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Traceability of processed animal proteins with varying texture in feed: determination with microscopic and polymerase chain reaction methods

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To test the traceability of different animal components that could enter the feed chain two methods for the determination of processed animal proteins (PAPs) in feed – classical microscopy and polymerase chain reaction (PCR)-analysis – were applied in the following study. To determine PAPs of varying but defined structure different animal meals were produced artificially and analysed after spiking to a set of 13 compound feed samples. The aims of the study were (i) to compare the capacity and the limits of both methods with respect to the determination of animal constituents of varying composition, (ii) to verify a correct interpretation of the results from each method and (iii) to determine an optimum application area for each method. Both methods complemented each other. The microscopic approach allowed a reproducible, high sensitive and quantitative determination of animal ingredients with morphological detectable structures, and in the presence of bone fragments a differentiation between fish and terrestrial animals was possible simultaneously. The PCR-analysis provided the detection of animal ingredients in feed even in absence of visible structures but fishmeal was not detected in a sufficient manner by the chosen screening setup. However, the PCR-method enabled to differentiate between animal groups or species and to identify animal species. The methods complemented each other not only in the analytical features but also regarding the results produced by the detection of two different analytical targets of PAPs, morphological structures and gene sequences, respectively. Suitable data regarding the presence of their analytical targets were produced by each method, but a combination of both methods enabled furthermore to report correct results regarding the presence of the artificially composed PAPs in the feed samples. It was concluded that a combination of microscopy and PCR-analysis is reasonable for special application purposes to determine PAPs in feed: while microscopy provides reliable results also in highly processed feed with well-preserved morphological animal structures even with highly degraded genomic material, PCR provides applicable results in feed samples with preserved genomic animal material even after the separation of morphological structures. These specialties have to be considered for the choice of capable analytical methods and even for a correct evaluation of the results obtained from these methods in highly processed feed. An interpretation scheme based on the results of the study was proposed.
Keywords. Processed animal proteins (PAP), meat and bone meal (MBM), feedingstuffs, classical microscopy, molecular biological methods (PCR and RFLP), interpretation of methods and results.

1. INTRODUCTION

The ban on use of animal meat and bone meal (MBM) in feed for farmed animals is considered to prevent the spread of bovine spongiform encephalopathy. At present a total ban is effective especially for ruminant feed (EC, 2000; EC, 2001) whereas in the past MBM was added to feed primarily as supplement of essential amino acids. The official term for MBM and other animal by-products is processed animal proteins (PAPs) which are produced mainly in the form of ground processed (rendered) slaughter by-products originating essentially from ruminants, pig, poultry or fish. Today the presence of animal material in the feed chain for ruminants results mainly from cross contaminations in feed production processes (Frick *et al.*, 2002).

In addition to the total ban on PAPs for ruminant feed at present meal from terrestrial animals is not allowed in feed for non-ruminants to prevent intra-species recycling (EC, 2002). To relax the complete ban on terrestrial PAPs in the non-ruminant nutrition sufficiently performing analytical techniques are needed to control such measures whilst maintaining the intra-species ban.

The fundamental problem to detect all animal constituents with one suitable method is firstly based on the very high heterogeneity of PAPs especially

regarding the source of the animal material, the constitution and the history of processing (**Figure 1**). Raw animal materials may vary with respect to the animal species composition or with respect to structural differences of animal bodies or parts thereof. Also the processing status of the material (fresh, decomposed or rendered) as well as the amendment of additives affect different analytical characteristics. The observed high heterogeneity of PAPs has effects on the characterisation of the animal material as target for determination.

In addition a very low analytical detection limit is needed because of the stipulated zero tolerance of PAPs in feed for ruminants and the need to be in compliance with the imposed thermal processing standards (EC, 2002). These requisites limit the variety of methods that are suitable for analytical inspection. Directive 98/88/EC (EC, 1998) defines rules for a microscopic identification of animal constituents in animal feeds which is up to now the only method recommended to control the ban of MBM in feed (EC, 2003). Main visible characters of animal origin that might be present in feeds are bone fragments and muscle fibers. Additionally cartilage, hairs, feather filaments, egg shells, fish scales and ligaments that may also be present and can be detected with microscopy. Because parts from organs, skin and other soft tissues have only a limited number of visible morphological characters, it can not be detected microscopically if additional morphological characters like muscle fibers are lacking. Bone fragments are very persistent particles so that MBM with its high contents of morphological characters is identified by microscopy even after the current EU rendering practice of sterilisation at 133°C and 3 bar for 20 min (EC, 2002; Engling *et al.*, 2000).

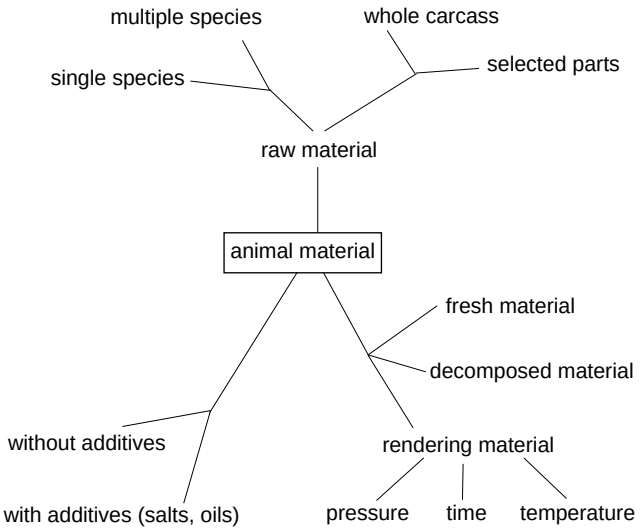

Figure 1. Heterogeneity of material of animal origin that should be detected analytically to control the ban on animal proteins in feed.

Several alternative analytical methods were described for the detection and identification of animal constituents in feed. Polymerase chain reaction (PCR) methods were established and applied mainly to determine animal constituents especially in meat used for food production, for example meat and heated meat products (Laube *et al.*, 2001; Meyer *et al.*, 1994). Some other methods like high performance liquid chromatography (HPLC), near infrared spectroscopy (NIRS) or near infrared microscopy (NIRM) are put into practise by a limited range of users up to now (Schönherr, 2002; Murray *et al.*, 2001; Piraux, Dardenne, 2000). An immunoassay created to control the adequate heating of MBM was not applicable for the determination of animal ingredients as it detected only animal material that did not fulfill the rendering conditions (Wolf *et al.*, 2001). But meanwhile immunoassays are in progress with antibodies that are raised against thermo stable antigens to enable a detection of animal components even after accurate rendering processes (Gizzi *et al.*, 2003). Further PCR-methods are also tested to detect animal material in highly processed samples and feed (Bellagamba *et al.*, 2001; Lahiff *et al.*, 2001).

Because of the great variety of animal components that could potentially enter the feed chain in the following study the analytical features of a method based on the determination of genetic targets (VDLUFA-Verbandsmethode, 2002) were compared with the features of the official microscopic method (EC, 2003) that are based on the microscopic determination of animal specific morphological structures. Special emphasis was placed on the impact of the composition and structure of the animal material even after treatment under the requested conditions (EC, 2002). For that aim animal meals consisting of different animal organs or tissues were prepared artificially. Different amounts of these defined PAPs were added to feed samples and were investigated subsequently to check abilities, limits and results of the methods, to verify a correct interpretation of the results from each method, and to determine an optimum scope of application for each method with respect to the considered animal constituents.

2. MATERIALS AND METHODS

2.1. Production of defined animal meals (PAPs) and preparation of the test samples

Approximately 1 kg fresh raw material from swine and cattle (in each case pure intestines, liver), sheep (butcher residues: cartilage and muscle fibers),

chicken and herring (in each case whole animals) was taken for the production of defined animal meals. Cattle is referred to as species A, swine as species C, sheep as species D, chicken as species B and herring is referred to as species E. The raw materials were hackled separately. Each type of animal material was put into an autoclaving bag, flattened and sterilised for 20 min at 133°C and 3 bar in a steam steriliser (H+P Labortechnik, Oberschleißheim, Germany) without stirring. After separation of the run-off fat the material was ground in a Thermomix (Vorwerk, Wuppertal, Germany), dried at ≤ 60°C, ground and dried again.

Feed samples were prepared on the basis of a feed for dairy cows with the following components: triticale, corn gluten feed, wheat gluten feed, wheat, barley, beet pulp, palm kernel meal, molasses, wheat bran, corn, rape expeller oil meal, limestone, vinasses, natrium chloride. The pelleted feed was homogenized in a Thermomix (Vorwerk, Germany) and tested for negative PCR-reactions with the animal primer pairs of the PCR-method, plant chloroplast-DNA was amplified with primer pair A1/A2 for positive amplification control (**Table 1**).

A set of 13 samples was prepared by spiking the compound feed meal with different amounts (0%, 0.1%, 0.5% and 2%) of the artificially produced animal meals. The samples were homogenised in the Thermomix (Vorwerk, Wuppertal, Germany). The material was mixed manually in intervals to minimise inhomogeneities in the blender.

2.2. Microscopy

Classical microscopy was done according to the EU guideline 98/88/EC and EU directive 2003/126/EG, respectively (EC, 1998; EC, 2003). With this method two sample fractions are checked for animal constituents in general (van Raamsdonk *et al.*, 2004): the sediment fraction contains all components with a specific gravity higher than the one of tetrachloroethylene. In this fraction for example bone fragments can be found besides lime or other feed minerals. As this sediment fraction concentrates bone fragments and other components with a high specific gravity it leads to a very low limit of detection of bones. In the sieved fractions the presence of muscle fibers and other animal components can be checked (Frick *et al.*, 2002). The practise of the microscopists doing the analyses is checked regularly in ring trials because special experience is needed to enforce the microscopic analysis. The analyses were done in duplicates: every sample of this study was investigated individually by two microscopists.

2.3. PCR

PCR-analysis was done according to the VDLUFA-method "Molecularbiological determination of animal ingredients – PCR-method" (VDLUFA-Verbands-methode, 2002). This method consists of different modules that can be combined individually. In a first step three different PCR-analyses are recommended to detect animal components in a screening (**Table 1**). Two targets are sequences of the cytochrome *b* gene and one target detects a fragment of the myostatin gene. A detection of highly processed animal material should be possible even after DNA-degradation processes because a very short DNA-target sequence is included in the screening. As additional module restriction fragment length polymorphism analyses can be done with primer pairs H15149M/ HM9 and K12-2/K13 to differentiate between animal species. The third module regarding species specific PCR enables the identification of diverse animal species in samples containing even several animal species.

For PCR-analysis, total DNA was extracted from the feed samples. Two grams of the feed samples were added to 10 ml standard lysis buffer for DNA extraction (Genescan, Freiburg, Germany) and incubated at 65°C for 1 h with slight shaking. Afterwards 200 µl of the supernatant were taken for DNA isolation with the DNEasy Mini Plant kit

Table 1. Specificity of primer pairs used for PCR-analyses (recommended by the VDLUFA-Verbandsmethode, 2002) to determine animal ingredients in feed.

Primer pairs	Amplification product [bp]*	Determination of target sequence	Literature
A1-F/2 A2-R	500–600	Plant (chloroplast gene)	Feldmann *et al.*, 1998
My-f/ My-r	97	Animal (myostatin gene)	Laube *et al.*, 2001
H15149M/ HM9	263	Warm blooded animal (cytochrome b gene)	VDLUFA-Verbandsmethode, 2002
K12-2/ K13	165	Warm blooded animal (cytochrome b gene)	VDLUFA-Verbandsmethode, 2002
bosPDE-r/ bosPDE-f	104	Cattle (phosphodiesterase gene)	Laube *et al.*, 2001
SW01/ SW02	108	Pig (porcine growth hormone gene)	Meyer *et al.*, 1994
galF/ galR	171	Chicken specific target gene	LUFA Augustenberg, pers. comm.

* bp = base pairs.

(Qiagen, Hilden, Germany) according to the manufacturer. The amplification of the target DNA-sequences (**Table 1**) was performed with the HotStar Master-Mix kit (Qiagen, Hilden, Germany).

PCR was performed with 40 cycles of amplification in a Mastercycler (Eppendorf, Hamburg, Germany) with temperature profiles for each primer pair according to the method (VDLUFA-Verbandsmethode, 2002). PCR-products of every sample were separated by agarose gel electrophoresis (1,5 %) and visualised with ethidiumbromide staining.

Every feed sample was investigated in duplicate (DNA-isolation and PCR-analysis) to ensure reproducibility. PCR-screening results were reported positive if one or more of the tested three screening primer pairs gave positive results in both duplicates of the extracted sample. Species-specific PCR-results were reported positive if every duplicate of the extracted samples gave a positive result. In case of non-conformity of the results within the duplicates the analysis was repeated until accordance of the results was determined.

3. RESULTS

3.1. Microscopy

Detailed results from the microscopic approach are listed in **table 2**. All samples containing visible animal structures (bone fragments or muscle fibers) were evaluated correctly by microscopy – independently of the concentration of PAPs in the feed sample and independently of the different starting materials of the PAPs (butcher residues, whole animals). No animal characteristic morphological structures were detected by microscopy in those samples containing 0.1% or 0.5% animal meal made from pure intestines (**Table 2**). Regarding the absence of animal characteristic morphological structures this result was correct. In sample n°4 that was spiked with 2.0% meal from pure intestines, animal characters were detected so that this sample was determined correctly as animal-positive. Thus the microscopic method allowed the detection of all added animal ingredients (0.1%, 0.5% and 2%) with morphological structures (bone particles, cartilage and/or muscle fibers) in this study. The results obtained from the samples that were spiked with PAPs made from pure intestines showed that the content of morphological structures in the animal meal had an impact on the traceability of this PAPs with microscopy.

The differentiation between fish and warm-blooded animals in the presence of bone fragments was done correctly as well. In sample n°13 (spiked with a mixture of 0.1 % of each five animal species) the concurrent presence of fish and warm blooded animals was detected. The microscopic approach allows to calculate the found animal particles and to give an assessment about the amount of MBM in the

Table 2. Detailed results of the microscopic analysis of feed samples contaminated with different artificially prepared animal meals. Every sample was investigated twice.

Sample n°.	Species	Content (%)	Components	Animal ingredients detected with microscopy			
				Qualitative	Detailed estimation		Overall microscopical findings
					1*	2**	
1	-	0	-	No	-	-	No animal ingredients
2	Warm	0.1	Intestines	No	-	-	No animal ingredients
3	blooded	0.5		No	-	-	No animal ingredients
4	animal A	2.0		Animal ingredients	<0.1%	-	<0,1% traces of animal
5	Warm	0.1	Bone fragments,	Warm	0.1–0.3%	0.1–0.2%	0.1–0,3% warm blooded animal
6	blooded animal B	0.5	muscle fibers	blooded animal	0.9–2.9%	1.0–1.5%	1.0–1,5% warm blooded animal
7	Warm	0.1	Intestines	No	-	-	No animal ingredients
8	blooded animal C	0.5		No	-	-	No animal ingredients
9	Warm	0.1	Cartilage, muscle	Animal	-	0.1–0.3%	0.1–0.3% animal
10	blooded animal D	0.5	fibers	Animal	-	0.7–1.3%	0,7–1.3% animal
11	Animal E	0.1	Bone fragments,	Fish	< 0.1%	ca. 0.1%	0.05–0.15% fish
12	(fish)	0,5	muscle fibers	Fish	0.4–1.0%	0.3–0.5%	0.3–0.7% fish
13	Mixture of all	0,5	Bone fragments, intestines, muscle fibers	Fish + warm blooded animal	0.2–1.4%	0.2–0.5%	0.2–0.6% fish + warm blooded animal

*determined from sediment fraction = bone fragments; **determined from sieved fraction = muscle fibers.

sample. On that basis, an estimation of the animal content from the particles observed in the two investigated fractions (sediment fraction and sieved fraction) was possible. In sample n°4 the content of animal meal was highly underestimated highly because of the low number of target structures in that animal meal.

The reproducibility of the qualitative test results (**Table 2**) were confirmed additionally by three independent microscopic laboratories (data not shown).

3.2. PCR

The results obtained by the PCR-method showed that all samples containing PAPs from terrestrial animals (0.1%, 0.5% and 2%) were determined positively, samples containing PAPs with morphological structures as well as samples with nearly no visible structures of animal origin (**Table 3**). This indicated that a determination of every tested animal material (pure intestines, whole animals and butcher residues) even at concentrations of 0.1% in the feed was possible despite the processing at 133°C and 3 bar for 20 min. Herring was not detected with the tested screening primer pairs. The chosen primer pairs seemed to be not specific for herring under the chosen conditions whereas other fish species not included in this study gave positive signals with the screening primers (data not shown).

The presence or absence of pig, cattle and chicken was determined correctly in the tested samples with species-specific PCR (data shown in **table 3**). Even in feed sample n°13 (spiked with a mixture of 0.1% of each five animal species) all tested single species (pig, cattle and chicken) were identified. Besides the species-specific PCR an additional module for RFLP-analyses allowed to distinguish between animal groups and to identify diverse animal species (data not shown).

The results presented in **table 3** were confirmed by independent analyses of other laboratories to control the reproducibility of the results even with varying laboratory equipment (data not shown).

3.3. Comparison of the information given by the different methods

The data presented in **table 2** and **table 3** show that microscopy and PCR analyses complemented each other with respect to the obtained information. Microscopy gave detailed data on different information levels (**Table 2**):

– information about the presence or absence of morphologically characteristic animal material (muscle fibers and bone fragments as typical structures of MBM);
– fish and warm-blooded animals could be distinguished if bone fragments were present in the sample;
– quantitative results were determined from two investigated sample fractions: the sediment fraction and the sieved fraction. Correct results on every

Table 3. Detailed results of the PCR analysis of the test feed samples contaminated with different artificially prepared animal meals (n.a. = not analysed). Every sample was investigated twice (DNA-extraction and PCR-analysis). Every sample was investigated twice. A positive animal-specific PCR-result is based on a positive result of both replicates for at least one of the three animal-specific PCR-analyses. A positive species-specific result is based on a positive PCR-result for both replicates.

Sample	Characterisation of the animal meal in the feed sample			Animal targets detected with PCR	
n°	Species	Content [%]	Components	Animal specific	Cattle, chicken, pig
1	-	0	-	No	Not detectable
2	Warm blooded	0.1	Intestines	Yes	n.a.
3	animal A	0.5		Yes	Cattle
4		2.0		Yes	n.a.
5	Warm blooded	0.1	Bone fragments,	Yes	n.a.
6	animal B	0.5	muscle fibers	Yes	Chicken
7	Warm blooded	0.1	Intestines	Yes	n.a.
8	animal C	0.5		Yes	Pig
9	Warm blooded	0.1	Cartilage, muscle fibers	Yes	n.a.
10	animal D	0.5		Yes	Not detectable
11	Animal E (fish)	0.1	Bone fragments,	No	n.a.
12		0.5	muscle fibers	No	Not detectable
13	Mixture of all	0.5	Bone fragments, intestines muscle fibers	Yes	Cattle, chicken, pig

level confirmed the suitability of the microscopic method to determine MBM in the official control of feedingstuffs.

The PCR-approach gave detailed data on the following information levels (**Table 3**):

– correct information was given with respect to the presence or absence of animal material independently of morphological structures (except for herring) ;
– correct information was given regarding the detected animal species. The tested PCR-method did not differentiate between particles of animal origin (muscle fibers, bone fragments) because the DNA-sequence that was chosen as analytical target is present in every animal cell.

The two methods complemented each other also in the analysis of the chosen test setup (**Table 4**). Although not every positive result obtained with one method could be confirmed by the other test method, all artificially composed animal ingredients added to the feed in this study were determined with at least one of the two test methods. Every method gave reliable results regarding the presence of its analytical target in the feed samples.

Table 4. Evaluation of the results obtained by microscopy and PCR-analysis in feed samples contaminated with varying amounts of different artificially prepared animal meals. + = right; - = wrong; * animal material without taxonomically relevant morphological structures.

Detection of animal ingredients in feed contaminated with differently structured animal meal (%)	**Method**	
	Microscopy	**PCR-analysis**
0		
0%	+	+
Warm blooded animal species		
A*		
0.1%	-	+
0.5%	-	+
2%	+	+
B		
0.1%	+	+
0.5%	+	+
C*		
0.1%	-	+
0.5%	-	+
D		
0.1%	+	+
0,5 %	+	+
Animal E (fish)		
0.1%	+	-
0.5%	+	-
Mix A, B, C, D, E		
0.5%	+	+

4. DISCUSSION

4.1. Capacity of the methods

Summarizing the methodological features from the results of the study the microscopic approach allowed a reproducible, highly sensitive and quantitative determination of animal ingredients with morphological detectable structures in feed. A differentiation between fish and terrestrial animals – only possible in the presence of bone fragments - was correctly done. The PCR-analysis (VDLUFA-Verbandsmethode, 2002) allowed the detection of animal ingredients in feed even in the absence of morphologically detectable structures. This method enabled also to differentiate between animal groups and species and to identify various species.

The microscopic results indicated that the traceability of its analytical target (morphologically specific structure) depends on the content of intact analytical target in the sample: while 0.1% PAPs were detected in all samples with animal meal containing adequate amounts of particles with morphological characteristics, in the sample with 2% pure intestines only traces of morphological visible structures of animal origin were detected microscopically. MBMs derived from rendering plants with contents of 60% sediment were identified and quantified reproducibly in ring trials even at concentrations of 0.02% in compound feed (Engling *et al.,* 2000). These data were confirmed by another ring trial (Jörgensen, 2003) and show that this method is suitable to screen even for very low concentrations of MBM in feed. It should be mentioned that in principle even a higher accumulation of bone fragments in the microscopic approach is possible by producing a larger sediment fraction (see 2.2).

In the presented study a differentiation of terrestrial animals and fish was possible in the sample spiked with 0.1% of each of the considered five animal meals. It has to be mentioned that only PAPs from two of these animal meals (herring and chicken) showed bone fragments that are needed to differentiate fish and terrestrial animals microscopically. This indicates that concentrations of 0.1% fish with visible characters (herring, whole body) and 0.1% warm blooded animal species B with visible characters (chicken, whole body) were detected and differentiated in this feed sample. The

capability of the microscopic method to discriminate even low concentrations of material of terrestrial animals in the presence of fish meal in feed was demonstrated in a ring trial where 0.2% MBM of terrestrial animals were reproducibly differentiated and reliably estimated in feed samples containing 3% fish meal (Jörgensen, 2004).

It has to be mentioned that the analytical discrimination of fish and terrestrial animals is affected by the individual composition and processing of the PAPs in the same manner as the traceability of animal components in general. Thus in another microscopic ring trial 0.1% MBM of terrestrial animals were not identified sufficiently in feed samples with 5% fish meal (von Holst *et al.*, 2004). Because the PAPs tested in different studies were not of the same composition the cited data from diverse studies cannot be compared directly, it can only be cited to give an impression about the possibility to discriminate even low concentrations of terrestrial animals in feed with fish meals with the microscopic method.

In the same way the data of this study demonstrate the analytical possibility to determine the different composed PAPs, but the data are not suited to derive general informations like detection limits for each method from it. First of all the study was not performed to determine exact analytical data to characterise a detection limit by investigating different concentrations of PAPs in feed. Secondly in contrast to chemical substances or other defined biological materials PAPs and other animal adulterations that could enter the feed chain can vary greatly regarding the amount and the quality of analytical targets – like the tested animal materials in this study – so that no general data for a detection limit of PAPs can be derived.

However, in principle the sterilisation conditions requested for the production of PAPs affect microscopy less than PCR and ELISA regarding the traceability of animal ingredients (Wolf *et al.*, 2001). For PCR it was shown in the present study that even 0.1% PAPs of different animal origin were detected by PCR. Similar sensitivity levels were reported for PCR-methods from other studies determining processed animal meals in feed (Gizzi *et al.*, 2003; Wolf *et al.*, 2001).

Because the ban on MBM to ruminant feed and the ban on intra-species recycling call for validated tests capable of recognizing the presence and the animal species of animal by-products in compound animal feed, the tested PCR-method seems to produce feasible results for this purpose in the presence of its intact analytical target.

4.2. Combining the methods

The test setup chosen for this study enabled to determine the limits of both methods with respect to the presence of their analytical target structures in the feed samples. The use of both methods enabled to determine all of the highly diverse structured animal materials tested in the feed samples in this study.

Microscopy provides reliable results also in highly processed feed with well-preserved morphological structures even when the genomic material is highly degraded. The PCR-method provides results in feed samples with preserved genomic animal material even after the separation of morphological structures.

Besides the choice of an appropriate method these specialties have to be considered also for the evaluation of the results obtained from investigations in highly processed feed.

To be able to interpret the results obtained by the two methods correctly (especially if the result of one method is not in accordance with the result of the other method) an interpretation scheme was proposed on the basis of the findings (**Figure 2**). If a positive result obtained by one method is confirmed by the other method, visible material can be found besides genomic material of animal origin. If the results of both methods are different, there are several possibilities to interpret the results: if the PCR produces negative results in contrast to microscopy, the genomic material as analytical target possibly was degraded by high processing like heat treatment or the material was possibly separated from the genomic material. It could be also possible that the chosen PCR-method was not specific for the animal component or showed a higher limit of detection than the microscopic approach.

If a positive result was produced by PCR in contrast to microscopy, animal ingredients without morphological characteristics were detected in the sample. As positive PCR-data can result from animal ingredients like milk products in the feed composition as well as from animal adulterations in the feed, a positive PCR-result cannot automatically be attributed to an adulteration of the feed with PAPs. For this reason feed samples containing animal ingredients like milk products can not be investigated with respect to animal adulterations with PCR-screening methods (Decastelli *et al.*, 2004). Considering these findings the determination of morphologically visible substances like bone fragments or muscle fibers gives a better hint with respect to the presence of adulterations like MBM in feed.

This study confirms on the basis of experimental data the statement of Gizzi *et al.* (2003) that there is no ultimate approach that would fulfil all requirements and that the methods complement each other. While

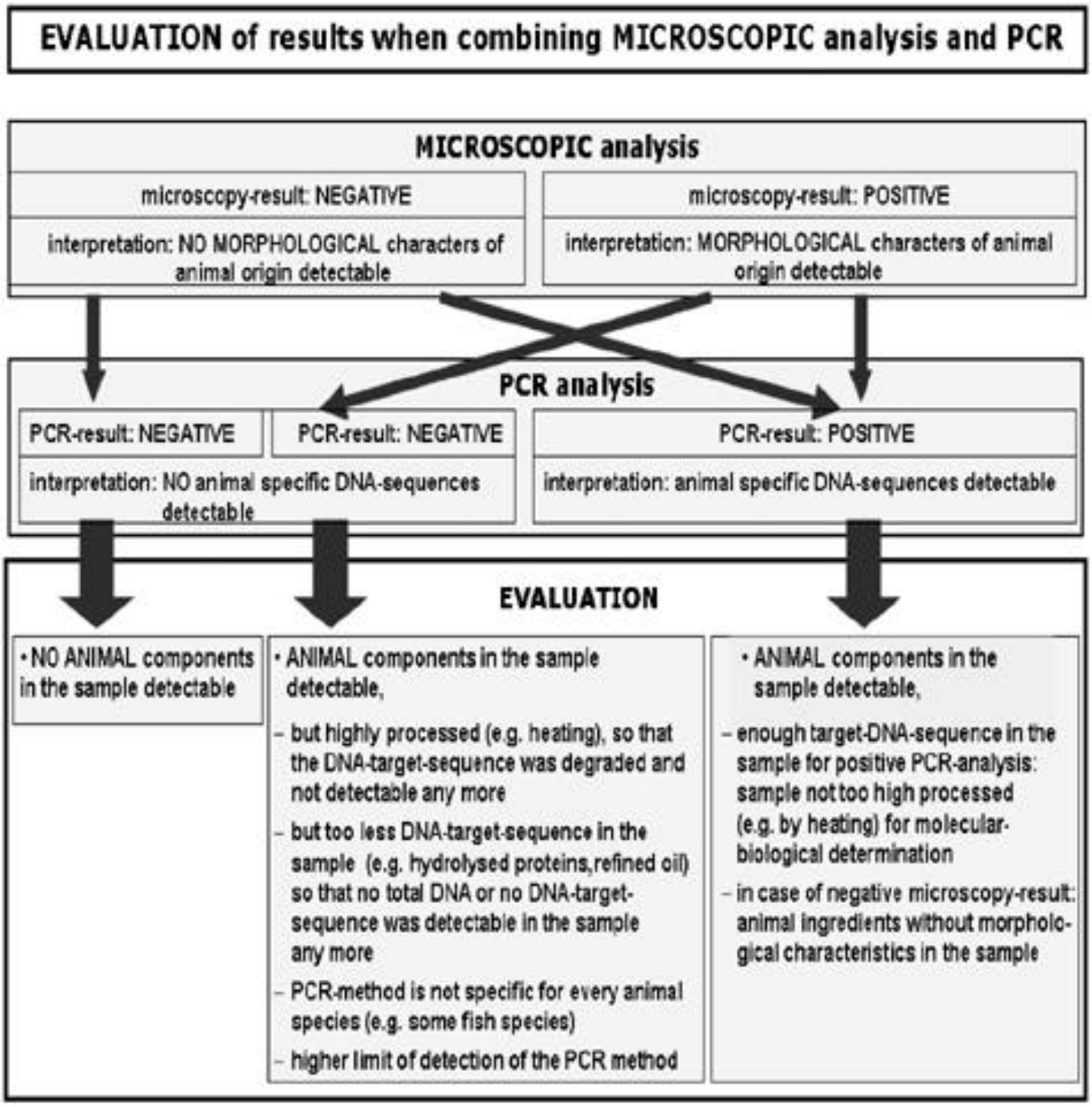

Figure 2. Interpretation scheme to evaluate the results of the determination of animal ingredients in feed with microscopy and PCR.

microscopy is a powerful methodology to detect even the slightest traces of MBM in feed, for special purposes a combination of other appropriate methodologies can be reasonable for a maximum of information (Frick *et al.*, 2002). Additional applications for PCR are needed so that a proofed identification of animal groups and animal species is available. Besides PCR other promising techniques to complement the microscopic data with respect to the detection of PAPs without morphological characters or the determination of animal species like HPLC, immunoassays, near infrared spectroscopy and near infrared microscopy (Gizzi *et al.*, 2003; Schönherr, 2002) should be tested further on to provide additional informations.

5. CONCLUSIONS

Because the ban on MBM in feed for ruminants and the ban on intra-species recycling (EC, 2002) call for validated tests capable of recognizing the presence and the animal species of animal by-products in compound

feed, the tested method combination seems to produce feasible results for this purpose even at the thermal processing standards.

The results presented here show that classical microscopy is a very effective screening tool for MBM in the official control because the typical morphological characters of MBM (bone fragments and muscle fibers) were determined in an absolutely reliable way in feed. This method provides a very low detection limit for morphologically detectable particles of animal origin which can additionally be differentiated from other animal ingredients in compound feed like milk components. These features emphasize the suitability of the microscopic approach for the control of the ban on MBM in feed for ruminants. For special application purposes microscopy can be combined with a demonstrated PCR-method to provide additional information like the presence of animal material without morphological structures or the characterisation of the animal material with respect to the animal species.

The presented data indicate that it is basically possible to control analytically even a relaxation of the total ban on PAPs derived from terrestrial animals on the feed for non-ruminants. Further investigations with the methods tested in this study will stress on the traceability of PAPs that are originally produced in rendering plants.

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