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Detection of banned meat and bone meal in feedstuffs by near-infrared microscopic analysis of the dense sediment fraction

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Abstract In this paper we present an alternative method for detection of meat and bone meal (MBM) in feedstuffs by near-infrared microscopic (NIRM) analysis of the particles in the sediment fraction (dense fraction ($d > 1.62$) from dichloroethylene) of compound feeds. To apply this method the particles of the sediment fraction are spread on a sample holder and presented to the NIR microscope. By using the pointer of the microscope the infrared beam is focussed on each particle and the NIR spectrum (1112–2500 nm) is collected. This method can be used to detect the presence of MBM at concentrations as low as 0.05% mass fraction. When results from the NIRM method were compared with the classical microscopic method, a coefficient of determination (R^2) of 0.87 was obtained. The results of this study demonstrated that this method could be proposed as a complementary tool for the detection of banned MBM in feedstuffs by reinforcement of the monitoring of feeds.

Keywords Meat and bone meal (MBM) · Bovine spongiform encephalopathy (BSE) · Near-infrared microscopy (NIRM) · Optical microscopy · Sediment fraction

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Introduction

The development of analytical methods to detect meat and bone meal (MBM) in feedstuff ingredients and compound feeds was one of the consequences of the so-called ''mad cow disease'' that appeared in the 1980s. Feed contaminated with MBM is commonly accepted as the main transmission carrier of the prion responsible for bovine spongiform encephalopathy (BSE) within the European bovine herd. To confront this problem many countries have banned MBM as a feed ingredient. In the European Union, this ban was first applied to feed for ruminants [1]. Currently, the MBM ban is regulated by EC Regulation 999/2001 [2], which was recently amended by EC Regulation [3], excluding MBM of all animals from feed for all farmed animals and allowing fishmeal only in the feed for non-ruminants. Because there is no legal limit for the presence of MBM in feed, violation of current legislation is confirmed when MBM is detected in feed whatever its concentration. Analytical methods are therefore useful to enforce European legislation when the technique enables reliable identification of banned animal tissue, whereas quantification is less important. It is generally accepted that an analytical method subjected to validation should have sufficient sensitivity at a 0.1% concentration of MBM in feed, because the only official European method, classical microscopy, reaches this level [13]. The rate of false negative results at this concentration level should be below 5%. The methods proposed are usually based on the detection of particles (i.e. optical microscopy [4–6]), proteins (i.e. immunological methods [7, 8]), or fragments of deoxyribonucleic acid (DNA) (i.e. methods based on the polymerase chain reaction (PCR; [9–11]) of animal origin. Other methods such as infrared spectroscopy use the global molecular fingerprint of the samples to detect the presence of MBM [12].

Optical microscopy is the reference method used in the European Union and in many other countries to monitor correct application of the legislation regarding

the MBM ban [13]. With the microscopic method the detection of animal ingredients is achieved by microscopic observation of specific characteristics of particles from different sieved and/or sediment fractions after adequate sample preparation. The test is based mainly on the detection of bones by observation of lacunae on the surface of particles present in the sediment of the sample. The identification of additional animal fragments, for example muscle fibres and hairs, facilitates the detection of particles of animal origin. A recent comparative study [14] demonstrated the performance characteristics of this method were sufficient, because most of the participating laboratories applying microscopy were able to detect the presence of MBM at 0.1% mass fraction in the feed. In addition, identification of MBM by this method is of high forensic value. However, a major drawback is that the analysis strongly depends on the experience and the training of the person conducting the trial.

Molecular biology methods include PCR and immunological techniques. The PCR methods, which have been widely proposed to tackle the problem, are based on the detection of DNA targets specific to one single species (e.g. bovine or pig) or to a class of species (e.g. mammal or ruminant) after amplification of the DNA extracted from the sample. The PCR methods applied to the detection of MBM in feed have striking advantages, for example substantial increase of sample throughput and the option of species-specific detection. However, analysis of samples containing MBM treated according to European legislation [15], i.e. steam sterilisation at 133°C, 3 bar and 20 min, revealed distinct decay of the target DNA [16]. In consequence, current PCR technology still fails [14] when analysing samples containing traces of MBM that have previously been heat-treated under severe conditions. In contrast, the immunology methods are based on the detection of specific proteins present in MBM or resulting from thermal degradation after the mandatory treatment of the MBM [17]. Currently available immunoassays are very sensitive and lend themselves perfectly to the analysis of a large number of samples. However, results from immunoassays do not have a high forensic value and therefore require samples with a positive result to be re-analysed by a confirmatory method such as microscopy.

The main spectroscopic methods proposed for identification of illegal addition or detection of cross-contamination of feedstuffs are based on NIR spectroscopy. The infrared spectra obtained with an NIR spectrometer from the raw sample [18, 19] or gathered with an NIR microscope from the particles making up the sample [20, 21] are used to identify samples containing banned MBM. These previous studies have demonstrated the high potential of the NIRM method to detect MBM at a concentration as low as 0.5%. When using an NIR microscope the subjective judgement of the scientist is replaced by a particle-specific spectrum which can be subjected to statistical analysis. However, applying this method is very time-consuming, because one technician can only process two samples per day. Moreover, detecting MBM at the very low concentration level of 0.1% requires many more particles to be analysed, leading to an overall duration of several days to conduct the analysis. This approach would make the method useless for routine monitoring. To increase the speed of the NIRM analysis we investigated the benefit of a sedimentation process conducted before the near-infrared microscopic analysis. The major aim of the sedimentation step was to concentrate the particles of MBM and to remove non-specific particles. In this study, the results from NIRM analysis of sediment fractions from several raw feed materials and spiked samples are presented and discussed. The method has been compared with the classical microscopic technique.

The objective of this study was to establish the suitability of NIRM for detection of MBM, irrespective of the species composition in feed, by determining the sensitivity and specificity at a concentration level of 0.1% of MBM in feed. In addition, we compared the performance characteristics of this method when used with two different techniques for preparation of the sediments.

Materials and methods

Instrumentation

In this study an Auto Image microscope connected to a Perkin–Elmer Fourier transform near-infrared spectrometer (FT-NIR) was used. This instrument enables the collection of spectra from small surfaces (50 m×50 m). The microscope includes a camera and a viewing system to magnify the visible-light image of the sample to observe, highlight, and isolate a point of interest. The particles of the sediment fraction are spread on a Spectralon plate and presented to the NIR microscope. Using the pointer of the microscope, the infrared beam is focussed on each particle and the NIR spectrum (1,112–2,500 nm) is collected. For this study, a resolution of 4 cm^{-1}, a gain of four, and a number of co-added scans of 10 were used. The spectra were obtained after determination of the ratio of the raw spectrum to the background obtained from measurement of the Spectralon plate. The software ''autoimage'' from Perkin–Elmer was used to collect and store the spectra.

Test material

We used different sample sets for conducting the trials. Sample set A, used to calibrate the NIR microscope, was made of feed ingredients and feedstuffs selected from the CRA-W sample bank, including samples of animal, vegetable, and mineral origin. Sample set A was obtained from Belgian feed producers and from the European sample bank generated in the framework of

the Stratfeed EU project [22]. Most of the samples were produced and collected during the 2000–2002 period and analysed by other techniques, for example optical microscopy and PCR, that enabled confirmation or correction of the declared composition. Sample set A consisted of a total of 180 samples.

Sample set B was used to test the method and included materials not used in the calibration stage. This sample set was produced at CRA-W and included five samples, four of which were spiked with MBM at a level in the 0.05–1% range. A compound feed free from MBM and MBM resulting from mixing of equal quantities of eight animal meals (including mainly bovine and porcine materials with traces of poultry material) from different sources were used to prepare this set.

Sample set C was also used to test the method and was prepared at the University of Cordoba (UCO). This set included 72 samples: 24 compound feeds free from MBM that had been produced by the Spanish feed plant Rendersur (Cordoba, Spain) at the end of 2001, according to different formulae commonly used in the feed industry, and 48 spiked samples obtained by mixing the 24 matrices with MBM at different levels (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8%). Eight different MBM were used to spike the samples. These animal feed ingredients were selected from the Stratfeed sample bank. The NIR spectra and the GH statistic (Global Mahalanobis distance) were used to select the most different types of MBM from a set of 102 samples. The spiked samples of set B were prepared by use of the dilution procedure described elsewhere [14]. After preparation the samples were vacuum packed in polyethylene and aluminium multilayer bags, and stored at 4°C. The grinders and mixers used for the preparation of the test sets were carefully cleaned between the two successive mixings.

Preparation of the sediment fraction

Two sedimentation methods were applied in this study and are based on the principle of concentration of the bone fraction using a high-density solvent. The first method applied uses only tetrachloroethylene ($Cl_2C=CCl_2$) as solvent and recovers the fraction that has a density higher than 1.62. The second method (also called the French method) uses two solvents, tetrachloroethylene and tetrabromoethane ($Br_2HC–CHBr_2$), and recovers the fraction that has a density within the 1.62–2.2 range [23]. This method results in a lower sediment weight, i.e. a more highly concentrated sample. With both methods 10 g of compound feed or 3 g of the MBM is transferred into a separation funnel with tetrachloroethylene applying the EU guideline [13] or with tetrachloroethylene and tetrabromoethane applying the French method (shaken for 20 s and sedimented for 5 min). The major part of the 10 g remains floating whereas the denser matter, comprising mainly bone particles, egg shell, fish scales and minerals, forms the sediment. This fraction is recovered on cellulose filters and dried. The sediment is then stored in paper envelopes at room temperature.

NIRM analysis

The NIRM analysis was performed on the fraction larger than 250 μm obtained after sieving the sediment fraction. About 100 and 400 particles of each sample were analysed. The methodology applied in the NIRM analysis of particles from the sediment is the same as that used to analyse raw material and has been described in previous papers [18, 19].

Mathematical and statistical analysis

"Convert Abs NM" software from Perkin–Elmer was used to convert the frequencies of the spectra from wavenumber (cm^{-1}) to wavelength (nm) and the Specconv software from Perkin–Elmer was applied to convert the spectra to an ASCI format. The ASCII data were imported in Matlab (Mathworks, USA). To construct and test the partial least-squares (PLS) discriminant equation, the WIMPLS algorithm adapted for wide data matrix was used [24, 25]. The leave-one-out cross validation method and the root-mean-square standard error (RMSE) statistic were used to determine the optimum model complexity.

Results and discussion

Construction of the discriminant models

To calibrate the NIR microscope a series of 34 sediment fractions (sample set A) prepared from 27 feed ingredients of animal origin (i.e. bovine, pig, sheep and poultry) and seven MBM-free compound feeds were analysed by NIRM, by following the procedure described in the "Materials and methods" section. The compound feeds were used to represent the wide range of mineral and vegetable feed ingredients used in the feed sector and were selected to be representative of those most frequently used by feed producers. The total training set consists of a total of 8,391 spectra resulting from NIRM analysis of particles. Figure 1 shows several spectra obtained from particles from the sediment fraction of animal origin (A), and of vegetable or mineral origin (B). The animal particle spectra contain bands with maxima near 1,945, 2,045, 2,175, and 2,290 nm. All spectra from particles of animal origin analysed in this study have bands in the region between 1,700 nm and 2,500 nm, mostly attributed to the protein, water and fat content of the particle.

To discriminate the particle spectra according to their origin, two strategies were applied. The first strategy was the construction of discriminant models

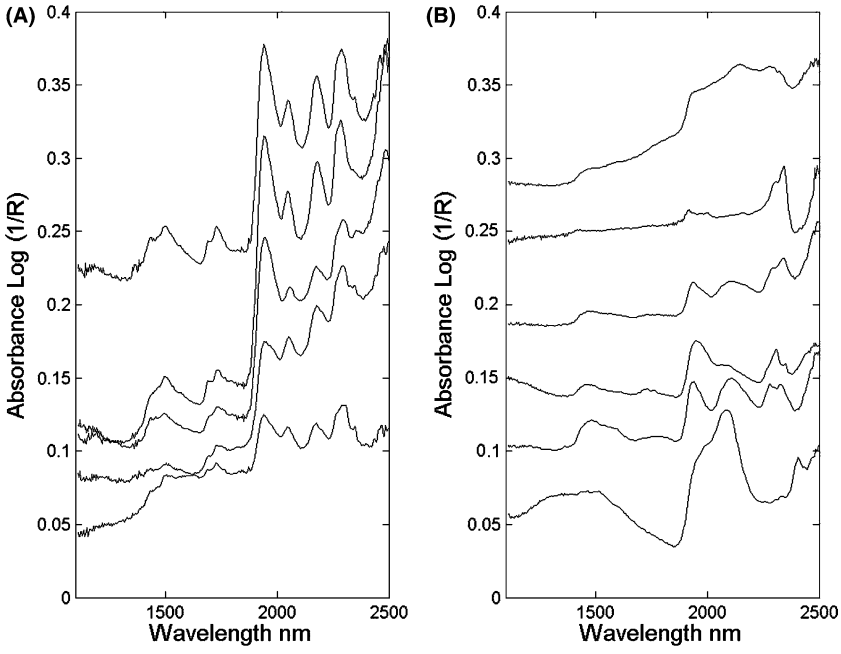

Fig. 1 Particle spectra from the sediment fraction of terrestrial animal origin (**A**) and non-animal (vegetable or mineral) origin (**B**)

using the PLS algorithm. The second strategy was the use of a decision rule based on absorbance values at different wavelengths. For construction of the PLS model, an arbitrary variable of unity was attributed to the group of particles of animal origin (=group I (Gr. I), 4923 spectra) whereas the variable of the group of particles from compound feed without MBM was −1 (=group II (Gr. II), 3468 spectra). These values were used as reference values in the calibration stage. A first PLS model was constructed with the 8391 spectra. The mean of the predicted values of the training samples of group I was 0.827 with a standard deviation (SD) of 0.251 and the mean of the predicted values of group II was −0.754 with an SD of 0.518. When the predicted value of a particle was outside the range mean ± 2 SD (2 SD interval) of a specific group, this particle was considered as an outlier and therefore withdrawn from this group. A total of 389 spectra (=4.64% of the spectra collected from sample set A) were detected as outliers and removed from Sample set A: 207 spectra from Gr. I and 182 spectra from Gr. II. The final PLS model was constructed using the remaining 8002 spectra. The model had a root-mean-square of calibration (RMSC) of 1.351. With the PLS model, approximately 97.5% of the animal particles were classified in the 2 SD interval of Gr. I and only three animal particles were classified in Gr. II (i.e. 0.064% misclassification). Two vegetable or mineral particles were wrongly classified in Gr. I (i.e. 0.061% misclassification). The second approach for the identification of MBM particles was based on a decision rule using absorbance values at three wavelengths (i.e. 1,944, 2,060 and 2,148 nm) from the first ($d\times1$) and second ($d\times2$) derivative spectra. The wavelengths were chosen as representative of the MBM spectra included in the training set. The following decision rule (i) was used to decide whether or not a particle i belonged to the animal group Gr. I.

If $dx1(i, 2148) > 0.001$ and $dx1(i,1944) > -0.001$ and $dx1(i,1944)<0.003$ and $dx2(i,2060)<0$ then i particle belongs to Gr. I.

Applying this decision rule to the 8002 spectra used to construct sample set A, about 96.6% of animal particles are classified in Gr. I, 160 animal particles are classified in Gr. II (i.e. 3.339% misclassification) and 31 vegetable or mineral particles are classified in Gr. I (i.e. 0.944% misclassification).

Validation of the equations constructed

Sample set B was designed to determine the limit of detection, to study the repeatability between measures, and to compare the two different sedimentation procedures as described in the experimental section and currently applied in European countries. The results from analysis of sample set B are displayed in Table 1. For each sub-sample, the true MBM percentage, the sediment fraction percentage, the number of sediment particles analysed, and the number of spectra detected as being of animal origin with the PLS model and with the decision rule (results in parentheses) are displayed. The percentage of particles of animal origin in the sediment, the weight of the bone fraction in the sediment (calculated as the percentage of bones included in the sediment and without taking into account the size and the density of the sediment particles from the different origin), the percentage of bone in the sample, and the percentage of

Table 1 Results from NIRM analysis of the samples of the sample set B

Sample number	True MBM (%)	Sediment[a] (%)	No. of particles analysed	No. of animal particles[b]	Bones in the sediment[c] (%)	Weight of bones in the sample[d] (g)	Animal ingredients in the sample[e] (%)	Conclusion mean
One-solvent method								
254	0	2.977	157	0 (11)	0.000	**0.000**	0.000	*0.000*
264	0	2.665	135	0 (5)	0.000	**0.000**	0.000	
363	0	2.673	161	0 (5)	0.000	**0.000**	0.000	
256	0.05	2.862	155	3 (20)	1.935	**0.006**	0.198	*0.186*
285	0.05	2.892	108	2 (7)	1.852	**0.005**	0.191	
365	0.05	2.753	116	2 (9)	1.724	**0.005**	0.170	
255	0.1	2.788	182	1 (19)	0.549	**0.002**	0.055	*0.266*
265	0.1	2.829	140	8 (13)	5.714	**0.016**	0.577	
364	0.1	2.857	122	2 (5)	1.639	**0.005**	0.167	
257	0.5	2.857	168	13 (20)	7.738	**0.022**	0.789	*0.932*
267	0.5	2.815	124	10 (12)	8.065	**0.023**	0.811	
366	0.5	2.876	103	12 (15)	11.650	**0.034**	1.197	
258	1	2.969	142	20 (31)	14.085	**0.042**	1.493	*1.508*
268	1	2.845	144	20 (29)	13.889	**0.040**	1.411	
367	1	2.835	100	16 (18)	16.000	**0.045**	1.620	
Two-solvent method								
249	0	0.554	144	0 (10)	0.000	**0.000**	0.000	*0.000*
259	0	0.511	159	0 (17)	0.000	**0.000**	0.000	
369	0	0.488	129	0 (21)	0.000	**0.000**	0.000	
251	0.05	0.588	158	3 (15)	1.899	**0.001**	0.047	*0.089*
261	0.05	0.571	105	5 (10)	4.762	**0.003**	0.113	
371	0.05	0.604	117	5 (14)	4.274	**0.003**	0.108	
250	0.1	0.623	156	8 (23)	5.128	**0.003**	0.133	*0.130*
260	0.1	0.609	150	7 (17)	4.667	**0.003**	0.118	
370	0.1	0.543	132	7 (19)	6.061	**0.003**	0.137	
252	0.5	0.766	140	19 (31)	13.571	**0.010**	0.433	*0.544*
262	0.5	0.717	132	26 (35)	19.697	**0.014**	0.588	
372	0.5	0.722	118	24 (32)	20.339	**0.015**	0.612	
253	1	0.851	137	39 (46)	28.467	**0.024**	1.009	*1.018*
263	1	0.936	136	36 (46)	26.471	**0.025**	1.032	
373	1	0.932	142	37 (45)	26.056	**0.024**	1.012	

[a]Sediment (%) = (weight sediment/weight sample)×100
[b]No. of animal particles = number of particles classified as being of animal origin. The results from the decision rule model are given in parentheses
[c]Bones in the sediment (%) = (No. of animal particles/No. of analysed particles)×100
[d]Weight of bones in the sample = [Bones in the sediment (%)/100×weight sediment (g)]
[e]Animal ingredients in the sample (%) = [weight bones in the sample (g)/% bones in the animal feed ingredient used to spike the sample (= *f* factor)]

animal ingredients in the sample, are also given in Table 1 and were calculated using the PLS model. The final conclusion of the analysis is obtained using the results of the PLS model and visual inspection that the particles classified as animal by the model had the typical profile shown in Fig. 1a. Using the PLS model, all samples containing MBM were correctly classified as "positive" and all samples with 0% MBM were correctly classified as "negative". By applying the decision rule, several spectra obtained from analysis of particles from MBM-free matrices were wrongly classified as being of "animal origin" although they did not correspond to the typical MBM spectra as shown in Fig. 1a. Comparing the results from both strategies revealed that the PLS model is more specific, because none of the particles from the blank samples were wrongly identified as "animal particle" which was true for the decision rule. In terms of sensitivity, both strategies seem to be comparable, because all samples containing MBM were correctly classified as positive.

The results from the PLS model showed that the NIRM method could detect MBM at a concentration level of 0.05%. Neither false positive nor false negative samples were observed. The relative standard deviation of the number of positively identified particles ranged from 0.186 to 1.508 for the one-solvent method and from 0.089 to 1.018 for the two-solvent method (Table 1). Given the low concentration of MBM in the feed these values were regarded as acceptable. Comparing the variability of both methods showed that the two-solvent method was slightly superior to the one-solvent method. Other differences between the two methods were observed. Indeed, the sediment percentage was, on average, 4.22 times higher with the two-solvent method, meaning that, for a determined sample, a higher number of particles had to be analysed to get the same limit of detection using the one-solvent method. Moreover, in this experiment and with the two-solvent method, the sediment percentage was directly proportional to the percentage of MBM ($R^2 = 0.97$), while no correlation was

observed between the sediment percentage and the one-solvent method results ($R^2 = 0.36$).

In addition, we used Sample set C to study the reliability of the method on a wide range of compound feeds and to compare the results of NIRM with those from optical microscopy (reference method). Tables 2 and 3 show, respectively, the results from the 48 spiked samples and from the 24 matrices included in Sample set C. The results are expressed in the same way as in Table 1. To calculate the percentage of animal ingredients in the sample, the measured *f* factor (i.e. the percentage of bones present in the samples) of the MBM sample present in the mixture was used. Table 2 shows that, generally, the number of particles detected as being of

Table 2 Results from NIRM analysis of the 48 samples in sample set C

Sample code	Matrix used	MBM used	Theoretical MBM (%)	Sediment[a] (%)	No. of particles analysed	No. of animal particles[b]	Bone in the sediment[c] (%)	Weight of bone in the sample[d] (g)	Animal ingredients in the sample[e] (%)	Conclusion
0466–01	6	1	0.5	2.73	185	7 (5)	**3.78**	0.01	0.90	Positive
0466–20	10	1	1.5	3.01	177	10 (10)	**5.65**	0.02	1.48	Positive
0466–56	20	1	2.5	5.52	220	25 (24)	**11.36**	0.06	5.47	Positive
0466–57	22	1	4.5	2.21	176	42 (48)	**23.86**	0.05	4.60	Positive
0466–09	14	1	4.5	4.05	190	24 (24)	**12.63**	0.05	4.46	Positive
0466–30	4	1	6.5	7.86	165	13 (14)	**7.88**	0.06	5.40	Positive
0466–17	7	2	1	2.87	165	16 (22)	**9.70**	0.03	1.11	Positive
0466–03	11	2	2	11.60	207	18 (23)	**8.70**	0.10	4.03	Positive
0466–53	21	2	3	4.88	169	43 (46)	**25.44**	0.12	4.95	Positive
0466–51	23	2	5	4.66	165	60 (61)	**36.36**	0.17	6.76	Positive
0466–71	15	2	5	3.89	163	71 (78)	**43.56**	0.17	6.76	Positive
0466–31	5	2	7	10.46	172	57(60)	**33.14**	0.35	13.84	Positive
0466–11	8	3	1.5	2.55	183	20 (27)	**10.93**	0.03	1.27	Positive
0466–19	12	3	2.5	3.67	172	31 (35)	**18.02**	0.07	3.01	Positive
0466–41	22	3	3.5	2.69	172	55 (58)	**31.98**	0.09	3.92	Positive
0466–7	24	3	5.5	2.48	160	109 (109)	**68.13**	0.17	7.68	Positive
0466–12	16	3	5.5	4.38	177	41 (42)	**23.16**	0.10	4.61	Positive
0466–32	6	3	7.5	4.61	185	64 (62)	**34.59**	0.16	7.25	Positive
0466–13	9	4	2	6.10	155	20 (23)	**12.90**	0.08	3.62	Positive
0466–36	13	4	3	4.16	179	56 (58)	**31.28**	0.13	5.99	Positive
0466–52	23	4	4	4.91	183	55 (58)	**30.05**	0.15	6.79	Positive
0466–64	1	4	6	4.03	167	80 (84)	**47.90**	0.19	8.89	Positive
0466–25	17	4	6	3.40	161	100 (101)	**62.11**	0.21	9.71	Positive
0466–42	7	4	8	4.56	161	79 (83)	**49.07**	0.22	10.29	Positive
0466–35	16	5	0.5	3.54	157	7 (7)	**4.46**	0.02	0.63	Positive
0466–22	10	5	2.5	4.08	172	16 (16)	**9.30**	0.04	1.51	Positive
0466–62	14	5	3.5	4.22	185	29 (33)	**15.68**	0.07	2.63	Positive
0466–15	24	5	4.5	2.10	174	84 (85)	**48.28**	0.10	4.03	Positive
0466–65	2	5	6.5	4.34	194	59 (59)	**30.41**	0.13	5.24	Positive
0466–26	18	5	6.5	2.78	153	69 (69)	**45.10**	0.13	4.99	Positive
0466–37	17	6	1	2.04	172	29 (31)	**16.86**	0.03	1.31	Positive
0466–24	11	6	3	12.51	175	21 (31)	**12.00**	0.15	5.71	Positive
0466–55	15	6	4	3.53	154	55 (55)	**35.71**	0.13	4.79	Positive
0466–16	1	6	5	3.65	175	70 (76)	**40.00**	0.15	5.55	Positive
0466–66	3	6	7	4.38	189	69 (66)	**36.51**	0.16	6.08	Positive
0466–28	19	6	7	6.83	167	45 (46)	**26.95**	0.18	7.00	Positive
0466–44	8	7	0.5	2.84	162	7 (7)	**4.32**	0.01	0.38	Positive
0466–33	18	7	1.5	1.94	180	39 (38)	**21.67**	0.04	1.31	Positive
0466–14	12	7	3.5	3.88	165	53 (54)	**32.12**	0.12	3.89	Positive
0466–21	2	7	5.5	4.12	160	55 (55)	**34.38**	0.14	4.41	Positive
0466–58	4	7	7.5	8.65	191	42 (43)	**21.99**	0.19	5.93	Positive
0466–38	20	7	7.5	6.40	186	73 (74)	**39.25**	0.25	7.83	Positive
0466–29	9	8	1	6.55	173	2 (2)	**1.16**	0.01	0.75	Positive
0466–34	19	8	2	4.75	164	6 (7)	**3.66**	0.02	1.72	Positive
0466–08	13	8	4	3.49	205	23 (28)	**11.22**	0.04	3.87	Positive
0466–23	3	8	6	3.38	158	14 (19)	**8.86**	0.03	2.96	Positive
0466–63	5	8	8	7.60	168	10 (19)	**5.95**	0.05	4.47	Positive
0466–46	21	8	8	4.59	193	24 (27)	**12.44**	0.06	5.64	Positive

[a]Sediment (%) = (weight sediment/weight sample)×100

[b]No. of animal particles = number of particles classified as being of animal origin. The results from the decision rule model are given in parentheses

[c]Bones in the sediment (%) = (No. of animal particles/No. of analysed particles)×100)

[d]Weight of bones in the sample = [(bones in the sediment (%)/100×weight sediment (g)]

[e]Animal ingredients in the sample (%) = [weight bones in the sample (g)/% bones in the animal feed ingredient used to spike the sample (= *f* factor)]

Table 3 Results from NIRM analysis of the feed matrices used for preparation of sample set C

Sample code	Theoretical (%)	Matrix used	Sediment[a] (%)	No. of particles analysed	No. of animal particles[b]	Conclusion[c]
0543–09	0	1	2.36	305	**0 (7)**	Negative
0543–10	0	2	2.31	313	**0 (3)**	Negative
0543–11	0	3	2.26	318	**0 (0)**	Negative
0543–12	0	4	6.08	333	**0 (1)**	Negative
0543–13	0	5	6.72	396	**0 (1)**	Negative
0543–14	0	6	2.70	315	**1 (0)**	Negative
0543–15	0	7	2.09	318	**0 (15)**	Negative
0543–16	0	8	1.89	309	**0 (0)**	Negative
0543–17	0	9	8.72	320	**0 (0)**	Negative
0543–18	0	10	3.07	319	**0 (6)**	Negative
0543–19	0	11	9.35	311	**0 (2)**	Negative
0543–20	0	12	2.91	310	**4 (2)**	Negative
0543–21	0	13	3.94	328	**0 (0)**	Negative
0543–22	0	14	3.60	315	**0 (3)**	Negative
0543–23	0	15	2.46	325	**0 (2)**	Negative
0543–24	0	16	0.31	346	**0 (1)**	Negative
0543–25	0	17	1.97	322	**2 (1)**	Negative
0543–26	0	18	1.09	174	**0 (0)**	Negative
0543–27	0	19	4.56	321	**0 (1)**	Negative
0543–28	0	20	5.44	344	**0 (0)**	Negative
0543–29	0	21	3.97	303	**0 (2)**	Negative
0543–30	0	22	2.13	331	**0 (1)**	Negative
0543–31	0	23	3.47	343	**0 (0)**	Negative
0543–32	0	24	0.96	190	**0 (0)**	Negative

[a]Sediment (%) = (weight sediment/weight sample)×100
[b]No. of animal particles = number of particles classified as being of animal origin. The results from the decision rule model are given in parentheses
[c]Conclusion = conclusion made after visual observation of the particle spectra classified as animal

"animal origin" is higher with the decision rule [1] than with the PLS model. Looking more carefully at the spectra classified as animal by the decision rule showed that most of the animal particles were correctly classified and that some vegetable or mineral particles were wrongly classified as animal particles. The results in Table 3 showed that for three samples (sample codes 0543–14, 0543–20 and 0543–25) the PLS model had classified a total of seven particles as animal whereas examination of their spectra revealed that they did not belong to this group, indicating a rate of false positive results of 0.093%. With the decision rule, 48 particles were wrongly classified as animal particles leading to a rate of false positive results of 0.639%. The error of classification of both models is in the same order of magnitude as those calculated on Sample set A (respectively, 0.061 and 0.944%).

Looking at the conclusions of the NIRM analysis based on the PLS model, all samples of sample set C were correctly detected as positive (as shown in Table 2) and all blank matrices were correctly detected as negative (Table 3) indicating that no false negatives and no false positives were observed. The evaluation revealed that the results from sample set C corresponded well with the results from Sample set B (Table 1). It is also important to emphasise that we included rather different formulas of compound feeds as indicated by the broad range of the percentage of sediment fraction of the samples varying from 1.94% to 12.51% (Table 2). Between 1.16% and 68.1% of the particles turned out to be of animal origin based on analysis of 153–220 particles per sample. The results also show that to avoid false negative results at least 200 particles should be analysed in cases in which the compound feed has a high sediment percentage. For instance, in sample 0466–29, only two bone particles were found in the 173 sediment particles analysed and the percentage adulteration was about 1%. If a sample were prepared with the same matrix and MBM material at a level of adulteration of 0.1%, the chance of detecting at least one animal particle by analysis of 200 particles would be very low. The low percentage of particles of animal origin detected in the samples containing MBM 8 (third column in Table 2) is because of the low bone content in this material, 10.12%, whereas the bone content varies from 11.46% for MBM 1 to 32.08% for MBM 7. Therefore, to avoid false negative results we recommend increasing the number of particles to be analysed when the sediment fraction of the sample is high. The same is true when using optical microscopy for analysis.

Looking at the correlation between the true MBM concentration in the compound feed and the MBM concentration established by the proposed procedure revealed a coefficient of determination (R^2) of about 0.57%, which was obtained when all samples were used for the assessment irrespective of the type of MBM. The correlation was higher when evaluation was done for each individual type of MBM, because R^2 for MBM 1 to MBM 8 was 0.64, 0.89, 0.82, 0.93, 0.97, 0.74, 0.93 and 0.82, respectively.

Table 4 Results from optical microscopic analysis of part of Sample set C

	Sample code	Theoretical MBM (%)	Sediment[a] (%)	No. of particles analysed	No. of animal particles[b]	Weight of bone in the sample[c] (g)	Animal ingredients in the sample[d] (%)	Conclusion
n	0466–44	0.5	2.1	598	47	**0.02**	0.51	Positive
p	0466–29	1	6.2	447	8	**0.01**	1.10	Positive
a	0466–20	1.5	2.3	220	16	**0.02**	1.46	Positive
c	0466–03	2	11	404	42	**0.11**	4.56	Positive
e	0466–19	2.5	2.8	376	107	**0.08**	3.62	Positive
g	0466–36	3	2.5	360	127	**0.09**	4.06	Positive
j	0466–62	3.5	3.2	400	114	**0.09**	3.62	Positive
h	0466–52	4	3.3	339	125	**0.12**	5.60	Positive
l	0466–55	4	2.8	486	251	**0.14**	5.50	Positive
k	0466–15	4.5	1.4	330	197	**0.08**	3.32	Positive
m	0466–16	5	2.1	506	221	**0.09**	3.49	Positive
o	0466–21	5.5	3	555	213	**0.12**	3.59	Positive
q	0466–23	6	2.4	628	128	**0.05**	4.84	Positive
b	0466–30	6.5	6.7	442	68	**0.10**	8.99	Positive
d	0466–31	7	7.4	418	149	**0.26**	10.53	Positive
f	0466–32	7.5	3.5	527	250	**0.17**	7.55	Positive
i	0466–42	8	3.6	162	81	**0.18**	8.28	Positive

[a]Sediment (%) = (weight sediment/weight sample)×100
[b]No. of animal particles = number of particles classified as being of animal origin
[c]Weight of bones in the sample = [(bones in the sediment (%)/100×weight sediment (g)]
[d]Animal ingredients in the sample (%) = [weight bones in the sample (g)/% bones in the animal feed ingredient used to spike the sample (= *f* factor)]

Comparison with the optical microscopy method

We applied the optical microscopy method as a reference method to establish whether the low value for R^2 (0.57) was because some particles of animal origin were not correctly identified. A set of 17 samples from sample set C was analysed by the reference method. Sedimentation and analysis on these samples was performed blind using the one-solvent sedimentation procedure. Table 4 displays the results of this analysis. As expected, all the samples were detected as positive. Comparing the results for the weight of the sediment revealed that the values obtained with optical microscopy were systematically inferior to those obtained with NIRM. The average portion obtained by optical microscopy was 3.90% (SD = 2.50) whereas for NIRM it was 5.01% (SD = 2.64). This systematic difference can be explained by differences between sedimentation times and/or differences between times during the drying step in the sediment extraction procedure. The weight of bones in the sediment obtained by the optical microscopy and NIRM methods are compared in Fig. 2. The correlation coefficient between the results was 0.87; this was regarded as high given the fact that the analyses were done in two different laboratories. Applying the Student *t*-test ($\alpha = 0.05$, $df = 16$) showed that there was no significant difference between the two results. Therefore we concluded that the NIRM method does not underestimate or overestimate the bone content of the sediment fraction.

Conclusion

In this study we showed the fitness for purpose of an alternative procedure based on NIRM analysis for detection of banned MBM in compound feed. The re-

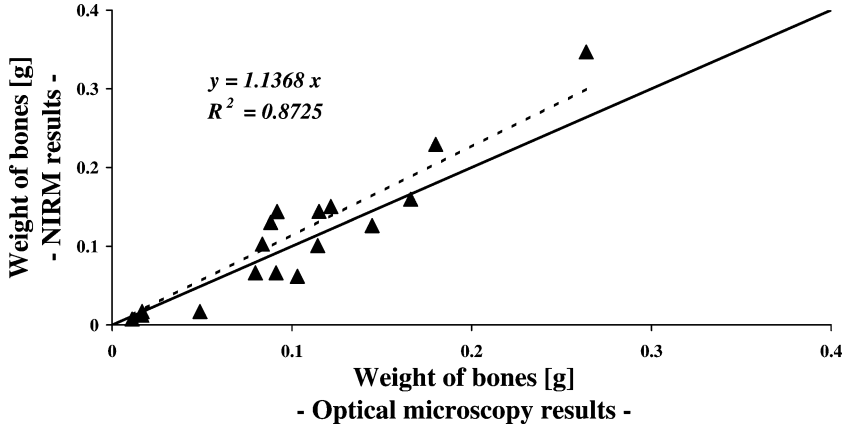

Fig. 2 Comparison of the weight of bones (g) obtained by use of the optical microscopy and NIRM methods (sample set C)

quired sensitivity of the method was achieved by focussing on the sediment of the samples containing a higher fraction of particles of animal origin. The results of the experiments demonstrate that:

1 this technique can be used for the detection of MBM in feed at a concentration of 0.05%; and
2 the NIRM method gives results equivalent to those obtained by optical microscopy, the official European Union method.

In contrast with optical microscopy, the proposed method does not require extremely experienced personnel to conduct the analysis because the particles are identified from their IR spectra and not by visual inspection of the particles.

The method depends on detecting bone fragments and studies must be undertaken to determine its capacity to distinguish animal species of bone. Further developments will also focus on increasing sample throughput by using more advanced technology, for example an IR camera [26]. In addition, we will establish whether the discriminant model presented in this study can be used by another laboratory to correctly classify feed samples containing MBM.

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