and fishmeal as well as compound feed without PAPs. The detailed composition of the samples used in the study is presented in Table 1.

For the analysis of the samples, the participants had to follow strictly the method protocol and to collect 100 spectra from each sample.

Prior to the actual validation phase, all the results from the training period were thoroughly evaluated and specific laboratories were contacted to inform them about how to improve the execution of the method. In the validation phase the laboratories had to analyse a set of eight blind samples, as shown in Table 1. For the analysis of the blind samples the participants were requested to follow strictly the protocol of the method and to provide the organisers with about 600 spectra per sample. This number is requested from the method protocol and based on a statistical assessment considering the required target limit of detection of 0.1% MBM in compound feed. For each sample, the participants had also to report on the exact total number of particles analysed, the number of particles recognised as being from animal origin and to give a final conclusion as regards animal material presence (positive or negative).

Test materials

Ten different materials containing typical compound feed, fortified or not with meat and bone meal at different concentration levels, were used in the two phases of the study (Table 1).

Three different compound feeds for bovine containing typical feed ingredients were used as a basis for the preparation of samples. The same blank material was used for the preparation of materials 5, 7, 8 and 10, whereas material 6 was prepared with a different blank. Samples 3 and 4 were prepared with the same blank material. The sample set for the training phase comprised four pure processed animal proteins (MBM and fish meal) and two compound feeds free of animal particles. In the validation phase the sample set was comprised of eight blind samples, four of which consisted of sedimented fractions and the other four were samples as such. Materials 6 and 9 were sent as blind duplicates (Table 1).

The target concentrations of PAPs in the raw fractions were 1% and 2% and in the sedimented fractions were 0.1% and 0.5%, respectively. It must be underlined here that the indicated PAPs concentrations of the sedimented fractions specified the concentrations in the compound feed samples, prior to the sedimentation. In consequence, the actual concentration of bones in the sedimented fraction was much higher than these levels as explained above.

The blank compound feeds were analysed by classical microscopy and polymerase chain reaction (PCR) confirming the absence of traces of animal origin. In the same way, terrestrial MBM (pure bovine) and fish meal were analysed by PCR to check the species present in the sample, confirming that the terrestrial MBM did not content any fish material and vice versa.

Fortified samples were prepared applying the stepwise dilution procedure (von Holst et al. 2006) in order to ensure homogeneity in the test materials. Sedimented test materials were obtained by treating the feed sample with tetrachloroethylene as described in

the European official microscopic method (European Union 2009; Veys and Baeten 2010; Liu et al. 2011). About 5–10 g of unsedimented materials and 0.1–0.2 g in the case of the sedimented samples were kept in glass vials for dispatching.

Method

Details of the method protocol are given in a previous publication on the detection of banned meat and bone meal in feedstuffs by NIRM analysis in the sediment fraction of compound feeds (Baeten et al. 2005). The detection of animal material is performed through the NIR analysis of individual particles. By using an NIR microscope, the light is focused onto the surface of a particle and the NIR reflected light from each individual particle in the sample is collected. NIR spectra are then evaluated by applying visual decision rules based on absorbance values at different wavelengths in order to establish whether or not the particles are from animal origin. In detail, the identification of spectra belonging to particles from animal origin is based on the following three criteria already described by Zengling et al. (2011):

- Presence of maxima in the 1920–1960, 2030–2070 and 2150–2200 nm regions.
- Presence of minima in the 2010–2030 (a), 2070–2150 (b) and 2210–2250 nm (c) regions.
- Value of
  [absorbance (line segment (a–c)]$_{\text{wavelength defined by b}}$ > absorbance (b).

A particle is only classified as originating from meat and bone meal (positive for animal presence) if its spectrum fulfils all three criteria described above.

Based on the number of positively identified spectra, the laboratories decided whether the respective sample was considered positive or negative. Positive in this context means the presence of animal particles in the sample. Here the laboratories employed their own criterion regarding the critical number of spectra above which the sample is considered as positive. In general, the number varied between the laboratories from one to five spectra. Included here in the method protocol was the flexibility of using the laboratories' own criteria because this approach reflects the current situation when applying classical microscopy and most of the attending apply this technique for routine analysis.

Data treatment

For each material the numbers of correct positive (CP), correct negative (CN), false-positive (FP) and false-negative (FN) results were calculated based on the pooled results provided by the laboratories. The target values were set according to the composition of the test materials. In order to obtain an overall estimation of the performance of the analytical method, sensitivity (SE) and specificity (SP) were calculated per each of the materials according to the following equations:

$$\text{Sensitivity } SE = \frac{CP}{CP + FN}$$

$$\text{Specificity } SP = \frac{CN}{FP + CN}$$

Sensitivity is defined as the ability of the method to detect animal presence when it is present in the samples, while specificity is the ability to classify a negative sample as negative. However, the sampling error due to the limited number of samples included in the study was not taken into account.

Based on the total number of spectra measured by each laboratory and the corresponding number of positive spectra, a quantitative estimate expressed as the percentage of animal particles in the samples was also calculated. The results from materials 6 and 9 delivering duplicate analyses were then subjected to analysis of variance (ANOVA) in order to establish major error sources contributing to the variation of the results and to compare the measurements from the raw fraction and the sediments.

Results and discussion

Seven out of eight laboratories participating in the validation study used Auto Image Microscopes connected to a Fourier transform NIR spectrometer from Perkin-Elmer, UK. The eighth instrument was a Hyperion 2000 from Bruker Optics (Belgium) with a tungsten source, CaF2 beam splitter and InGaS detector.

Training phase

The results from the first step demonstrated that the proposed protocol can be applied in different laboratories, independently of the location, device, analyst or software used to acquire spectra. Spectral libraries coming from different instruments can be matched by means of some standardisation procedures demonstrating sufficient performance of the various instruments (Fernández Pierna et al. Submitted).

The results from the second training step, which concerned the correct classification of spectra measured by the organisers of the study, revealed that all laboratories were able to apply correctly the rules as described in the method protocol.

The evaluation of the results from the third training step, in which the laboratories had to measure and interpret different samples as indicated in Table 1, confirmed the laboratories' capability to identify

correctly spectra from terrestrial MBM and fishmeal as coming from animal particles. For the blank samples all laboratories classified material 3 correctly as negative, whereas all laboratories except laboratories 2 and 6 reported low levels of animal content in material 4. The possible reason for these results is contamination during sample handling at the participant's laboratory, as this material had been previously analysed by classical microscopy and PCR and the results were negative for animal presence. As these false-positive results were detected in the training period, it was considered as a chance for improvement and the participants were advised to pay extra attention during the process of manipulation of samples. This is also a key issue in the application of the classical microscopy technique (Veys and Baeten 2010).

Validation phase

Based on the reported results, all eight laboratories were invited to participate in the validation study. The results provided by the individual laboratories are summarised in Table 2. Due to technical difficulties related to the software of the instrument that occurred after the training phase, laboratory 6 could not collect 600 spectra by focusing each individual particle. To overcome this problem, the spectra were collected by another technique, which is called mapping, where different particles were measured by applying the same focus adjustment. Since this type of measurement is considered a major deviation from the protocol, the corresponding data provided by laboratory 6 were excluded from the evaluation. In consequence, results from seven laboratories have been finally considered for the evaluation of the method performance.

Blank samples

All laboratories classified correctly the raw fraction from blank compound feed (material 10) as negative, since none of the spectra measured by the laboratories was considered as coming from animal origin. For the sediment from blank compound feed (material 7), five out of seven laboratories classified all measured spectra correctly as negative, whereas laboratories 3 and 7 classified a very small portion of the measured spectra as positive. Interestingly, laboratory 3 considered three positive spectra out 750 as not sufficient to trigger a positive conclusion. On the other hand, laboratory 7 just classified one out of 607 samples as positive and concluded on the sample that it was positive. It is obvious that the use of different threshold values for the number of positive spectra triggering a positive conclusion on the sample contributed to the inconsistent conclusion on the sample. In addition, we checked the four positive spectra from laboratories 3 and 7 and confirmed that positive spectra from laboratory 3 were wrongly classified due to the incorrect application of the decision rules.

Positive samples, sediments

All laboratories identified a sufficiently high number of positive spectra in the sedimented samples containing MBM at 0.5% and 0.1% (materials 5 and 6). Therefore all laboratories concluded correctly that the samples were positive. For instance, for material 6 which was prepared from compound feed containing MBM at 0.1%, the minimum number of positive spectra was eight out 600, whereas for material 5 which was prepared from compound feed containing MBM at 0.5% the minimum number of positive spectra was 102 out of 600. These results clearly demonstrate that the sensitivity of the NIRM method evaluated in this study is in line with the requirements of the European legislation.

Positive samples, raw fractions

The results from the raw fraction of compound feed containing MBM at 1% and a mixture of MBM and fishmeal at 2% showed that the number of positive spectra is much lower compared with the corresponding number in the sedimented samples. In consequence, some of the positive samples have been wrongly classified as negative. For instance, the raw fraction of compound feed containing 1% MBM (material 9) was sent as blind duplicates and laboratory 3 classified all 717 spectra measured from one sample as negative. In the other sample this laboratory identified two out of 725 spectra as positive. Also laboratory 4 classified in one sample two spectra as positive and in the other sample all spectra as negative. Again, the laboratories applied different threshold levels, since for laboratory 4 two spectra were enough to reach a positive conclusion on the sample which was not the case for laboratory 3. Similarly, laboratory 1 classified one and two spectra in the duplicate samples as positive, but concluded in both cases that the sample was negative. By increasing the level of concentration of animal proteins in the sample up to 2% (material 8), the number of false-negatives decreased and only laboratory 3 concluded that the sample was negative even if two spectra out of 901 were classified as positive.

The results from the study clearly highlight the importance of setting harmonised criteria for the final conclusion of samples as being positive or negative depending on the number of spectra classified as positive. This is particularly important when measuring blank sediments with a risk of false-positive results (material 7) and the raw fraction containing 1% MBM with a risk of false-negative results (material 9). In this context we need to underline that we are discussing the impact of a very small number of particles identified as

Table 2. Details of the results for the blind samples used in the validation phase and reported by the eight participants (laboratory code 1–8). Results are classified by material analysed (5–10).

Lab code	Sample code	Mat	Sediment	Material composition	No. total spectra	Positive spectra	Animal (%)	Conclusion
1	343	5	Yes	0.5% terrestrial	610	183	30.00	+
2	109	5	Yes	0.5% terrestrial	640	301	47.03	+
3	408	5	Yes	0.5% terrestrial	864	212	24.54	+
4	317	5	Yes	0.5% terrestrial	600	102	17.00	+
5	5	5	Yes	0.5% terrestrial	636	340	53.46	+
7	226	5	Yes	0.5% terrestrial	661	180	27.23	+
8	135	5	Yes	0.5% terrestrial	600	149	24.83	+
1	214	6	Yes	0.1% terrestrial	610	20	3.28	+
1	240	6	Yes	0.1% terrestrial	610	12	1.97	+
2	149	6	Yes	0.1% terrestrial	644	40	6.21	+
2	318	6	Yes	0.1% terrestrial	645	36	5.58	+
3	84	6	Yes	0.1% terrestrial	928	9	0.97	+
3	409	6	Yes	0.1% terrestrial	928	10	1.08	+
4	110	6	Yes	0.1% terrestrial	600	25	4.17	+
4	370	6	Yes	0.1% terrestrial	600	37	6.17	+
5	45	6	Yes	0.1% terrestrial	624	31	4.97	+
5	344	6	Yes	0.1% terrestrial	611	25	4.09	+
7	123	6	Yes	0.1% terrestrial	605	30	4.96	+
7	279	6	Yes	0.1% terrestrial	615	33	5.37	+
8	19	6	Yes	0.1% terrestrial	600	19	3.17	+
8	266	6	Yes	0.1% terrestrial	600	8	1.33	+
1	397	7	Yes	Blank	610	0	0.00	–
2	345	7	Yes	Blank	624	0	0.00	–
3	59	7	Yes	Blank	750	3	0.40	–
4	384	7	Yes	Blank	600	0	0.00	–
5	20	7	Yes	Blank	604	0	0.00	–
7	358	7	Yes	Blank	607	1	0.16	+
8	293	7	Yes	Blank	600	0	0.00	–
1	164	8	No	1% terrestrial + 1% fish	610	4	0.66	+
2	60	8	No	1% terrestrial + 1% fish	609	11	1.81	+
3	112	8	No	1% terrestrial + 1% fish	901	2	0.22	–
4	138	8	No	1% terrestrial + 1% fish	600	2	0.33	+
5	8	8	No	1% terrestrial + 1% fish	627	8	1.28	+
7	268	8	No	1% terrestrial + 1% fish	606	4	0.66	+
8	281	8	No	1% terrestrial + 1% fish	600	2	0.33	+
1	22	9	No	1% terrestrial	610	2	0.33	–
1	152	9	No	1% terrestrial	610	1	0.16	–
2	321	9	No	1% terrestrial	635	4	0.63	+
2	425	9	No	1% terrestrial	685	3	0.44	+
3	178	9	No	1% terrestrial	717	0	0.00	–
3	269	9	No	1% terrestrial	725	2	0.28	–
4	230	9	No	1% terrestrial	600	0	0.00	–
4	256	9	No	1% terrestrial	600	2	0.33	+
5	35	9	No	1% terrestrial	688	3	0.44	+
5	295	9	No	1% terrestrial	649	6	0.92	+
7	386	9	No	1% terrestrial	600	3	0.50	+
7	399	9	No	1% terrestrial	605	3	0.50	+
8	113	9	No	1% terrestrial	600	3	0.50	+
8	204	9	No	1% terrestrial	600	4	0.67	+
1	374	10	No	Blank	610	0	0.00	–
2	322	10	No	Blank	588	0	0.00	–
3	335	10	No	Blank	810	0	0.00	–
4	348	10	No	Blank	600	0	0.00	–
5	296	10	No	Blank	643	0	0.00	–
7	179	10	No	Blank	608	0	0.00	–
8	140	10	No	Blank	600	0	0.00	–

Notes: Animal (%) specifies the ratio of the number of positive spectra divided by the total number of spectra measured. "+" and "–" indicate that the laboratories concluded on the sample as containing animal particles or not, respectively.

Table 3. Results expressed in specificity (SP) and sensitivity (SE).

	Material 5		Material 6		Material 7		Material 8		Material 9		Material 10	
	0.5% MBM (sed.)		0.1% MBM (sed.)		Blank (sed.)		1% MBM + 1% Fish (not sed.)		1% MBM (not sed.)		Blank (not sed.)	
n	7		14		7		7		14		7	
	CP	FN	CP	FN	CN	FP	CP	FN	CP	FN	CN	FP
	7	0	14	0	6	1	6	1	9	5	7	0
SE (%)	100		100				86		64			
SP (%)					86						100	

Notes: *n*, total number of observations; CP, samples correctly classified as positive; FN, false-negative results; CN, samples correctly classified as negative; FP, false-positive results.

Table 4. Results from an ANOVA performed on the laboratories' duplicate analyses.

Error source	Sediment (material 6)	Raw fraction (material 9)
Within laboratory variation (RSD%)	23	47
Between laboratory variation (RSD%)	45	42
Total variation (RSD%)	51	63
Average portion of positive spectra (%)	3.8	0.41
Average absolute number of positive spectra	24	3

Notes: Data presented under "Animal (%)" were used for the ANOVA calculation.
RSD%, relative percentage standard deviation.

positive (between one and three) present in a high number of particles identified as negative (at least 600). In fact, a harmonised strategy on how to handle such as a small fraction of positive spectra need also to include a decision rule establishing under which conditions the whole analysis has to be repeated and/or the sample reanalysed by the European official method.

The objective would be to reach consistent conclusions between laboratories and to decrease further the risk of wrong results.

Table 3 shows a summary of the obtained method performance characteristics. A sensitivity of 100% was obtained for the sedimented samples containing 0.1% and 0.5% MBM, respectively, since all samples were correctly identified as positive. When analysing the raw fractions of the compound feed samples, the sensitivity was lower, namely 86% and 64% for materials 8 and 9, respectively. This was mainly related to the very low number of positive spectra. The sensitivity could be improved by increasing the number of particles analysed per sample. The specificity of the method for the blank raw fraction was 100%, because all samples were considered as negative. This was a remarkable result, since the raw fraction of compound feed is an extremely complex matrix, but nevertheless none of the 4459 spectra measured by all laboratories was erroneously classified as positive. For the sedimented blank sample one laboratory reported one positive result, resulting in a specificity of 86%. This decision is just based on the classification of a single spectrum out of 607 spectra measured. Therefore, it can be expected that harmonisation of the rules regarding the interpretation of a small number of positive samples will improve the specificity of the method.

The results from ANOVA shown in Table 4 revealed that the total variation of the percentage positive spectra expressed as relative standard deviation was 51% for sediments (material 6) and 63% for the raw fraction (material 9). The closeness of these values is quite surprising, since the raw fraction of compound feed is much more complex compared with the sedimented fraction. Nevertheless, the partition of the variance into the error components showed that for the sediment fraction (material 6) the contribution of the within laboratory variation was 23% and therefore smaller compared with within laboratory variation of the raw fraction (material 9), which was 47%. Since the between laboratory variations were almost identical for the sediments and the raw fraction, it can be concluded that the influence of the laboratory effect on the overall variation was very similar for both matrices. In consequence, it can be assumed that sample specific aspects such as the much lower number of animal particles identified in the raw fraction compared with the sediments contributed to the differences of the *within* laboratory variation. In fact,

the average number of animal particles identified in the sediment (material 6) is about 24 whereas the corresponding number for the raw fraction is about three. In consequence, just the intrinsic higher sampling error associated with the lower number of animal particles in the raw fraction may have affected the higher within laboratory variation observed with this matrix.

Conclusions

An NIRM method for the detection of animal products in feedingstuffs was successfully validated via a collaborative study in which seven laboratories from four European Union member states and one laboratory from China participated. The specificity varied from 86% to 100%. In sedimented samples the limit of detection was 0.1%, since all laboratories correctly identified the positive samples, while in samples which were not sedimented the percentage of MBM has to be increased up to 2%. The required sensitivity for official control is therefore achieved in sedimented samples where the method can be applied for the detection of animal presence. This characteristic is absolutely in line with the performance of classical microscopy. Criteria for classification of samples when fewer than four spectra are found as being of animal origin need to be set in order to harmonise the approach taken by the laboratories when applying NIRM for the detection of animal presence in feed. When combining the very good results from the current study with those on the standardisation cell demonstrating that spectra from different laboratories are interchangeable, the creation of a laboratory-independent spectra library may be considered. Such a tool has the potential of further improving the correct classification of analysed particles.

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