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# In-house validation of a method for detection of animal meals in ruminant feeds by microscopy

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**Abstract**

The aims of this work were the optimization and the in-house validation of a qualitative method for detection of meat and bone, steamed bone, blood, poultry offal, feather and fish meals in ruminant feeds by microscopy. Specificity, detection limit and fitness for purpose were evaluated by procedures with reference materials, sample blanks and samples spiked with each analyte at 0.025%, 0.05%, 0.10%, 0.20%, 0.40% and 0.80%, in at least three independent replicates. Detection limits were 0.05% for meat and bone, steamed bone, blood, feather and fish meals and 0.10% for poultry offal meal.


*Keywords:* Feed; Microscopy; Animal meals

## 1. Introduction

The transmissible spongiform encephalopathy (TSE) is responsible for fatal neurodegenerative diseases in humans and animals. Human TSE includes Creutzfeldt–Jakob Disease (CJD), fatal familial insomnia (FFI), Gerstmann–Sträussles–Scheinker syndrome (GSS) and kuru, which is transmitted through ritual cannibalism. In animals, TSE includes natural scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) or mad-cow disease, feline spongiform encephalopathy (FSE), transmissible mink encephalopathy (TME) and chronic wasting disease (CWD) (Concepcion & Padlan, 2003; Dormont, 2002).

The first cases of BSE were diagnosed in the United Kingdom in 1986. It is believed that BSE is a form of scrapie that has been acquired by cattle after ingestion of scrapie-infected sheep matter that had been added to cattle feed (Kimberlin, 1993).

A new variant of CJD (vCJD) began to appear about 10 years after BSE was first identified in cattle in the United Kingdom and was described in March 1996. In contrast to the traditional form of CJD, it affects young patients (Brown, 1997; Fishbein, 1998; Will, Ironside, & Zeidler, 1996; Will, Zeidler, & Brown, 1996). It is also believed that a vCJD may have been caused by BSE being transmitted to humans by consumption of infected beef (Bruce et al., 1997; Concepcion & Padlan, 2003; Scott et al., 1999).

The occurrence of BSE in cattle and its transmissibility to humans is quite serious, mainly due to the fact that there is no therapy applicable to TSE in humans. Therefore, one should try to minimize the exposure of the

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human population to the BSE agent through food practices and the usage of biological products of bovine origin (Dormont, 2002).

Recommendations on the measures to protect public health agreed by the World Health Organization (WHO), Organization International des Epizooties (OIE) and Food and Agricultural Organization (FAO) include: (1) no part or product of any animal which has shown signs of TSE, nor tissues that are likely to contain BSE agent should enter into the food chain (human or animal); (2) all countries should establish surveillance and compulsory notification of BSE; (3) all countries should ban the use of ruminant tissues in ruminant feed; (4) milk and milk products are considered safe; (5) gelatin and tallow are only considered safe if effective rendering procedures are used; and (6) medicinal products and medical devices should be obtained from countries with no sporadic cases of BSE and measures recommended to minimize the risk (WHO, 1996).

In consequence, many countries formalized regulations that prohibit the use of proteins derived from animal by-products in feeding ruminants. These regulations defined a need for analytical methods to allow the control of the mentioned subjects.

Several methods have been described for the determination of animal material in feed. They are based on the analysis of proteins, e.g. isoelectric focussing (Jemmi & Schlosser, 1991; Jemmi & Schlosser, 1993; Wintero, Thomsen, & Davies, 1990), immunoassays (Ansfield, 1994; Hofmann, 1996; Hofmann, 1997; Hsieh, Sheu, & Bridgman, 1998; Morales et al., 1994) or gel diffusion (Waiblinger, Weber, & Kleinert, 1998).

DNA-based techniques are also described. DNA hybridization (Ebbehoj & Thomsen, 1991; Hunt, Parker, & Lumley, 1997) and polymerase chain reaction systems (PCR) can be animal specific, e.g. beef (Brodmann & Moor, 2003; Matsunaga et al., 1999; Meyer, Höfelein, Lüthy, & Candrian, 1995), pig (Meyer, Candrian, & Lüthy, 1994; Wolf & Lüthy, 2001) or sheep (Chikuni, Tabata, Kosugiyama, & Monma, 1994). It was shown that a species specific detection of the central nervous tissue by gas chromatography–mass spectrometry detection of sphingolipids is possible (Niederer & Bollhalder, 2001).

Feed microscopy, based on tissue analysis, is an extensively used technique for detection of animal material in feeds for ruminants. The detection and identification is based on the observation of the morphological conformation of rough fragments with a stereomicroscope and on the examination of histological structure of fine particles with an optical microscope (AOAC, 1998).

Independently of the analytical technique employed, the reliability of the generated results should be guaranteed by validation procedures (Eurachem, 1998; ISO, 1999; Thompson, Ellison, & Wood, 2002). The aims of this work were the optimization and the in-house validation of a qualitative method for detection of animal meals (meat and bone, steamed bone, blood, poultry offal, feather and fish meals) in ruminant feeds by microscopy, for use of the control activities of the Brazilian Ministry of Agriculture and Livestock.

## 2. Materials and methods

### 2.1. Samples

Sample blanks of ruminant feeds were obtained from processes that did not contain animal meal in their formulations. These samples were homogenized, identified and stored at room temperature, protected from humidity and light, until the moment of the analysis.

### 2.2. Reference materials

Reference materials (meat and bone, steamed bone, blood, poultry offal, feather and fish meals) were obtained from industries inspected by the Brazilian Ministry of Agriculture and Livestock official service. At least three different reference materials of each analyte were evaluated.

The production processes used were done according to the Brazilian standards to particle size (ANFAR, 1998). Steamed bone, blood, poultry offal, feather and fish meals were obtained with maximum of 5% of retention when passed through a 2 mm sieve. Meat and bone meal was produced with maximum of 5% and 10% of retention when passed through sieves of 2 mm and 1.68 mm, respectively.

### 2.3. Chemicals

ACS/ISO grade petroleum ether, chloroform and acetone were obtained from Fisher (NJ, USA).

### 2.4. Instrumentation

The stereoscopic microscope (magnification of 10×–100×, with reflected/transmitted light and polarization) and the compound microscope (magnification of 40×–600×, with polarization) were from Carl Zeiss Jena Docuval (Germany).

### 2.5. Method procedures

The optimized and validated method was based on evaluation of the morphological characteristics through microscopy, with aid of qualitative chemical and physical tests (AAFM, 1992; Commission, 1998; Ferrando & Henri, 1966; Huss, 1975).

*2.5.1. Sample preparation*

Pellet samples were previously humidified until complete disintegration, cleared by washing with water under vacuum in a sintered glass funnel (0.1 mm–0.2 mm porosity), dried with acetone and broken into fragments with a pestle. Aliquots of 15 g of the samples and 50 mL of chloroform were placed in a separatory funnel, homogenized, and allowed to stand for phase separation. The precipitate was drained on to a capsule. The intermediate solvent phase was discarded. The upper was treated with 50 mL of petroleum ether + chloroform (10 + 90, v/v), homogenized and allowed to stand until precipitation from the lower phase. The precipitate was drained on to a capsule and the procedures were repeated with petroleum ether + chloroform 20 + 80, 30 + 70, 40 + 60 and 50 + 50 (v/v). After evaporation of the solvent and drying, the collected fractions were separately sieved and transferred to previously identified Petri plates.

*2.5.2. Microscopic detection*

The fractions contained in the Petri plates were analyzed under stereoscope microscope beginning with the thickest fraction. A compound microscope was used for evaluation of the fine particles.

The analytes were identified according to size, shape, color, texture, hardness, brightness and other morphological characteristics by comparison with reference materials. Confirmation by chemical and physical tests was performed.

*2.6. Method optimization and validation*

The selectivity and detection limit were defined by in-house validation procedures employing assays with reference materials, sample blanks and spiked samples. The fitness for purpose of this method was discussed based on the results of the performance characteristics evaluated (Thompson et al., 2002).

*2.6.1. Reference material assays*

To evaluate the distribution of the analytes in the different solvent mixtures, 15 g of at least three different reference materials of each analyte were submitted to the treatment with petroleum ether + chloroform 0 + 100, 10 + 90, 20 + 80, 30 + 70, 40 + 60 and 50 + 50 (v/v). The analyte concentrates in the solvent mixtures were submitted to the microscopic analysis to establish the properties (morphological characteristics) that could be attributed to each analyte.

*2.6.2. Sample assays*

Sample blanks and spiked samples at six different concentrations 0.025%, 0.05%, 0.10%, 0.20%, 0.40% and 0.80%, in at least three independent replicates, were analyzed. All of the samples were blind for the analysts.

The ability of the method to determine the analytes without interference from other components was evaluated.

The sample blanks were analyzed, those cases with absence of false positive in all replicates were considered acceptable. In the analysis of the spiked samples, cases with absence of false negative results in all replicates were considered acceptable.

Detection limits were defined for each analyte separately. Detected or non-detected results versus concentrations were evaluated to determine the threshold concentration at which the method became unreliable. The smallest concentration level at which the analyte was detected in all replicates was considered as limit of detection. At least ten independent replicates were analyzed in the concentration level corresponding to the detection limit.

## 3. Results and discussion

As it can be observed in Fig. 1, there were larger concentrations of particles of meat and bone meal and steamed bone meal when petroleum ether + chloroform 0 + 100 (v/v) was used ($p < 0.05$). For the blood and feather meals, the largest concentration of particles was found in petroleum ether + chloroform 30 + 70 (v/v) ($p < 0.05$). In the case of poultry offal and fish meals no concentration of particles in any of the different solvent mixtures studied was observed ($p > 0.05$). The concentration profiles were similar for all the reference materials of the same analyte.

The fines in meat and bone meal were granular. Particles of various colors were found. Bone particles in the wet rendering process occurred in small, opaque, white and dull pieces, with a rough surface and hard to break with tweezers (Fig. 2A). Tendon and meat particles appeared as small, irregularly shaped, semi-transparent, yellow to yellowish brown, hard and dull pieces, with a rough or smooth surface. Good quality meat and bone meal contain only small quantities of undesirable impurities, but a wide variety of contaminants. Hoof, horn, hair and leather were observed (Fig. 2B). Contamination by vegetal fiber could occur through inclusion of some paunch and intestinal contents of the animal.

Fines in steamed bone meal appeared as a white powder. Larger particles were chunky white to gray with smooth to medium rough surfaces (Fig. 3A). Small quantities of hoof, hair, leather and vegetal fiber were sometimes present (Fig. 3B).

Drum dried blood fines were mostly spheres or broken spheres. Spray or ring dried blood consisted mostly of fine spherical particles of irregular shape, black or deep purple, hard to break, with a smooth surface but dull or lacking luster (Fig. 4A). Blood sometimes

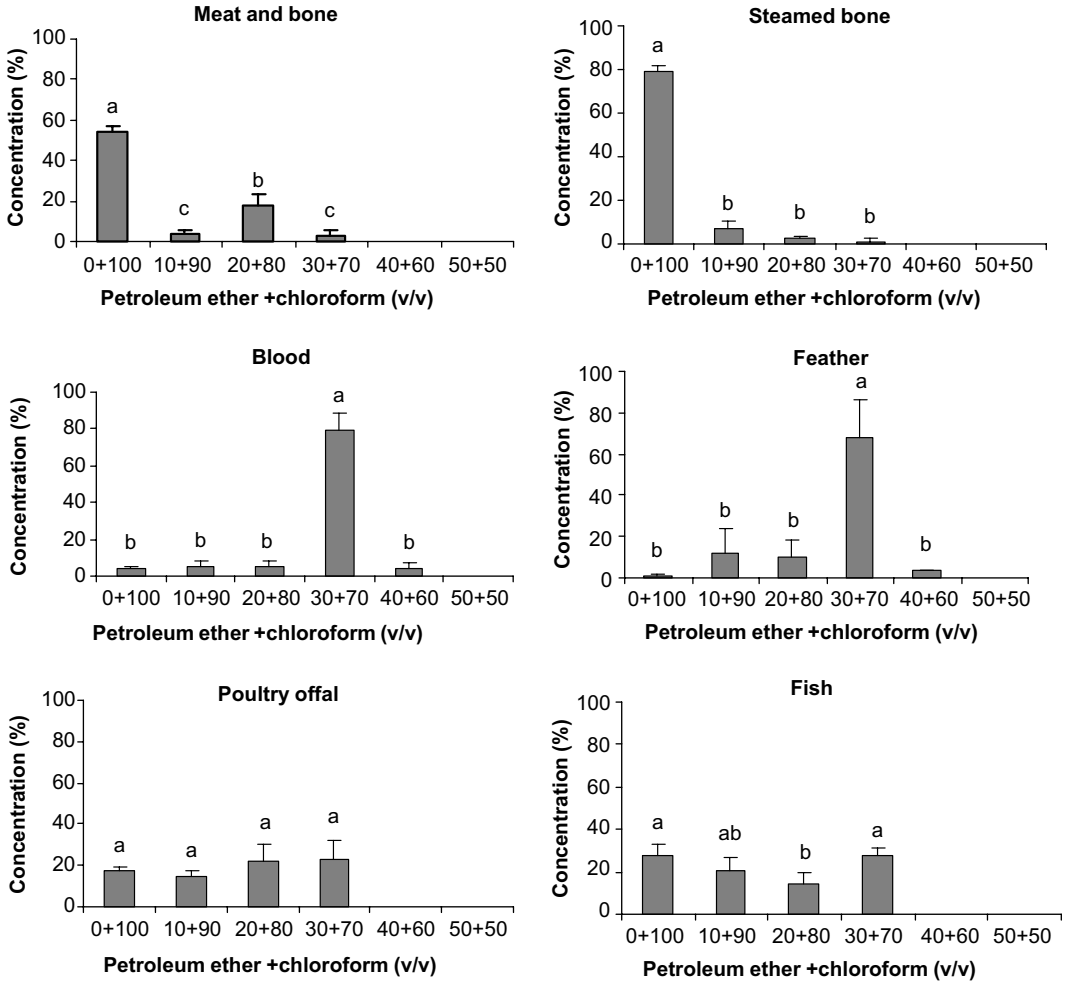


Fig. 1. Concentration profile of the analytes in different solvent mixtures. Means indicated with the same character were not significantly different by the test of Tukey at 5% level of probability. The error bars represent standard deviations.

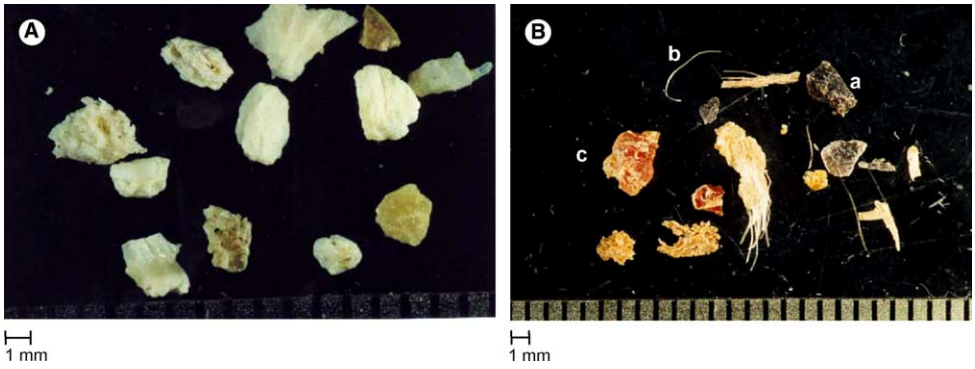


Fig. 2. Fines in meat and bone meal. A: fraction petroleum ether + chloroform 0 + 100, v/v—bone particles. B: fraction petroleum ether + chloroform 20 + 80, v/v—hoof / horn (a), hair (b) and leather (c).

contained bone and occasionally small quantities of vegetal fiber and hair (Fig. 4B).

Fines in poultry offal appeared as small particles, irregularly shaped, slightly greasy and with a rough surface. Some of the larger pieces of bone appear honeycombed on the inner surface and are not as thick as in bone from cattle and swine (Fig. 5A and B).

Some feathers resembled hair (Fig. 6A), others resembled compressed clear plastic tubes (Fig. 6B). The rachis of contour feathers was encountered with its jagged, ser-

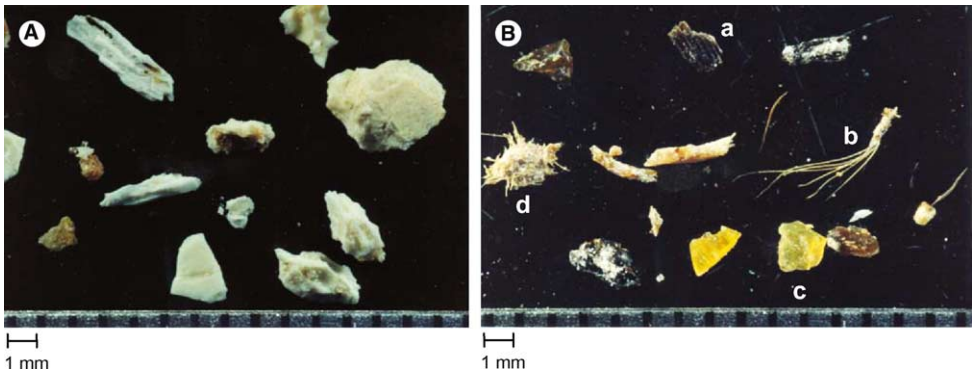


Fig. 3. Fines in steamed bone meal. A: fraction petroleum ether + chloroform 0 + 100, v/v—larger particles. B: fraction petroleum ether + chloroform 20 + 80, v/v—hoof (a), hair (b), leather (c) and vegetal fiber (d).

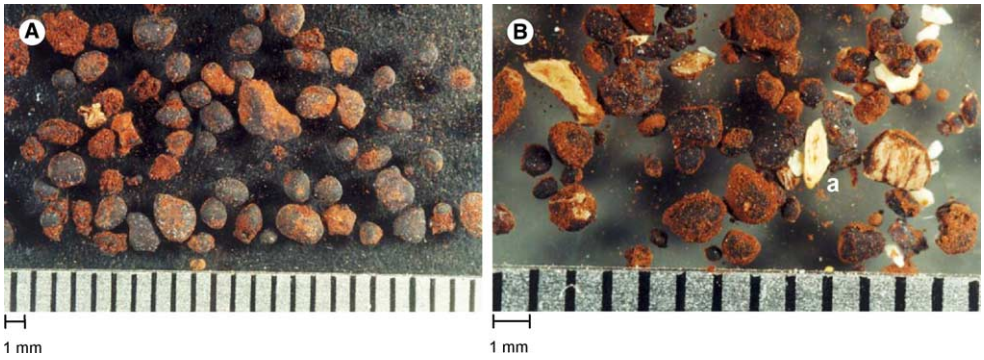


Fig. 4. Fines in blood meal. A: fraction petroleum ether + chloroform 30 + 70, v/v—spherical particles. B: fraction petroleum ether + chloroform 0 + 100, v/v—bone (a).

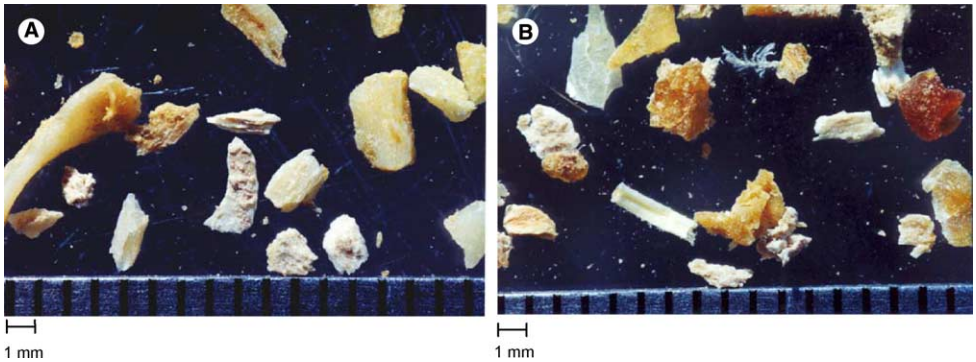


Fig. 5. Fines in poultry offal meal. A: fraction petroleum ether + chloroform 0 + 100, v/v. B: fraction petroleum ether + chloroform 30 + 70, v/v.

rated edges where the barbs have been broken off. Scale-like structures from the legs and feet could be detected. These appeared broader than long, slightly curved at the edges and were clear to shiny. Some particles from the beak and claw could be detected. These resembled bone in texture and hardness, but have a pearly luster similar to teeth. Complete hydrolysis removed all evidence of normal feather structure; however detection of raw feathers seldom occurred.

Fines in fish meal appeared granular. Larger particles were somewhat rough surfaced, partially retaining a fibrous structure. These particles were similar to expeller meat meal but lighter in color. The most unmistakable characteristics of fish meal were the bones and scales. Fish bones gave evidence of anatomical structure, even when in small pieces. Many were slender and pointed, while others show typical vertebral form (Fig. 7A). Scales were flat or curled almost transparent pieces with

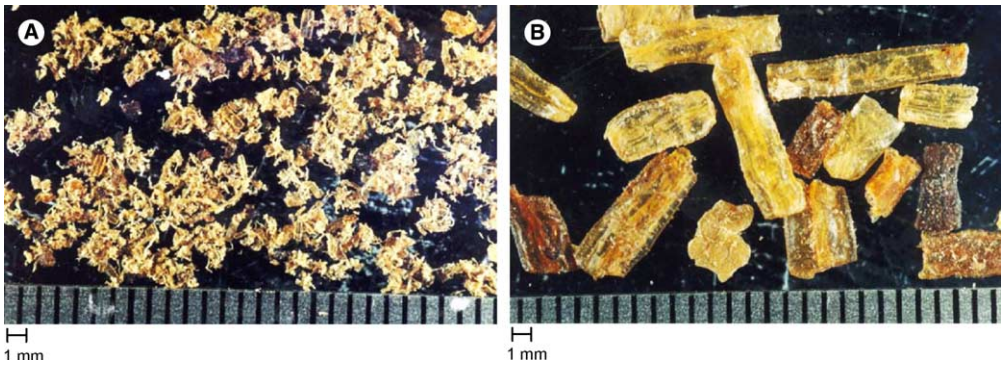


Fig. 6. Fines in feather meal. A: fraction petroleum ether + chloroform 30 + 70, v/v—resembled hair. B: fraction petroleum ether + chloroform 30 + 70, v/v—resembled plastic tubes.

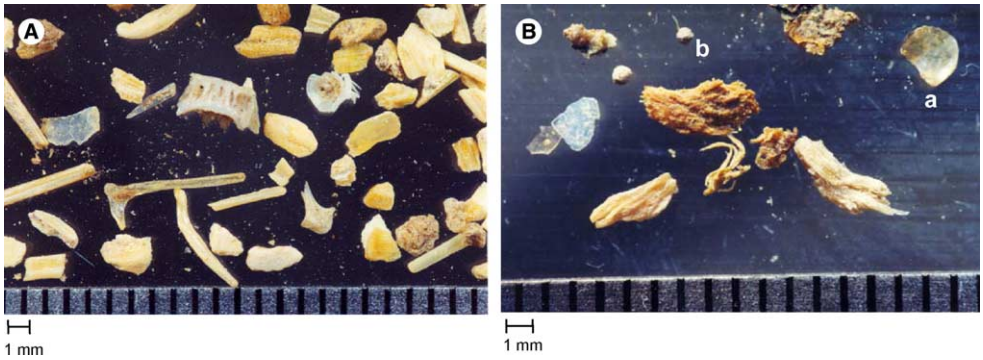


Fig. 7. Fines in fish meal. A: fraction petroleum ether + chloroform 0 + 100, v/v—bone particles. B: fraction petroleum ether + chloroform 30 + 70, v/v—scale (a) and eye lenses (b).

concentric markings. Small scale, eye lenses and roe were sometimes present (Fig. 7B).

All the sample blanks analyzed had non-detected results for the studied analytes. In assays employing spiked samples at 0.05%–0.80% for meat and bone, steamed bone, blood, feather and fish meals and at 0.10%–0.80% for poultry offal meal, all of the samples had detected results (Table 1). The smallest concentration levels at which the analytes were detected in all replicates were 0.05% for meat and bone, steamed bone, blood, feather and fish meals and 0.10% for poultry offal meal. These levels, the detection limits of the method for the respective analytes, were better than the 1% described by Brodmann and Moor (2003) for beef and mammalian detection in feed by PCR and than the 0.10% obtained in an interlaboratory study in the European Union (Momcilovic & Rasooly, 2000).

The rendering process in feed preparation involves heat treatment that denatures and degrades proteins and DNA. This is a significant limitation for techniques

Table 1
Detected and non-detected results in assays with sample blanks and spiked samples

| Concentration (%) | Analyte | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Meat and bone | | Steamed bone | | Blood | | Feather | | Poultry offal | | Fish | |
| | *n*[a] | D/ND[b] | *n* | D/ND | *n* | D/ND | *n* | D/ND | *n* | D/ND | *n* | D/ND |
| 0.80 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 |
| 0.40 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 |
| 0.20 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 |
| 0.10 | 10 | 10/0 | 3 | 3/0 | 3 | 3/0 | 3 | 3/0 | 12 | 12/0 | 3 | 3/0 |
| 0.05 | 10 | 10/0 | 10 | 10/0 | 10 | 10/0 | 10 | 10/0 | 3 | 2/1 | 10 | 10/0 |
| 0.025 | 3 | 2/1 | 3 | 2/1 | 3 | 2/1 | 3 | 2/1 | – | – | 3 | 2/1 |
| 0.00 | 6 | 0/6 | 4 | 0/4 | 6 | 0/6 | 5 | 0/5 | 4 | 0/4 | 4 | 0/4 |

[a] *n* = number of replicates.
[b] D/ND: number of detected results / number of non-detected results.

designed to analyze heat-treated proteins or DNA (Momcilovic & Rasooly, 2000).

Immunological techniques are simple, convenient and allow detection of very small amounts of proteins. However, most of them may cross-react with certain materials such as gelatin, giving rise to false-positive readings. Various approaches have been suggested to overcome this problem, but no data are available regarding reproducibility (Ansfield, 1994; Hofmann, 1996; Hofmann, 1997; Hsieh et al., 1998; Morales et al., 1994).

DNA-based techniques have accepted detection limits and are species specific, but cannot distinguish different kinds of tissues, since each cell from every tissue in an organism has identical DNA (Momcilovic & Rasooly, 2000).

Although microscopic methods present difficulty to distinguish bones of mammalian origin from those of poultry origin, this technique is the only test that the Commission of the European Communities endorses (Commission, 1998). In addition, there are many advantages to detection of animal meals in feed by microscopy: being unaffected by heat treatment of the sample, accepted detection limits, low costs, simplicity and speed when the operator had experience in identifying animal structures (Momcilovic & Rasooly, 2000).

## 4. Conclusions

The optimized and validated method showed sufficient selectivity on determining the analytes of interest in the presence of matrix interferents. The capability to distinguish allowed and prohibited animal meals, in the range of 0.05%–0.80% to meat and bone, steamed bone, blood, feather and fish meals and in the range of 0.10%–0.80% to poultry offal meal was also observed. The performance characteristics of the method presented in this paper indicated its fitness for use in feed control by the Brazilian Ministry of Agriculture and Livestock.

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