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TECHNICAL NOTE

Transferability study of a near-infrared microscopic method for the detection of banned meat and bone meal in feedingstuffs

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Abstract Near-infrared microscopy (NIRM) has been proved to be a powerful tool for the detection of banned meat and bone meal (MBM) in feed. The identification of MBM traces and its ability to differentiate animal from vegetable feed ingredients is based on the evaluation of near-infrared spectra obtained from individual particles present in the sample. This evaluation is supported by appropriate decision rules for the absorbances at specific wavelengths. Here we show that the method and the corresponding decision rules can be successfully transferred from the laboratory which constructed the decision rules to two independent laboratories that were not involved in the calibration process of the method. The analytical results from blind feed samples containing MBM (positive samples) and feed samples without MBM (negative samples) revealed a very good agreement between the three laboratories, thus demonstrating the transferability of the method.

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Introduction

The development and validation of analytical methods for the detection of the presence of species-specific animal proteins in animal feed was described in the European Commission's TSE (transmissible spongiform encephalopathies) roadmap [1] as the main condition for lifting the extended feed ban established through Commission Regulation 1234/2003 [2] amending Regulation 999/2001 [3].

Classical microscopy is at the moment the only official method in the European Union for the detection of constituents of animal origin in feed (Directive 126/2003/EC) [4]. This official method is capable of discriminating between fish and terrestrial processed animal proteins (PAPs), including those in meat and bone meal (MBM), but its sensitivity regarding detection of terrestrial PAPs decreases in the presence of significant amounts of fishmeal. More details about the criteria for this differentiation as applied in classical microscopy are described in a recent review article on the detection of MBM in feed [5].

Classical microscopy is conducted on the feed sample as such and on the sediment fraction, which is obtained after treatment of the sample with a solvent of high density. The investigation of the sediment is crucial for the required sensitivity of the method, because all bone particles are concentrated in this fraction. Since the feed ban includes a zero-tolerance policy, the presence of one single bone specula in feed is considered a positive sample and therefore an infringement of the feed ban. Another limitation of classical

microscopy is the fact that its performance depends significantly on the expertise of the operator conducting the microscopic analysis.

Other methods are applied to the analysis of feed samples for the presence of banned PAPs [5]. Since PAPs represent a complex mixture of different substances and constituents, none of these methods measure PAPs "as such", but they detect specific "targets" indicating the presence of PAPs. In fact, classical microscopy and near-infrared microscopy (NIRM) focus mainly on animal particles, whereas immunoassays and polymerase chain reaction analyse for specific proteins and DNA targets, respectively.

NIRM is based on the use of the near-infrared (NIR) spectra of individual particles to discriminate the origin of the feed compounds in a sample. The method allows the collection of spectra of up to thousands of particles from the analysis of one compound feed. Various chemometric tools, such as partial least squares, neural networks, and support vector machines, have been successfully developed and applied to NIR spectra in order to establish whether individual particles are of animal origin or not [6]. Another objective of the development of these tools is to allow for a discrimination of particles at different taxonomic levels. NIRM follows exactly the same protocol for sample preparation as classical microscopy and previous studies have demonstrated that there is no difference between the results obtained by classical microscopy and those obtained by NIRM. Both methods achieved a limit of detection of 0.1 wt% MBM in feed [7].

Recently, the Walloon Agricultural Research Centre (CRA-W) has developed new decision criteria for the identification of MBM in feedingstuffs. These criteria are based on the visual inspection of the NIR spectra obtained at specific wavelengths and can be applied alternatively to multivariate statistics. Here we report the results of the transferability study in which these decision criteria were applied to measurements obtained by two independent laboratories of the Joint Research Centre, namely the Institute for Health and Consumer Protection (IHCP) in Ispra (Italy) and the Institute for Reference Materials and Measurements (IRMM) in Geel (Belgium). In particular it was of interest to establish whether the decision criteria for the evaluation of the NIR spectra obtained as developed by CRA-W were still valid when utilised in two different laboratories. For the comparison of the results from the three laboratories, each laboratory determined the proportion of particles identified as MBM by analysing a set of positive and negative samples. The main target of the comparison was to check the performance of the methods in terms of the correct *identification* of bone particles (qualitative analysis). In addition, we also evaluated the method regarding its capability of estimating the MBM content. In the course of method validation, proven transferability of a new analytical concept is an important performance characteristic and is often considered as a prerequisite before launching a full interlaboratory study.

Materials and methods

Instrumentation

The three laboratories utilised Fourier transform (FT) infrared microscopes from PerkinElmer, but the models and the specifications were different. The instruments utilised in the study enabled the collection of spectra from small surfaces using an aperture size of 50 μm×50 μm. The microscopes include a camera and a viewing system to magnify the visible-light image of the sample to observe, highlight and isolate a point interest. For this study ten coadded scans were used. The spectra were obtained after determination of the ratio of the raw spectrum to the background obtained from measurement of a sample disc made of Spectralon.

CRA-W utilised a PerkinElmer Spectrum IdentiCheck FT-NIR system, IHCP measured with a PerkinElmer FT infrared Spectrum 2000 system and IRMM used a PerkinElmer Spectrum One NTS system. All the spectrometers were equipped with a PerkinElmer autoimage or a PerkinElmer Spotlight microscope.

Sample preparation and measurement of the NIR spectra

The analytical method including the sample preparation, the measurement and the evaluation of the NIR spectra has been described elsewhere [7].

The NIRM analysis was performed on the particles of the sediment fraction, which represents the fraction of the feed sample with a density above 1.62 g/ml. The sediment fraction was obtained by treating the feed sample with tetrachloroethylene as described in the European official microscopic method [4]. In short, at least 5 g—in this study 10 g—of the compound feed samples is transferred into a separation funnel and tetrachloroethylene is added. The mixture is first shaken and then left to stand for sufficient time, so the sediment containing the bone particles can be separated off. The particles of the sediment are then spread on the Spectralon plate of the FT-NIR microscopes, which allows the measurement of small surfaces (10 μm×10 μm). The spectra (1,100–2,500 nm) are collected by selecting visually individual particles and focussing the infrared beam on each of the selected particles. Spectra from at least 150 particles from each sample are measured and evaluated by applying decision rules on absorbance values at different wavelengths in order to establish whether the particles represent material of animal origin such as MBM or not. The estimation of the MBM content in feed is based

on three factors, namely (1) the weight of the sediment and the corresponding feed sample, (2) the proportion (weight percent) of bone in the constituents of animal origin and (3) the portion of identified bones in the sediment. In this study, we focused on the determination of the bone content (factor 3), which was estimated by the percentage number of identified bones divided by the total number of particles, from which NIR spectra were obtained. This is due to the fact that this part of the analysis is generally considered as most critical regarding the correct identification of bone particles and the estimation of the content.

Decision criteria for the evaluation of the NIR spectra

In the framework of the accreditation of the NIRM method according to ISO 17025, CRA-W has developed a new decision rule based on the visual observation of the spectrum in order to assess the animal origin. The rule is based on the following three criteria:

1. Presence of maxima in the 1,920–1,960-nm (Fig. 1, region a), 2,030–2,070-nm (Fig. 1, region c) and 2,150–2,200-nm (Fig. 1, region e) regions
2. Presence of minima in the 2,010–2,030-nm (Fig. 1, region b), 2,070–2,150-nm (Fig. 1, region d) and 2,210–2,250-nm (Fig. 1, region f) regions
3. A value of the bracket expression (absorbance at the minima observed in region b + absorbance at the minima observed in region f)/2 higher than the absorbance at the minima observed in region d (dashed horizontal line in Fig. 1).

A particle is only classified as originating from MBM (positive particles) if its spectrum fulfils *all* three criteria.

Test material

The test samples utilised in this study were prepared within the frame of the European FP5 project STRATFEED [8]. They consisted of industrial compound feedingstuffs that did not contain MBM and which were afterwards fortified with eight different types of MBMs at various concentrations ranging from 0.5 to 8 wt%. The MBMs included in the study cover quite different types of MBMs as shown by the corresponding NIR spectra, the global Mahalanobis distance statistics and the percentage bone fraction of the MBMs. Also some blank compound feed samples were measured in this transferability study. More details about the production of the test samples are given by Garrido-Varo et al. [9]. In order to focus on the impact of the spectroscopic analysis, CRA-W did the sedimentation of all test samples according to the European method [4]. Three representative subsamples were taken from the sediments and analysed by the three laboratories involved in this study.

Results and discussion

Each laboratory analysed in total 20 samples, corresponding to 3,500 spectra for CRA-W and IHCP and about 5,500 spectra for IRMM, respectively. The visual evaluation of the spectra applying the aforementioned criteria was straightforward and did not pose any problems. Table 1 shows the number of particles measured and the number of particles that were positively identified as MBM. All positive and negative samples were correctly identified by the three laboratories; thus, no false-positive or false-negative results were observed. The outcome of the quantitative estimate of the percentage bone content in the sediment is shown

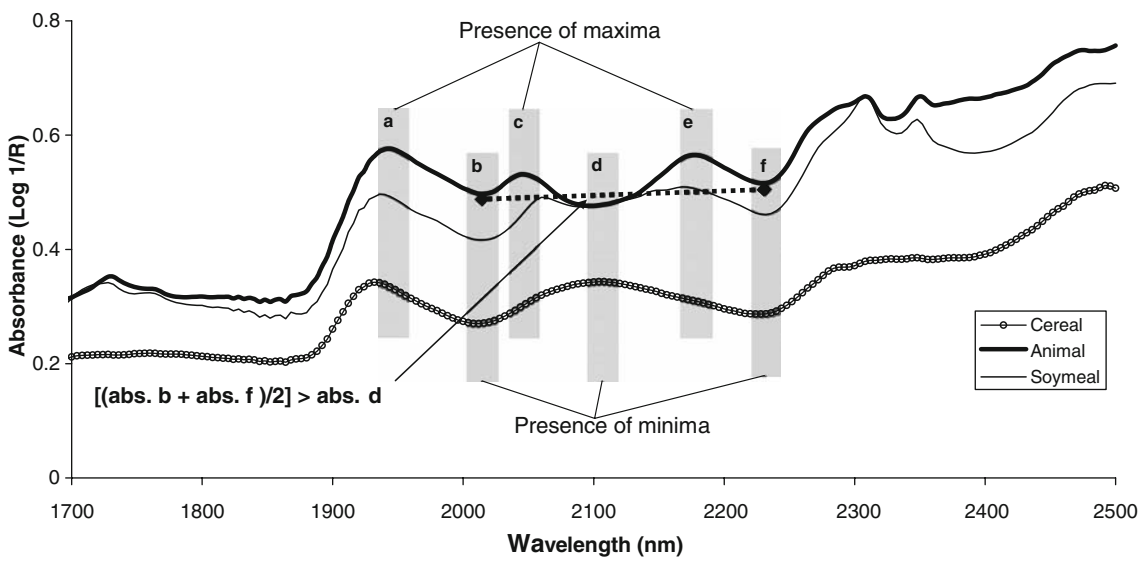

Fig. 1 Spectral conditions to be fulfilled by a spectrum for it to be from a particle of animal origin

Table 1 Analytical results from the three laboratories

		CRA-W		IHCP		IRMM	
Sample code	MBM (%)	Number of particles analysed	Number of positive particles	Number of particles analysed	Number of positive particles	Number of particles analysed	Number of positive particles
1	0.5	157	7	201	12	337	12
2	1	172	31	154	31	275	42
3	1.5	180	39	153	37	251	50
4	2	164	3	208	4	286	2
5	8	161	81	154	94	310	135
6	0.5	162	7	200	13	314	7
7	7	189	70	152	60	313	102
8	7.5	191	42	152	28	317	66
9	8	168	10	206	17	316	24
10	0.5	185	5	200	6	324	3
11	1	165	17	158	22	201	28
12	1.5	183	20	152	31	250	38
13	2	155	20	152	21	249	26
14	4	175	21	151	12	235	30
15	3	205	22	156	23	255	28
16	2.5	177	42	202	29	282	35
17	8	193	21	152	23	333	39
18	0	150	0	206	0	275	0
19	0	150	0	202	0	262	0
20	0	150	0	202	0	241	0

The "number of positive particles" is the number of particles identified as meat and bone meal on the basis of the evaluation of the corresponding spectra. *CRA-W* Walloon Agricultural Research Centre, *IHCP* Institute for Health and Consumer Protection, *IRMM* Institute for Reference Materials and Measurements, *MBM* target weight concentration of meat and bone meal in the animal feed

in Fig. 2, indicating sufficient correspondence amongst the three laboratories. We also applied the paired *t* test and the outcome of the statistical assessment indicated that the differences between the results of CRA-W and those of the two other laboratories were not significant (α=0.05).

It is important to emphasise that the sensitivity of the method can be adjusted to the target limit of detection by

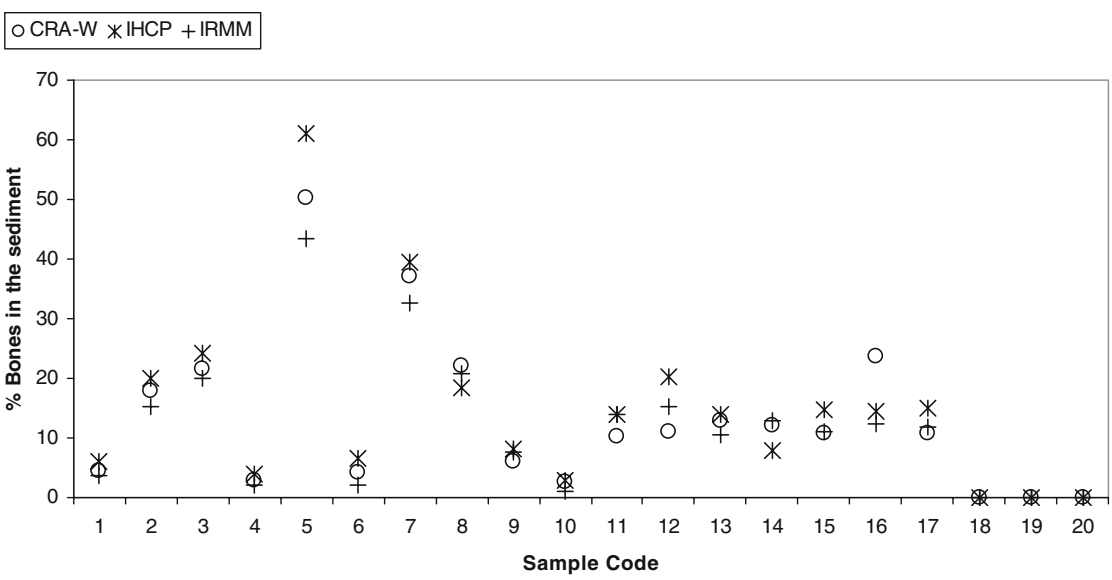

Fig. 2 Comparison of the measured percentage bone concentrations in the sediments obtained by the three laboratories. The bone concentration is expressed in terms of the percentage number of particles identified as meat and bone meal related to the total number of particles analysed. CRA-W Walloon Agricultural Research Centre, IHCP Institute for Health and Consumer Protection, IRMM Institute for Reference Materials and Measurements

including a sufficient number of particles in the measurement. Consider the example in which the target MBM concentration in feed is 0.1 wt% and samples containing MBM at this level should be positively identified with a probability of 95%. In this example we employ the Poisson distribution, since the positive identification of MBM in feed at this concentration is confirmed if *one* particle in the analysis of the sediment fraction is positively identified as MBM. Assuming that the percentage bone fraction in MBM is 20%, the sediment fraction is 2% and the sample size to be analysed is 10 g, the minimal number of particles to be analysed in the sediment should be at least 300. When utilising the method for quantitative analyses, one needs to measure even more particles in order to ensure sufficient precision of the estimate.

Conclusion

In this study we have demonstrated that NIRM is a powerful tool for the detection of MBM in the sediment fraction of animal feedingstuff samples. Identification of particles originating from MBM was obtained by applying a set of recently established criteria for the visual inspection of the measured NIR spectra. The method described here has been applied by two independent laboratories analysing blind samples and correct results were obtained. The successful transfer of the method to two independent laboratories demonstrated the ruggedness of the protocol. The ruggedness is an important method performance characteristic, especially when considering the method as an additional tool in the frame of official control. On the basis of these encouraging results further research activities have been launched to exploit the discrimination power of NIR spectroscopy for the detection of traces of animal origin in animal feedingstuffs at a higher taxonomic level. The potential of the NIRM method for the quantification of MBM in feed should be considered also to support the eventual introduction of a tolerance level in the feed ban.

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