Determination of processed animal proteins in feed: the performance characteristics of classical microscopy and immunoassays.

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Table 1. The ban on Processed Animal Proteins (PAPs) in animal nutrition and the corresponding European regulation. For instance, EC Regulation 999/2001 prohibits the use of PAPs from ruminants and pigs in the feed for ruminants. Regulations written in bold have permanent character. The ban on PAPs according to Regulations written in italic may be lifted provided that animal specific tests are available. For instance, pigs could be fed on PAPs from poultry if analytical methods allow for specific detection of porcine PAPs in the presence of PAPs from poultry.

Feeding intended for these animals but prohibited by European law				
	<i>Ruminants</i>	<i>Pigs</i>	<i>Poultry</i>	<i>Fish</i>
<i>PAPs from Ruminants</i>	EC Reg. 999/2001	<i>EC Reg. 1234/2003⁽¹⁾</i>	<i>ECReg.1234/2003⁽¹⁾</i>	<i>ECReg.1234/2003⁽¹⁾</i>
<i>PAPs from Pigs</i>		EC Reg. 1774/2002		
<i>PAPs from Poultry</i>	<i>ECReg.1234/2003⁽¹⁾</i>	<i>EC Reg. 1234/2003⁽¹⁾</i>	EC Reg. 1774/2002	
<i>PAPs from Fish</i>				ECReg.1774/2002⁽²⁾

(1) Amending Annex IV to the Reg. 999/2001
(2) Derogation for PAPs from fish as specified in Decision 811/2003/EC.

or Peer Review Only

Table 3. A schematic overview of the most important features of two different methods.

	EU Directive 2003/126 (Commission Directive EC/2003/126)	Two-solvent Method (Michard and Ziebal, 1999)
Sample amount	≥5 g	10 g
Sample milling	Grinding mill or mortar	Coffee mill, Retsch ZM100 or equivalent
Sedimentation funnel/beaker	Separation funnel or conical bottomed settling beaker	Separation funnel
Concentrating agent	TCE	TCE/TBE (42/58 v/v)
Sedimentation time (minutes)	Funnel: ≥ 2x3' Beaker: ≥ 5'	45' +30'
Sediment staining (alizarine)	Yes or No	Yes
Observed fractions (sediment, sieve fraction)	Sediment and Sieve fraction	Sediment

Table 4. Characteristics of the methods applied in the immunoassay study. Method B utilises the same immunoassay than method A, but applies a different sample preparation procedure thereby obtaining an improved sensitivity.

	Method A	Method B	Method C	Method D
Target PAPs	Ruminant	Ruminant	Terrestrial animals, Ruminant, Porcine, Poultry	All animals, mammalian
Target tissue	Skeletal Muscle	Skeletal Muscle	Muscle, bone and offal	All animals: connective tissue Mammalian: muscle
Design of the test	Lateral flow immunoassay	Lateral flow immunoassay	ELISA	Lateral flow immunoassay

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Table 5. Performance of methods expressed in terms of sensitivity (%) for PAPs from terrestrial animals.

Method applied	Study	0.1% MMBM in feed	0.1% MMBM in feed in the presence of 5% fishmeal
Two-solvent Method	This report	93	60
European Method (Directive EC/2003/126)	This report	98	72
European Method (Directive EC/2003/126)	Stratfeed validation study (von Holst et al., 2005)	99	77
European Method (Directive EC/2003/126)	RIKILT (Van Ramsdonk & Van der Voet, 2003)	100	98
Former European Method (Directive EC/88/1998)	DG-SANCO A (Gizzi et al., 2004)	92	44

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Table 6. Results of analysis from method D. P = Positive; FP = False Positive; N = Negative; FN = False Negative; NR = No results. Details of the methods are given in

Blank material	MMBM 0.1%, 133°C + fishmeal, 5%	Fishmeal, 5%	Bovine MBM 0.1%, 133°C	Bovine PAPs 0.1%, 129°C	Porcine PAPs 5%, 133°C	2% DCP
MAT I	MAT IV	MAT VI	MAT VII	MAT VIII	MAT IX	MAT X
FP N NR	P FN NR	FP N NR	P FN NR	P FN NR	P FN NR	P N NR
0 6 0	0 6 0	0 6 0	6 4 0	0 6 0	10 0 0	0 6 0

Table 2. Description of the samples used in the study. MMBM = Mammalian meat and bone meal; DCP = dicalcium phosphate; Concentrations are expressed in terms of percent of mass fraction. Total number of samples per laboratory was 24 for microscopy and 50 for immunoassay analysis.

	MAT I	MAT II	MAT III	MAT IV	MAT V	MAT VI	MAT VII	MAT VIII	MAT IX	MAT X	MAT XI	MAT XII
MMBM, 133°C (%)	-	0.1	0.1	0.1	-	-	-	-	-	-	0.5	-
Bovine MBM 133°C (%)	-	-	-	-	-	-	0.1	-	-	-	-	-
Bovine PAP 129°C (%)	-	-	-	-	-	-	-	0.1	-	-	-	-
Porcine MBM (%)	-	-	-	-	-	-	-	-	5	-	-	-
Fishmeal (%)	-	-	-	5	-	5	-	-	-	-	-	-
Feather meal (%)	-	-	5	-	0.5	-	-	-	-	-	5	5
DCP (%)	-	-	-	-	-	-	-	-	-	2	-	-
Microscopy: Number of samples per laboratory	3	3	3	3	3	3	-	-	-	-	3	3
Immunoassay: Number of samples per laboratory	6	-	-	6	-	6	10	6	10	6	-	-
Mean Ct with 2 x SD interval (and targeted animal species)	-	-	-	33,21 ± 2.01 (cattle)	-	-	34,77 ± 2.29 (cattle)	33,89 ± 1.96 (cattle)	-	-	-	-

Deleted: chicken

Determination of processed animal proteins in feed: the performance characteristics of classical microscopy and immunoassay methods

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Abstract

Species-specific detection and detection of groups of species such as ruminants is required according to European legislation dealing with the safe use of animal by-products in animal nutrition. Various methods are applied to the analysis of feed samples for the presence of banned processed animal proteins (PAPs) including meat and bone meal (MBM). Classical microscopy as described in the Commission Directive EC/2003/126 is the only official method to detect the presence of constituents of animal origin in feed, nevertheless some deviating protocols allowed under the old Directive (EC/88/1988) claim to gain comparable results. An inherent limitation of the microscopic method is the lack of species specificity. Immunoassays showed the most promising potential in research projects or intercomparison studies being able to detect ruminant PAPs at a concentration level of 0.5%. The aim of this paper is to present the results of the intercomparison study conducted on behalf of DG-Health and Consumer Protection (SANCO) in 2004 to establish whether the two-solvent Method would gain comparable results to the current European Method and to evaluate the current capability of immunoassays of determining the species in PAPs present in feed.

Keywords: Animal feed, Bovine spongiform encephalopathy, Processed animal proteins (PAPs), Meat-and-bone-meal (MBM), Species identification, Microscopy, Immunoassays.

Introduction

The required sensitivity and selectivity of analytical methods applied to the detection of processed animal proteins (PAPs) in feed are specified, taking into account current and up-coming European legislation regarding the use of PAPs in animal nutrition. Focusing on the BSE-related aspect, EC Regulation 999/2001 prohibits the use of *mammalian* PAPs in ruminant feed. General rules regarding the safe use of animal by-products are laid down by EC Regulation 1774/2002, prohibiting feeding of animals with processed proteins from the same species. Enforcing these regulations would therefore require that analytical methods are capable of *specifically* detecting mammalian PAPs, PAPs from pigs and from poultry. The latter aspect is important to avoid intra-species recycling. Since BSE is linked to the presence of *ruminant* PAPs in ruminant feed, the specific detection of ruminant PAPs is also extremely important.

However, due to various problems such as the lack of methods that fulfil the above mentioned criteria, EC Regulation 999/2001 was amended by EC Regulation 1234/2003 introducing a ban on almost all PAPs in animal nutrition. It is important to note that this Regulation clearly indicates the need for a reappraisal of the total MBM ban once new and more specific control methods are available. In particular, there is a strong need for ruminant specific tests in order to establish whether traces of MBM in feed or feed ingredients that are most likely present in the material due to cross contamination contain ruminant PAPs. An overview of the current legal situation regarding the use of PAPs is given in Table 1.

[Insert Table 1 about here]

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4 The detection of traces of PAPs including meat and bone meal (MBM) in feed is still a
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6 challenging task. Laboratories apply various methodological approaches for the
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8 detection of MBM, but a former intercomparison study (DG SANCO 2003) showed that
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10 classical microscopy is still the most reliable tool compared to polymerase chain
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12 reaction (PCR) and immunoassays (Gizzi et al. 2004). In addition, a more careful
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14 examination of the results from the laboratories applying microscopy revealed that quite
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16 different protocols for the microscopy were applied, which were all covered by the
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18 European method valid until 30 June 2004 (Commission Directive EC/88/1998).
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27 Since 2001 the European project “Stratfeed” (STRATFEED EC project 2001) was
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29 working on the development and validation of analytical methods based on PCR,
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31 spectroscopy and microscopy proposing a modified protocol for microscopy.
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33 Comparing the results of the Stratfeed project with the results from the DG SANCO
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35 2003 study demonstrated that the protocol parameters of the microscopic method
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37 applied by the best performing laboratories coincided partly with the Stratfeed protocol
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39 indicating the superiority of the latter method. Based on the results from the DG
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41 SANCO 2003 study the European Commission discussed with Member States’ experts
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43 a revision of the European method (Commission Directive EC/88/1988) leading to a
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45 modified protocol (Commission Directive EC/2003/126) which also reflects the method
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47 modifications proposed by the “Stratfeed” project. The modified protocol is the only
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49 official European method since 1st July 2004 and has been recently validated by an
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51 intercomparison study (von Holst et al. 2005). Comparing the results of this study with
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53 the results of DG SANCO 2003 study (Gizzi et al. 2004) corroborated that the
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55 sensitivity of the more specified method as required by Commission Directive
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EC/2003/126 is superior to the method as described in Commission Directive EC/88/1988 which allows more variability of the protocol applied. For instance, a very challenging task is the detection of *terrestrial* MBM at 0.1 % in the presence of 5 % of fishmeal. These levels have been selected taking into account the current requirements for analytical methods applied in feed analysis for this purpose as outlined in the chapter “Experimental” of this paper. The pooled results from the various microscopic protocols that were covered by the former official method (Commission Directive EC/88/1998) revealed a rate of correct positive detections of 45 %. In contrast, the validation of the microscopic method as described by Commission Directive EC/2003/126 confirmed a significant improvement since the rate of correct positive results increased to about 77 % (von Holst et al. 2005).

Nevertheless, there are still claims that major and minor deviations from the improved protocol would gain comparable result. This applies especially to the two-solvent Method (also called the “French Method”), which uses two instead of one solvent in the sedimentation part of the protocol (Michard and Ziebal 1999). This modification was allowed under the old Directive (Commission Directive EC/88/1988) but is not covered anymore by the new Directive (Commission Directive EC/2003/126). In order to establish whether the two-solvent Method would gain comparable results to the current European method, an intercomparison study was conducted in which a part of laboratories applied the two-solvent Method and the other group applied the official European one-solvent method analysing the same test samples.

Though microscopy is the only official method for the detection of PAPs in feed, an inherent limitation of this method is the lack of animal (e.g. ruminants) or even species specificity. For instance, the results from a former study revealed (Gizzi et al. 2004) that the detection of mammalian PAPs in the presence of poultry meal is too demanding for the majority of European control laboratories. In the same study laboratories applied other methods such as PCR or immunoassays on the same samples because these approaches were expected to be more suitable for species detection in PAPs. However, the study showed that most of the various PCR methods also experienced problems in detecting mammalian and ruminant PAPs, whereas some of the immunoassays reported promising results. In fact, some of the immunoassays were able to detect ruminant PAPs at a concentration level of 0.5 %, but failed when the samples contained 0.1 % of ruminant PAPs. It was therefore considered as important to know more about the current capability of immunoassays of determining the species in PAPs present in feed and to establish whether the sensitivity of the methods has been improved. Four laboratories which participated in the former study (Gizzi et al. 2004) were asked to participate in a second study analysing 50 samples containing PAPs of cattle and pork separately. This study was designed as pre-validation in order to establish the most promising tests which merit further investigation by conducting a full validation study. In particular it was of interest to know whether these tests allow for the detection of PAPs at different taxonomic levels such as PAPs from mammals and ruminants at 0.1 %. It was also important to establish the selectivity of the immunoassays. For instance, a test for ruminant PAPs should give a negative result when the feed samples only contain porcine material.

The availability of ruminant specific tests is extremely important to handle the presence of traces of bones in feed ingredients of plant origin as recently happened in the European Union (European Commission 2005). Due to the current lack of animal specific tests, the sole identification of terrestrial bones can justify the exclusion of the feed concerned from being used in animal nutrition. However, such a decision could be reappraised if a ruminant specific test was able to confirm that the detected residues from animal origin do not derive from ruminant.

Another aspect was the impact of organic dicalcium phosphate (DCP) on the detection of PAPs. DCP is permitted in feed for non-ruminant farmed animals and may test positive by immunoassay due to the presence of proteins in it. . We need, however, to place emphasis that such a result would need to be considered as false positive regarding the parameter banned PAPs. Feed samples containing DCP were therefore included in the sample set to examine the impact of the presence of DCP on the control of the MBM ban.

The objective of this paper is to present the results of these intercomparison studies conducted in 2004 on behalf of DG Health and Consumer Protection (SANCO). The target parameters for microscopy were (1) “PAPs from all terrestrial animals and fish” (total PAPs), (2) PAPs from terrestrial animals and (3) mammalian PAPs. For the immunoassay methods the target parameters were mammalian PAPs and ruminant PAPs. Though some of the immunoassays included in this study measured also other targets such as PAPs from pig or poultry, we report exclusively on the analytical results

for ruminant PAPs due to the particular focus of the sample design on this target.

Experimental

Organisation of the study

For the intercomparison study each participant received:

(1) A protocol of the study containing the required information for the participants of the exercise.

(2) Laboratories applying microscopic analysis:

- A set of 24 coded feed samples consisting of 8 different materials each in blind triplicate as shown in Table 2.

Laboratories applying immunoassays analysis:

- A set of 50 coded feed samples consisting of 7 different materials in various numbers of replicates as shown in Table 2.

(3) A report template in Excel format in which the participants had to fill the results of their analysis for each parameter. In addition the laboratories were also requested to provide the organiser of the study with information about the method applied using an electronic form.

[Insert Table 2 about here]

Since there is *no* legal limit for PAPs in feed, the laboratories were requested to express their results in terms of “present”, “not present” or “no results” without indicating the concentration of the MBM in the samples. The latter response corresponded to inconsistent results or was indicated by laboratories when the method did not allow determining a specific parameter (e.g. the detection of porcine PAPs by a

ruminant specific immunoassay). The performance characteristics of the methods were therefore calculated based on the number of correct negatives and false positive results of the samples without PAPs and the number of false negative and corrective *positive* results of the samples containing PAPs at two concentration levels included in the study.

Statistics calculated

Important method characteristics of the microscopic methods were calculated according to Langton et al. 2002, which are the sensitivity indicating the rate of false negative results and the specificity giving information on the rate of false positive results. The following equations were applied:

$$\text{Sensitivity } SE = \frac{PA}{PA + ND} \text{ and}$$

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics were expressed as percentages after multiplication of the sensitivity and specificity by 100.

Since the evaluation of the immunoassays included in this study is based on less data compared to the intercomparison study of the microscopic methods, the analytical results of the immunoassays were expressed in terms of correct positive, correct negative, false positive and false negative results.

Test material

Twelve different samples containing typical compound feed and PAPs from various animals at different concentration levels have been prepared as shown in Table 2.

The composition was established taking into account the following aspects:

- Two target concentrations of the processed animal proteins were selected which were (1) 0.1 %, reflecting the sensitivity of the European method as required in and (2) 0.5 % according to a recommendation of the European Commission's Scientific Steering Committee that cross-contamination with mammalian MBM (MMBM) should be condemned at this level (Scientific Steering Committee 1998).
- Presence of fishmeal at a realistic level of 5 % that could interfere with the detection of constituents from terrestrial animals when using microscopy and immunoassays.
- Presence of feather meal that could interfere with the detection of constituents (at 0.1 % and 0.5 % respectively) from mammalian animals when using microscopy.
- Presence of meat and bone meal from pig by-products could interfere with the detection of constituents from ruminants when using immunoassay techniques.
- Presence of dicalcium phosphate from organic origin could interfere with the detection of constituents from mammals and ruminants when using immunoassay techniques.

The base of the test material (MAT I) was a compound feed for bovine containing

corn, soya, beet, straw, molasses, sunflower, palmkernel, coconut, wheat, fat and various feed additives such as minerals and vitamins. The sediment content of the compound feed was about 1.16 % (Standard deviation (SD) = 0.27). Prior to use the material was tested by polymerase chain reaction (PCR), immunoassay kits and near-infrared microscopy (NIRM) confirming the absence of any interfering substances from animal origin.

Different processed animal proteins were included in the study. The mammalian MBM used in the study was a mix of bovine and porcine processed material with a bone content (of) about 15.31 % (SD = 1.24) and is described elsewhere (Gizzi et al. 2004). The MBM feed ingredients were analysed by PCR in order to check the species present in the sample, confirming that these samples mainly contained bovine and porcine materials with low amounts of poultry material. The content of bovine MBM was about 45 %. The pure cattle and pig MBMs representing a typical mixture of different proteins as present in carcasses and the feather meal were obtained from a pilot plant using pure animal by-products of each species. The meals were treated at temperature 133°C and 3 bar for 20 minutes. Afterwards the material was dried under atmospheric conditions until the moisture content was below 10%. Finally the product was pressed and ground. Applying PCR confirmed that the cattle, pig and feather materials contained exclusively PAPs from the indicated species. The PAPs of MAT VIII were pure cattle meat that was treated at temperature at 129°C for 20 min. The meat was processed in a laboratory autoclave applying a procedure that mimics the sterilisation conditions in an industrial batch type rendering system quite well as described elsewhere (Pallaroni et al. 2001). The process, in short, consists of mincing the meat and adding water to form a slurry.

The material and a temperature probe were transferred in a plastic bag and placed in the autoclave ensuring that the bag was completely surrounded by water. After completion of the process the material was dried in an oven over-night at 70°C and finally ground in a centrifugal mill. The indicated temperature refers to the value measured by the temperature probe.

The fishmeal was obtained directly from a fishmeal producer and its proportion of bones was about 12.99 % (SD = 0.04). The feather meal was obtained from a pilot plant and produced from poultry by-products and its proportion of bones was about 0.06 % (SD = 0.03). The material was treated at 133°C and 3 bars for 20 min. Afterwards the material was dried under atmospheric conditions until the moisture content was below 10%. Finally the product was pressed and ground. The fishmeal and the feather meal were analysed by PCR confirming that the fishmeal did not contain bovine, porcine and poultry materials and that the feather meal did not contain bovine and porcine materials.

The different MBMs, which were mammalian MBM (MMBM), cattle meal, pig meal, poultry meal and fishmeal, DCP and the compound feed were used as raw product without grinding to a fine powder prior to the mixing procedure. This was required since grinding the sample is part of the microscopic analysis. Preparing the samples using the raw material is very demanding, especially when considering the low target concentration of 0.1 % MBM in some of the test materials. In order to have an acceptable homogeneity for the fortified samples (MAT II-XII) the *stepwise* dilution procedure as described in a former study (Gizzi et al. 2004) was utilized. The amount of

each sample sent to the laboratory was set at 12 g.

Sufficient homogeneity of each material was established by analysing five to ten randomly selected bags using the near-infrared microscopic method (NIRM) and a PCR method that proved to be suitable for the purpose of this analysis (Gizzi et al. 2003). NIRM is based on the analysis of several hundreds of particles of the sediment fraction using a near-infrared spectrometer coupled to an adapted microscope. Details of the method are given elsewhere (Baeten et al. 2005). In particular it was important to demonstrate that each of the sample bags of the test materials containing 0.1% MBM (i.e. MAT II, MAT III, MAT IV, MAT VII in Table 2) contained enough bones for microscopy. The test material was regarded as fit for purpose when the analysis of all test materials containing MBM at this level would gain a positive result of this method. The analyses of the test material confirmed that in all test items investigated the MBM specific fraction of the sediment was sufficiently constant.

The homogeneity of the test materials MAT IV, MAT VII and MAT VIII were also analysed by PCR. The results are expressed as the Mean Ct value (threshold cycle) and the standard deviation calculated on the three or four replicates per bag as well as for the mean values of the five bags analysed (Table 2). All the samples were detected as positive regarding the presence of bovine DNA.

Information on the microscopic methods applied

In microscopic analysis the samples are ground by various means and subjected to the sedimentation procedure by treating the samples with a halogenated solvent. Bones

concentrate in the sediment and are detected by microscopic analysis of the sediment. According to the official European method only tetrachloroethylene (density 1.62 g/ml) is used. Therefore all particles such as bones and minerals with a higher density form the sediment. In contrast, the two-solvent Method utilised two solvents for the sedimentation, which are tetrachloroethylene (density 1.62 g/ml) and a mixture (density 2.4 g/ml) of tetrachloroethylene and tetrabromoethane (42/58, v/v). All particles with a density between 1.62 and 2.4 g/ml including bones concentrate in the layer between the two organic solvents (sediment fraction I), whereas minerals with a higher density than 2.4 g/ml concentrate at the bottom of the two solvents (sediment fraction II). Therefore sediment fraction I contains less interfering particles thereby facilitating the identification of animal specific constituents. Careful handling of the sedimentation is required to avoid mixing of both organic solvents.

For the sedimentation process the following glassware is applied. As described in the European official method (Commission Directive EC/2003/126) two types of sedimentation glassware can be used: a conical bottomed settling beaker (Figure 1) or a separation funnel.

[Insert Figure 1 about here]

Two types of beakers can be used: an open beaker without cock and an open beaker with cock. In the beaker without cock, the sedimentation solvent is poured out carefully while the sediment remains on the bottom. In the beaker with cock, the sediment is caught in a small tray that is closed by turning the cock. Since the two-solvent Method uses two organic solvents for the sedimentation, this method requires a flexible tube to

enclose the intermediate sediment fraction as shown in Figure 2.

[Insert Figure 2 about here]

Important aspects of the two methods investigated in this study are summarised in Table 3. When comparing the two protocols it is clear that the use of *two* different organic solvents of the two-solvent Method renders the execution of the method more complicated compared to the European official method in which only one solvent used.

[Insert Table 3 about here]

Information on the immunoassays included in the study.

Four methods (A, B, C and D) developed by three institutes were included in the study. As shown in Table 4 the immunoassays differed considerably in terms of the target animal, the target tissue and the design of the test. Method A and B were lateral flow immunochromatographic assays and from the same institute but had different sample preparation procedures thereby achieving a different sensitivity. Both tests had ruminant proteins as target. The design of method C was a classical ELISA and allowed the detection of proteins from various animals including ruminants. Method D was developed as dip stick immunoassay that is suitable for the detection of PAPs from all animals without any distinction between species. In addition, the tests measured PAPs exclusively from mammals.

[Insert Table 4 about here]

Results and discussion

Intercomparison study of microscopic methods

In this discussion the participating laboratories are subdivided into two groups based on

the protocol they used:

- Evaluation of the official European method as described in Commission Directive EC/2003/126.
- Evaluation of the two-solvent Method as described in Michard and Ziebal 1999.

The content of the samples has been reported at three systematic levels: presence of total PAPs, presence of PAPs with distinction between constituents originated from terrestrial animals and fish and a further distinction to mammalian or avian origin.

Evaluation of the official European method as directive EC/2003/126. Eighteen laboratories, including eleven laboratories from new European Member States, reported results of the samples. Two laboratories with major deviations towards the protocol from the Directive were excluded from the study. One laboratory used chloroform as sedimentation agent, another laboratory applied a sedimentation time of 1 minute whereas a minimum of 2x3 min is required for a closed separation funnel (Commission Directive EC/2003/126).

A first target parameter was whether laboratories are able to detect the presence of PAPs from all terrestrial animals (mammals and poultry) and fish as shown in Figure 3. For this overview the results from all materials that contained PAPs were pooled, i.e. all materials with the exception of MAT I which was the blank material. Most of the samples were correctly classified. Sixteen samples out of 336 were wrongly classified as negative (SE = 95%). Seven of these sixteen negative deviations have been found to be contradictory results (e.g. positive for fishmeal but negative for total PAPs) produced by two laboratories including five results by one laboratory due to an error against the

definition of PAPs and two results by another laboratory due to a lack for a final check for plausibility. Seven other negative reported results were from MAT V (feather meal 0.5%), the matrix with the lowest amount of bones of all the samples with animal material with SE = 83%.

[Insert Figure 3 about here]

For the discussion of the next level (PAPs from terrestrial animals) the results from all samples that contained terrestrial PAPs (MBM or feathermeal) were pooled, obtaining 90 % of correct positive results. Focusing on the results from MAT IV that contained 0.1 % of MBM and 5 % of fishmeal revealed that due to the presence of fishmeal the sensitivity decreased from 90 % to 70 % confirming that large amounts of fishmeal can make the detection of terrestrial PAPs more difficult. In Table 5 the results of this study were compared with the results from other studies. The sensitivity of the method according to the new Directive EC/2003/126 (this study and the Stratfeed validation study) for MAT IV (0.1% MMBM in feed in the presence of 5% fishmeal) was 70 % and 77 %, respectively. Both studies, therefore, obtained comparable results but the sensitivity was higher compared to the two-solvent Method and the former Directive EC/98/88 (DG SANCO study 2003), confirming the superiority of the current European method. The results of these studies were comparable since the trial conditions such as the characteristics of the test material were almost identical. In another study (van Raamsdonk & van der Voet, 2003) that also complies with the new European method even higher values for the sensitivity were obtained (98%). However these results were not completely comparable with the results from the other studies due to a different design of the exercise, namely the bone fraction of the ruminant MBM used in the study (van Raamsdonk & van der Voet, 2003) was 54% and therefore higher than in the other

studies where the bone fraction was about 15%.

[Insert Table 5 about here]

The correct identification of the negative samples for the parameter terrestrial PAPs was calculated evaluating the pooled results from MAT I and MAT VI (Table 2), obtaining 90 % for the specificity. This value was higher compared to the specificity for the parameter total PAPs which was 77 % and calculated from the correct negative results of MAT I. This means that in the latter case more particles were erroneously identified as positive for the parameter total PAPs than in the case when exclusively looking for terrestrial PAPs. This result reflects the fact that the criteria for the identification of *terrestrial* PAPs are more strict compared to the identification of PAPs irrespective of the species. However, this corresponds to a lower sensitivity of the positive samples for total PAPs compared to terrestrial PAPs as shown in Figure 3.

Mammalian material was identified with a sensitivity of 68% (MAT II, III, IV, XI) with a high number of no results (77 of 192 samples). In the absence of feather meal or fishmeal (MAT II), mammalian material was detected with a sensitivity of 93%. The sensitivity for mammalian material drops from 80% in MAT XI (0.5% MMBM in 5% feather meal) to 62% in MAT III (0.1% MMBM in 5% feather meal) confirming that the mammalian material is more readily identified in higher concentrations. The sensitivity for mammalian material in the presence of fishmeal was only 38%, but the already low sensitivity for terrestrial animal material, which is the previous level, has to be taken in account. These results also demonstrated that the identification of mammalian PAPs, especially in the presence of PAPs from poultry is not feasible under

routine conditions.

Evaluation of the two-solvent Method. Five laboratories sent in their results. No contradictory results have been reported. Based on the protocol the laboratories applied, no labs were excluded. The laboratories were able to detect the presence of total PAPs with a sensitivity of 96% (Figure 4). This result is comparable with the sensitivity of the laboratories applying the directive EC/2003/126.

With two false positive results for the blank sample, the specificity for the detection of total PAPs is 86%, which is higher than the specificity for laboratories applying the official method.

[Insert Figure 4 about here]

On the next level (PAPs from terrestrial animals) 88% of the samples are correctly classified. For MAT IV (MMBM 0.1% + fish meal 5%), the sensitivity is 60% which is lower than the sensitivity calculated for the laboratories using the directive as already mentioned above. A possible explanation can be that bones are overlooked because of the higher concentration of the sediment which contains predominantly fishmeal and a possible partial sedimentation of the bone material into sediment fraction II. The fact that the execution of the two-solvent Method is more complicated compared to the official method could have contributed to lower sensitivity of the two-solvent Method.

Pre-validation study for the detection of PAPs in feed by immunoassays

Three laboratories reported results of analysis of the samples. Since one laboratory applied two different analytical approaches, results from four different methods were

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4 included in this study. The characteristics of the immunoassays applied in this study
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6 (Table 4) indicate that methods A, B and C measured ruminant, whereas method D had
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8 two targets which were PAPs from all animals and mammalian PAPs. All immunoassay
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10 were evaluated only according to the claimed targets but not on results from parameters
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12 that were deduced from the target PAPs. For instance, the detection of ruminant PAPs
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14 also indicates the presence of mammalian animals but a *negative* result on ruminant
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16 PAPs does not necessarily demonstrate that there are *no* mammalian PAPs in the
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18 sample. This is due to the fact that mammalian PAPs include materials from animals
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20 that are *not* covered by this parameter. Therefore, the methods A, B and C were
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22 evaluated for the ruminant PAPs and method D for mammalian PAPs.
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29 *Immunoassay tests for the detection of ruminant PAPs.* Figure 5 shows the results of
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31 analysis for immunoassay A, B and C for the test samples MAT VII and MAT VIII.
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33 These samples contain ruminant PAPs at the target concentration of 0.1 % (Table 2) but
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35 the heat treatment and the protein composition was different since MAT VII contained
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37 bovine proteins from various tissues sterilised at 133 °C and MAT VIII contained
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39 bovine muscle protein sterilised at 129°C.
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44 [Insert Figure 5 about here]
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47 Method B turned out to be the most sensitive method, since 9 out of 10 results for
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49 ruminant MBM, treated at 133 °C (MAT VII), and all samples from muscle proteins
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51 treated at 129°C (MAT VIII) were correctly classified as positive.
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57 In contrast, method A failed in correctly classifying the ruminant MBM (MAT VII),
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59 since 9 out 10 were false negative results. However, the results of all samples from the
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processed muscle proteins (MAT VIII) were correctly positive. Both the lower heat treatment and the different protein composition of both test samples could have been the reason for the positive results on MAT VIII. More specifically, method A was targeted against muscle protein, which coincides with the composition of MAT VIII. The test samples MAT VII, however, was MBM from different animal by-products, thereby containing the target muscle protein of method A at a lower concentration.

Method C was able to detect all samples from the ruminant MBM (MAT VII), whereas failed on the processed muscle proteins (MAT VIII), since all results were false negative. This was a surprising result given the lower heat treatment of the processed muscle proteins compared to the ruminant MBM samples. On the other hand, the protein profile of the ruminant MBM fit better to the targets of method C than the pure processed muscle proteins, since this method was raised against proteins from *different* tissues.

All methods failed when analysing the test samples from MAT IV that contained mammalian MBM at 0.1 %. However, since this MBM contained only 50 % ruminant PAPs, the true concentration of ruminant MBM in feed was 0.05 % and therefore below the target level of 0.1 %. The results of the samples that did not contain ruminant PAPs are shown in Figure 6. The applicability of the tests requires that unspecific proteins in blank feed and proteins from animals other than ruminants do not interfere with the detection of ruminant PAPs. In particular, false positive results should be avoided. The outcome of the analyses revealed that the methods A and C did not report any false

positive result, whereas method B reported as positive for the presence of ruminant PAPs one out of 6 of the samples containing 5 % fishmeal and 1 out of 10 samples containing 5 % porcine. Interestingly, the method showing the highest sensitivity on the samples containing ruminant PAPs also reported two positive samples on samples that did not contain ruminant PAPs. However, the overall specificity of method B was still quite promising when pooling the results from all samples without ruminant PAPs, since 20 out 22 samples were correctly classified as negative.

[Insert Figure 6 about here]

Regarding the analyses of the samples containing DCP, all results from method A and C were negative, whereas one sample out six from method B were positive. These results coincide with the results from the other materials, since method B turned out to be again as the most sensitive method. In addition the results indicate that DCP can be used in animal nutrition since the probability of “false positive results” is considered as low.

Immunoassay test for the detection of mammalian PAPs. The results of method D that was the only method in this study analysing for the presence of mammalian PAPs are shown in Table 6. The outcome of the experiments hinted at an insufficient sensitivity of this test, since only 6 out of 22 results were correctly classified as positive when pooling the results from MAT IV (MMBM 0.1%, 133 °C + fishmeal, 5%), MAT VII (Bovine MBM 0.1%, 133 °C) and MAT VIII (Bovine PAPs 0.1%, 129 °C). All 10 samples from MAT IX (Porcine PAPs 5%, 133 °C) were correctly classified as positive. However, the concentration of porcine PAPs was 5 % and was therefore not a challenging task of the immunoassay.

[Insert Table 6 about here]

Conclusions

Comparing the results from the two microscopic based methods for total PAPs and PAPs from terrestrial animals the results of both methods are comparable with respectively 95% and 90% of the results correctly classified for the laboratories applying the Directive and respectively 96% and 88% for laboratories applying the two-solvent Method.

The performance of both methods in the presence of fishmeal is reduced, but still higher than the results obtained in the DG SANCO 2003 study since the rate of correct positive results is 60% for the two-solvent Method compared to 70% of the official European Method.

The detection of mammalian MBM by means of microscopy is very difficult. A high number of “no results” is reported due to the fact that laboratories cannot classify the detected bones (mammalian or poultry). Only in the case when no other animal material than MMBM is present in feed, identification is possible. None of the laboratories applying the two-solvent Method reports a result for mammalian MMBM.

For most of the materials, the results for the detection of the two-solvent Method are comparable with the results of the Directive EC/2003/126, however the two-solvent Method which fulfils the conditions of the old Directive EC/88/1998 but not of the more strict new Directive EC/2003/126 does not justify a change of the Directive EC/2003/126.

The results of the pre-validation of immunassays revealed that the characteristics and performance profiles of immunochemical assays vary considerably between the

currently available tests. Differences exist in respect to the selected animal targets and linked to it the tissue (or organ) range specificity, the taxonomic level of detection and the sensitivity. Method B that measures exclusively a ruminant-specific epitope on a skeletal muscle protein turned out to be the most sensitive test evaluated in this study. It is also important to note that this test identified almost all samples (15 out of 16 samples) containing bovine PAPs at 0.1 %. Sufficient sensitivity and selectivity regarding this target is of great importance when considering a modification of the current MBM ban. Similarly, method C showed an interesting performance profile due to its sensitivity towards various targets including ruminant PAPs. In contrast, method A did not show enough sensitivity for this target, since it failed on several samples that contained 0.1 % ruminant PAPs. Results of method D demonstrated that this test is not sensitive enough for the parameter Mammalian PAPs.

The results of this study and of former studies also show that *quantification* of PAPs in feed is almost impossible to achieve given the lack of additional information regarding the composition of the PAPs and the corresponding rendering conditions the material underwent. In particular, the quantification of PAPs applying microscopy would require knowledge on the corresponding bone fraction whereas other methods such as immunoassays and PCR would need to have PAPs available that are identical to the PAPs detected in the feed samples and which can be used for calibration purposes.

All methods evaluated in this study did not only demonstrate *animal* specificity but also *tissue* specificity which could be an important feature when considering the differentiation between allowed proteins such as milk powder and banned processed proteins. However, the proven tissue selectivity of the test included in this study could necessitate the addition of supplementary information on the label of the test indicating

minimum criteria regarding the composition of the PAPs present in the feed. For instance, applying method B requires that the PAPs present in feed also contains proteins from skeletal muscles.

The follow-up of this study will include a ruggedness test focusing on the impact of various parameters such as the feed composition on the outcome of analysis. These additional experiments do not need to be designed as an intercomparison study but can be conducted in a single laboratory. Next to this study, the final step would be conducting a validation by an intercomparison exercise, in which laboratories—preferably from the feed control field—will analyse the same set of samples.

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