Chapter 13
Combination methods for PAP detection and species determination of animal particles

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COMBINATION METHODS FOR PAP DETECTION AND SPECIES DETERMINATION OF ANIMAL PARTICLES

ABBREVIATIONS

AP	Alkaline Phosphatase
CAHEC	China Animal Health and Epidemiology Center
D	Density
DNA	Deoxyribonucleic Acid
EFPRA	European Fat Processors and Renderers Association
ELISA	Enzyme linked Immunosorbent Assay
FT-NIR	
GAM	Goat Anti- Mouse
HRP	Horse Radish Peroxidase
MAb	Monoclonal Antibody
NBT	
NIR	Near Infra Red
NIRM	Near Infra Red Microscopy
PAP	Processed Animal Protein
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
TCE	Tetrachloroethylene
TRIS	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

SUMMARY

Two novel methods for the detection of processed animal proteins based on a combination of optical microscopy with immunochemical detection techniques, and on a combination of Near Infrared Microscopy (NIRM) with polymerase chain reaction (PCR) are presented. These combinations of methods add the possibility of obtaining species information on individual particles at a low level of detection. Such an on the spot identification of microscopically identified particles with respect to the source (species) would strongly enhance the support of the legislation, especially the species-to-species ban.

In this chapter experimental details on sample treatment and clean-up of individual particles as well as optimisation of detection procedures for both combinations of methods will be discussed in particular.

Keywords: Immunoassay, PCR, optical microscopy, NIR microscopy, antibody, DNA, troponin I, muscle fiber, bone particle

13.1 INTRODUCTION

The use of processed animal proteins (PAPs), with the exception of fish meals, in feed for farmed animals is prohibited in the European Union according to the extended feed ban (Regulation 1234/2003/EC). This measure was apparently effective in preventing a further spreading of BSE. However, it also excludes valuable and sustainable sources of proteins from the feed market. Therefore, a safe introduction of non-ruminant PAPs in the feed chain is desired. This would involve a replacement of the extended feed ban with the species-to-species ban, prohibiting only the feeding of non-ruminant animals with proteins of the same species (Regulation 1774/2002/EC to be replaced by Regulation 1069/2009/EC). To enforce these provisions there is an urgent need for species-specific methods for detection of PAPs from several species in animal feed and in PAPs from other species.

Currently, optical microscopy is the only EU accepted method for detection of PAPs, since it has a very low detection level and one can easily distinguish microscopically the prohibited materials from authorised ingredients (van Raamsdonk et al., 2009; see also chapter 5). Microscopic identification of these prohibited materials is mainly based on searching bone particles in the sediment fraction of the analyzed feed. However, it does not provide sufficient information on the species of origin of the detected particles for enforcement of the species-to-species ban. Alternatively, methods like Real Time PCR and immunoassays do provide information on the animal species composition of PAPs (latest overview in Fumière et al., 2009; see other contributions in this volume), but using these methods alone gives rise to new specificity and sensitivity problems. Using PCR on an extract of a complete sample might give false positive results when authorised ingredients like milk and blood meals are present. When using immunochemical detection on a sample, cross-reactivity with matrix components cannot be excluded entirely. Therefore, the necessity of reliable and faster methods led to the development of alternative methods, such as the ones based on spectroscopy (e.g. near infrared (NIR) spectroscopy) or near infrared microscopy method (NIRM) to analyse compound feeds (Baeten et al., 2004; De La Roza et al. 2007, Piraux & Dardenne, 1999). This latter technique involves the analysis of several hundreds of particles obtained from the grinding of compound feedstuffs. The major advantage of this technique is that the recognition is based on the measured NIR spectrum and therefore does not depend on the expertise of the analyst as when applying optical microscopy. NIRM combines the optical microscopy

benefits (detection based on the presence of animal particles and not affected by the rendering process applied to the PAP and the spectroscopic benefits (detection based on the specific chemical composition of animal tissues).

The combination of optical and NIR microscopy with either immunoassay analysis or PCR adds the possibility of obtaining animal species related information on individual particles even at low level of contamination and helps in improving the microscopic method for identification of the animal species composition of the material. Such an '*in situ'* identification of the animal species origin on microscopically identified particles would strongly enhance the support of the legislation, especially with respect to the species-to-species ban.

This chapter describes the development and state of the art of two novel method combinations, namely the combination of optical microscopy with immunochemical detection techniques and NIRM with PCR.

For the optical microscopy method combined with immunochemical detection, muscle fibres were chosen as the target tissue because of their relative abundance in PAP and their relatively high content of species specific proteins. Also, such a method would be helpful in cases where feed samples contain only muscle fibres and no bone fragments. Furthermore, for immunochemical detection, the procedures are easy and do not require a high level of expertise or specialized lab personnel and no expensive equipment or chemicals are needed. The muscle fibres only need to be concentrated from the feed sample and immobilised on a microscopic slide. Optimal conditions have to be established for incubation of the first (and when applicable second) antibody, and for the staining procedure. Experiments for optimisation of these steps will be discussed.

In the combination of the NIR microscopy with the PCR, the short mitochondrial targets assessed in previous studies (Fumière et al., 2006; Prado et al., 2007) were used. The challenges were mainly in the pre-PCR steps, i.e. the isolation of the suspicious animal particles after the NIRM analysis and the DNA extraction from a single particle. In parallel with the development of an adapted DNA extraction protocol, an optimised treatment to clean the external part of the suspicious particle from environmental DNA has been developed. This cleaning should allow PCR analysis of only the target DNA originating from the core of the particle.

13.2 CLASSICAL MICROSCOPY AND IMMUNO-CHEMICAL DETECTION COMBINATION METHOD

13.2.1 Method description

Concentration of muscle fibres

Roughly 3 grams of feed spiked with meat meal is mixed (shaken) with 100 ml of a mixture of TCE:Heptane 7:3 (v/v). There is a limit to the amount of material that can be used in 100 ml separation liquid. Above 25 g of feed per 100 ml separation is less apparent. After 10-30 minutes, when a good separation between pellet and flotation is achieved, the flotation (where most of the muscle fibres are concentrated) and a large part of the liquid column (still containing some muscle fibres) is poured on a Whatman 42 filter. After drying of the filter (approximately one hour), the brown coloured flotation is scraped off**.** The whole procedure is carried out in a fume hood.

Immobilisation on the slide

Microscope slides are coated with a thin layer of Norland optical adhesive 81. The dried flotation is sprinkled on the coated microscope slide, with the aid of a sieve (355 µ mesh), positioned about 5 cm above the slide. For best results, the coating should be applied in a very thin layer, in order to prevent muscle fibres from sinking too deep into the coating and thereby impairing the accessibility to the antibody binding sites. The prepared slides are hardened with UV light (366 nm) for a few minutes only**.** Excess material is carefully rinsed off with demineralised water and slides are stored at room temperature until further use. Slides can be stored for at least three weeks at room temperature without apparent loss of detection signal.

Antibody incubation and detection

Slides are blocked for non specific binding with 50 mM Tris (pH 7,4) buffer with 1 % ovalbumine for 30 minutes. Subsequently, the slides are incubated for 1 hour with a primary antibody in 50 mM Tris (pH 7,4) with 0.3 % Tween-20 and 0.3 % skimmed milk powder. Next, slides are incubated with a secondary Goat anti-Mouse antibody, labelled with alkaline phosphatase (GAM-AP), for 1 hour. For colour development sufficient Vector blue substrate containing Levamisole (for reduction of endogenous AP activity) is added to cover the microscope slides (roughly 40 ml solution for 10-20 slides). Colour development takes place for 60 minutes. The reaction is stopped by rinsing with tap water. Between all incubations slides are washed with 50 mM Tris buffer (pH=7.4), three times for five minutes. After staining the slides are dried and covered with some drops of Faramount mounting medium from Dako, or with Norland resin and a cover glass prior to examination. The resin is hardened with UV light as described before.

Three primary monoclonal anti-troponin I antibodies were tested:

A non species specific antibody:

- AbD Serotec 9202-0537, Bio-connect

Two ruminant specific antibodies:

- Chinese mouse monoclonal antibody 1G11 provided by CAHEC (Qingdao, P.R. China)

- RIKILT mouse monoclonal antibody 86-2F1

Specificity and sensitivity of the applied antibodies are tested in an inhibition ELISA format.

Examination

The quality of the final result depends on several parameters, such as the depth of the coating at the slide, the sensitivity and specificity of the antibody and the heat treatment of the original animal proteins. After sprinkling, muscle fibres might be soaked in a coating of Norland adhesive. If the adhesive layer is too thick, colour reaction of the immersed (part of the) fibre might be impaired. On the other hand, when the adhesive layer is too thin, muscle fibres may detach during the subsequent treatments, which is visible by empty footprints in the coating. Both situations are clearly visible during examination.

Due to non-homogeneity of the heat treatment of animal proteins and suboptimal sensitivity and specificity of antibodies, the colour reaction is unevenly distributed along a muscle fibre. At a magnification of 400x, the sarcomers, where the troponin I is located, become especially visible through colouring. A non-specific reaction is sometimes observed at the outer membrane of muscle fibres of fish. However, specific and non-specific reactions can easily be distinguished by focussing through the muscle fibres. Non-specific staining is only located at the outside of the fish muscle whereas the specific staining is found throughout the whole muscle and the sarcomers are clearly coloured.

13.2.2 State of the art

Concentration of muscle fibres

Considering the density of muscle fibres, several solutions have been tested to separate most of the matrix (plant ingredients) from muscle material. The density of the solutions was chosen in such a way that besides the heavy bone particles also the lighter matrix materials, such as premixes and minerals are sedimented. For this, a relatively low density of the solvent is required, which allows for flotation of the muscle fibres, and sedimentation of the majority of the matrix particles. Petroleum ether (D=0.62-0.66), pentane (D=0.63), hexane (D=0.66), heptane (D=0.68), ethanol (D=0.79), ethylacetate (0.93), and dichloromethane (D=1.3) have been tested. All densities of these solvents are lower than that of the usual solvent in microscopy, tetrachloroethylene (D=1.62), where all ingredients remain floating except for the heavy ingredients (minerals, bone fragments; see chapter 5). After applying mixtures with TCE, all solvents with the density of or equivalent to ethanol and higher did not result in acceptable separation. Much material remained floating in the solvent, or formed a large flotation (dichloromethane). The best results have been achieved with a mixture of TCE : heptane in a combination of 7 : 3 (v/v). To test the applicability of the concentration step, feeds were spiked with meat meals from different species and different feeds were spiked with porcine meat meal. Typical results are presented in figure 13.1.

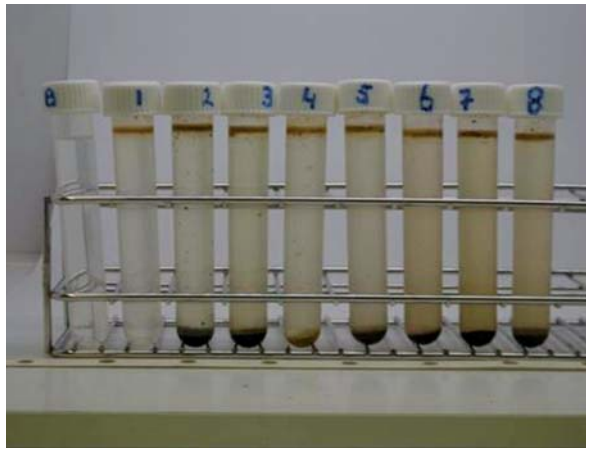

Figure 13.1. Concentration of muscle fibres from porcine meat meal (PMM) in different animal feeds using a mixture of TCE:heptane 7:3 (v/v). B Blank (no PMM and no feed), 1 PMM, 2-3 and 7-8 PMM in different cattle feeds, 4 PMM in poultry feed, 5-6 PMM in pig feeds.

In all experiments the muscle fibres were predominantly found in the flotation after a microscopic check. The flotation mainly consisted of muscle fibres with hardly any feed particles present. Hence, a rather pure muscle powder was obtained for immobilisation on the microscopic slides.

Immobilisation on the slide

A dedicated optical adhesive was selected that sets in a few minutes when exposed to ultraviolet light and that provides good light transmission over a wide spectral range. For optimal results it was found to be essential that the final layer of the adhesive was thin, in order to avoid muscle fibres from sinking too far into the adhesive. This obstructed binding of the antibodies to the muscle proteins.

Antibody incubation and detection

In general, limits of detection are lower when using fluorescent substrates than using a substrate leading to a coloured precipitate. Unfortunately, a bright auto-fluorescence of muscle material was observed over a broad range of wavelengths. This fluorescence was not located at a specific part of the muscle but occurred throughout the whole muscle fibre, when observed with confocal microscopy. The extent of the auto-fluorescence forced us to make a choice for using only enzyme substrates leading

to a coloured precipitate and consequently, for examination in visible light.

To yield different colours, Horse Radish Peroxidase (HRP) and AP labelled antibodies have been studied with several substrates [for AP (Vector Blue, BCIP/NBT en Liquid Permanent Red, LPR, DAKO K0640), and for HRP (Dab+, DAKO K3468, AEC+, DAKO K3461)] using AbD as primary antibody on porcine meat meal. Staining with Vector blue is the primary choice as the bright blue colour gives the best contrast with the natural brownish colour of the muscle fibres. An acceptable alternative is staining with AEC+. An advantage of using these staining methods is the possibility of combining Vector blue and AEC+ in a two component system, provided that a second primary antibody directed against a different species is available.

All antibodies were tested for sensitivity and specificity in inhibition ELISA. Using the AbD antibody, meat meal extract could be detected at roughly 5 ppm (protein concentration). This antibody cross-reacted with meat meal extracts of all species tested. Using the Chinese and RIKILT antibodies, ruminant meat meal extracts could be detected at 500 ppm (protein concentration). These antibodies were species-group specific and gave only a reaction with ruminant meat meals. No reaction with meals from other species was observed. In comparison with the antibody applied in a commercial kit (MELISA-TEK-Ruminant), the Chinese and RIKILT antibodies were less sensitive by a factor of 5.

Subsequently, the antibodies were tested with bovine, porcine and avian meat meal as well as with fish meal using the combination method. Initially, some non-specific binding was observed for all antibodies with all types (species) of muscles. By optimising blocking and incubation buffers this non-specific binding was prevented for all meals except for the fish meals. Even using the final protocol substantial non-specific background staining was observed with fish meals.

In figure 13.2 results are shown for incubation of the RIKILT antibody with muscle fibres concentrated from feed spiked with 50% w/w meat meals. Incubation with the RIKILT antibody, as well as the Chinese antibody, gave a clear staining with bovine muscles and lack colouring with muscles of porcine and avian meals. The non-specific staining in fish material is caused by the binding of the primary antibody to the membrane of the fish muscle fibres, as incubation with only the secondary antibody and subsequent staining did not result in any staining. The plaques of dye at the outer membrane can easily be recognised by focussing through the muscle fibres. The sarcomeres of the fish muscle fibres, where the troponin I is located, remain uncoloured.
About 80% of all bovine muscle fibres in a slide appear to be stained. This shows clearly the potential of the method. Within the near future, feeds spiked with lower concentrations of PAPs will be tested to establish the limit of detection.

13.3 NIR MICROSCOPY AND PCR COMBINATION METHOD

13.3.1 Method description

NIRM analysis
The NIRM instrument (Perkin Elmer) used is a Microscope AutoIMAGE System connected to a spectrometer Spectrum Identycheck FT-NIR System (see Chapter 8). The device allows for the recording of spectra of particles on a small surface (50 µm x 50 µm). The microscope includes a camera and a viewing system to magnify the visible light image of the sample, as well as to highlight and isolate a point of interest. See chapter 8 for more information concerning the NIRM instrument.

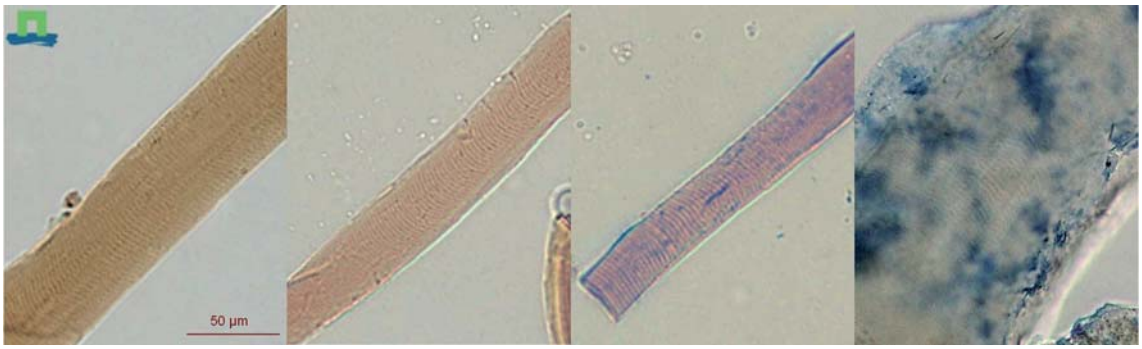

Figure 13.2. Result of the combination method with *in situ* immunochemical staining of muscle fibres. From left to right: negative control (porcine muscle fibre; full method applied), positive control (bovine muscle fibre; method applied without specific antibody), positive result with specific staining (bovine muscle fibre, full method applied), a-specific staining (fish muscle fibre, full method applied). All images at a magnification of 200 x.

After mixing the sample with the help of a slice, 4 sub-samples of about 100 mg are taken. The sub-samples are combined and placed in the sieve of 250 µm, which is then placed over an agitator for 2 minutes. The particles (>250 µm) are then taken from the sieve with the help of a stainless steel spoon, and placed on the sample holder (e.g. Spectralon). About 70 particles are spread out in the sample holder in a thin layer. Then the particles are presented to the NIR microscope. The prompter of the microscope ensures the light beam is directed onto every particle and spectra are acquired in the 4000-7800 cm^{-1} range with a resolution of 8 cm^{-1} and a number of scans equal to 10. The spectra are obtained following background correction performed using an empty spectralon plate. Spectra are considered as fingerprints that are based on the chemical composition of the analysed particles. For the identification of the measured spectra several decision criteria based on the visual observation of the spectrum are applied in order to assess the animal or vegetable origin (von Holst et al., 2008).

The particles analysed by NIRM that will also be analysed by PCR are manually recovered one by one with tweezers and kept individually in plastic *Eppendorf* tubes. The rest of the particles were preserved in a laboratory flask for further analysis if necessary.

Cleaning of the particles

Authorised ingredients such as dried whey, fat or blood may also be sources of the DNA target that can be detected by PCR and this is a limitation of the technique as these authorised materials may have contaminated the outer surface of the particle. In a previous study, Toyoda et al. (2004) already proposed to wash bone particles isolated from the sediment fraction with a sodium hypochlorite solution. The protocol described in their paper was unsuccessfully tested on single particles and various other reagents for cleaning and/or DNA denaturing properties such as RBS (Chemical Products R. Borghgraef S.A., Brussels, Belgium), hydrogen peroxide (H_2O_2), acetone and isopropanol were tested. The retained protocol consists of cleaning of particles with DNA Erase (MP Biomedicals, Solon, Ohio) and a final rinsing with milliQ water (Fumière et al., 2010). The particle is then dried and can be submitted to the DNA extraction protocol.

DNA extraction from a single particle

Considering the very limited amount of sample submitted to the DNA extraction, it was mandatory to develop a protocol without purification and loss of DNA. The first attempts placed the particle directly in the PCR medium but problems due to PCR inhibitors and impurities were identified. Different commercial kits usually used in forensic analysis for DNA traces identification were then tested. Finally, the most flexible and efficient solution was provided by the Direct PCR® Lysis Reagent from Viagen Biotech Inc. (Los Angeles, USA) (Fumière et al., 2008). This reagent is initially dedicated to mouse tail cells lysis and gives a DNA extract ready-to-use for the PCR, thanks to components inactivating possible PCR inhibitors.

The protocol adapted to the application is very simple and rapid: Proteinase K (Invitrogen, Carlsbad, CA, USA) is freshly dissolved in the Direct PCR® Lysis Reagent at a concentration of 0.4 µg/µl.

Seven µl of the prepared lysis buffer are added to the particle and incubated at 85 °C for 45 minutes.

The extract with the particle is briefly centrifuged and 6 µl of supernatant are diluted in 24 µl of water.

Real time PCR analysis

Real-Time PCR conditions, primers and probes were the ones optimised on a GeneAmp 5700 Real-Time PCR device (Applied Biosystems, Foster City, CA, USA) (Fumière et al., 2006) and were successfully transferred to a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) without any modification (Fumière et al., 2010). The usual PCR conditions were: after an initial incubation step at 50 °C for 2 minutes followed by a denaturation step at 95°C for 10 minutes, cycling parameters were as follows: 50 cycles including a melting step at 95 °C for 15 s, and an annealing/elongation step at 50 °C for 60 s. The amplifications were performed in a total volume of 35 µl containing 5 µl of the DNA extract, 17.5 µl of *Real Time PCR mastermix for Probe Assay* (Diagenode, Seraing, Belgium), 1.25 µl of each primer (Eurogentec, Ougrée, Belgium) at 3 µM for the cattle and pig PCR or at 10 µM for the sheep and chicken PCR, 1.25 µl of the appropriate TaqMan probe at 10 µM.

13.3.2 State of the art

NIRM analysis

As the NIRM is a non destructive analysis, it can be combined with Real Time PCR to determine the origin of the particle at a given classification level. The spectra of particles were acquired by NIRM as previously explained (see 13.3.1) . These data were collected on pure species PAPs obtained from EFPRA rendering plants.

The species origin of such particles was confirmed by PCR analysis with five animal probes (cattle, sheep, pig, chicken and fish). Only the spectra from particles giving a PCR signal with the expected probe were used to build authenticated species specific spectral databases.

Spectral databases gather PCR authenticated spectra of cattle, sheep, pig, chicken and fish particles from samples of different origins. Chapter 8 describes with a complete protocol the use of such spectral databases and gives an overview of the NIRM technique applied to the discrimination of the species origin of animal by-products.

Cleaning of the particles and Real Time PCR results

The PCR analysis of a single PAP particle is only interesting if any external DNA source especially from possible authorised ingredients is removed (eg. Milk) and if the PCR signal is due to the amplification of the DNA coming from the core of the particle. The possible presence of DNA molecules stuck to the surface of the particle was checked by preparing 9 mixes containing ingredients from two different animal species. The DNAs of the 9 mixes extracted from a test portion of 100 mg according to the Promega protocol described by Fumière et al. (2006) were firstly analysed by PCR. The results showed the simultaneous detection of all ingredients present in the mixes. The compositions of the 9 mixes are presented in table 13.1.

Table 13.1. Composition of nine mixes containing 2 animal ingredients and analysed by PCR

Mixes	% in weight of ingredients
Mix 1	92 % soybean, 5 % Chicken PAPs #1, 3 % milk powder #1
Mix 2	92 % soybean, 5 % Chicken PAPs #1, 3 % milk powder #2
Mix 3	92 % soybean, 5 % Chicken PAPs #2, 3 % milk powder #2
Mix 4	92 % maize, 5 % Cattle PAPs, 3 % egg powder
Mix 5	92 % maize, 5 % Pig PAPs, 3 % egg powder
Mix 6	50 % chicken PAPs, 50 % milk powder #2
Mix 7	92 % maize, 5 % Chicken PAPs, 3 % Cattle blood
Mix 8	92 % maize, 5 % Chicken PAPs, 3 % Pig blood
Mix 9	92 % soybean, 5 % Cattle PAPs, 3 % Pig blood)

From each mix, twenty particles detected as of animal origin by NIRM analysis (PAP particles) were directly extracted with the Direct PCR Lysis Reagent and analysed by PCR. The results obtained for the mixes from 1 to 5; show that the impact of presence of an authorised ingredient such as milk powder or egg powder in a mix can be limited. The percentages of the PAPs particles correctly detected (giving only a positive signal with target corresponding to their species) were between 90 and 100 %. Nevertheless, in the case of mix 3, this percentage decreased to 75 % because of simultaneous detection of chicken and cattle DNA on some particles and thus confirming the usefulness of a cleaning protocol. Table 13.2 presents the percentages of PAPs particles giving a positive PCR signal only with the probe corresponding to their species.

Table 13.2. PCR analysis of 20 particles of PAPs detected as from animal origin after NIRM analysis from 5 mixes. Percentages of correct detection after PCR analysis with the targets of animal species present in the mix.

Mixes (% in weight of ingredients)	Percentages of correct detection
Mix 1 (92 % soybean, 5 % Chicken PAPs #1, 3 % milk powder #1)	100 %
Mix 2 (92 % soybean, 5 % Chicken PAPs #1, 3 % milk powder #2)	95 %
Mix 3 (92 % soybean, 5 % Chicken PAPs #2, 3 % milk powder #2)	75 %
Mix 4 (92 % maize, 5 % Cattle PAPs, 3 % egg powder)	100 %
Mix 5 (92 % maize, 5 % Pig PAPs, 3 % egg powder)	90 %

In order to test the efficiency of the cleaning protocol with DNA Erase, particles coming from 5 mixes were analysed with and without a cleaning step. Four of these five mixes are composed of 92 % of vegetal meal, 5 % of PAPs and 3 % of milk powder or blood meal. The fifth mix tested with the cleaning protocol is composed of 50 % of PAPs and 50 % of milk powder (less realistic composition). This composition was selected in order to have more PAPs particles giving a positive result with the cattle target because of the presence of milk powder. Table 13.3 shows the PCR results obtained with PAPs particles when a cleaning step is used or not before the DNA extraction.

Table 13.3. PCR analysis of 20 particles of PAPs from 5 mixes. Percentages of correct PCR detection with the targets of animal species present in the mix with and without a DNA Erase cleaning protocol of the particles previous to the DNA extraction.

Mixes (% in weight of ingredients)	**Percentages of correct detection without particle cleaning**	**Percentages of correct detection with particle cleaning**
Mix 3 (92 % soybean, 5 % Chicken PAPs #2, 3 % milk powder #2)	75 %	100 %
Mix 7 (92 % maize, 5 % Chicken PAPs, 3 % Cattle blood)	40 %	100 %
Mix 8 (92 % maize, 5 % Chicken PAPs, 3 % Pig blood)	0 %	25 %
Mix 9 (92 % soybean, 5 % Cattle PAPs, 3 % Pig blood)	25 %	90 %
Mix 6 (50 % chicken PAPs, 50 % milk powder #2)	30 %	95 %

The currently used cleaning protocol of the particles using DNA Erase reduces greatly the occurrence of PCR signals due to the presence of another animal ingredient in the sample. Nevertheless, its efficiency is largely dependent on the sample. For the mixes containing pig blood powder, 90 % (mix 9) of the PAPs particles are detected only with the target expected after cleaning but in the case of mix 8, the rate falls to only 25 %. It must also be pointed out that the particles of pig blood still give a positive detection with the pig probe. However, this result can be attenuated looking at the Ct values obtained (table 13.4). The signals obtained indicate that considering similar PCR efficiencies with both probes, the number of targets coming from the core of the particle is higher than the number of targets coming from the environment of the particle. This data could be considered in itself as a first evidence of the origin of the PAPs particle. Moreover, the cleaning protocol does not only or slightly affect (a delay of less than 2 cycles) the signals with the probe for the animal species from which the PAPs particle originates, whereas the signals obtained with the other probes are strongly delayed (more than 6 cycles) or completely eliminated.

Table 13.4. PCR analysis of 20 particles of PAPs detected as from animal origin after NIRM analysis from 3 mixes. Comparison of mean Ct values in number of cycles with the chicken probe, the cattle probe and the pig probe with and without a DNA Erase cleaning protocol of the particles.

	Without particle cleaning			**With particle cleaning**		
Mixes (% in weight of ingredients)	Chicken	Cattle	Pig	Chicken	Cattle	Pig
Mix 7 (92 % maize, 5 % Chicken PAPs, 3 % Cattle blood)	23.27	39.00	NT*	25.10	Not detected	NT*
Mix 8 (92 % maize, 5 % Chicken PAPs, 3 % Pig blood)	21.64	NT*	31.90	21.52	NT*	37.55
Mix 9 (92 % soybean, 5 % Cattle PAPs, 3 % Pig blood)	NT*	25.09	30.04	NT*	25.19	39.18

* Not tested

The cleaning protocol as well is tested on cattle blood particles for the mix 7. Indeed, the identification of species origin of the particles blood is interesting. The results obtained on the cattle blood particles are presented in table 13.5. After cleaning, 30% of the cattle blood particles still give a positive detection with the chicken probe. These results must also be attenuated by looking at the Ct values obtained with and without the DNA Erase cleaning protocol (table 13.6). The signals obtained for the particles without cleaning are comparable for both species while for the particles with cleaning, the results obtained with the cattle probe are significantly earlier than with the chicken probe.

Finally, the cleaning protocol was applied to vegetable particles (soybean or maize), even if in a global analysis process combining NIRM and PCR analysis these types of particles would not be analysed because plant particles can be easily distinguished from PAPs and blood during the NIRM analysis. After cleaning, each particle is tested with animal and vegetable targets present in the mix. The results obtained on the vegetable particles are presented in table 13.7. After cleaning, a majority of the vegetable particles are correctly identified.

Table 13.5. PCR analysis of 20 particles of blood. Percentages of correct detection after analyse with the targets of animal species present in the mix with and without a DNA Erase cleaning protocol of the particles.

Mixes (% in weight of ingredients)	**Percentages of correct detection without particle cleaning**	**Percentages of correct detection with particle cleaning**
Mix 7 (92 % maize, 5 % Chicken PAPs, 3 % Cattle blood)	0 %	70 %

Table 13.6. PCR analysis of 20 particles of blood. Comparison of mean Ct values in number of cycles with the chicken probe and the cattle probe with and without a DNA Erase cleaning protocol of the particles.

	Without particle cleaning		With particle cleaning	
Mixes (% in weight of ingredients)	**Chicken**	Cattle	Chicken	Cattle
Mix 7 (92 % maize, 5 % Chicken PAPs, 3 % Cattle blood)	29.54	29.47	36.74	29.74

Table 13.7. PCR analysis of the vegetal composition of 20 particles from 4 mixes. Particles detected as from vegetal origin after NIRM analysis. Percentages of correct detection after analysis with the targets of animal and plant species present in the mix with and without a DNA Erase cleaning protocol of the particles.

Mixes (% in weight of ingredients)	**Percentages of correct detection without particle cleaning**	**Percentages of correct detection with particle cleaning**
Mix 3 (92 % soybean, 5 % Chicken PAPs #2, 3 % milk powder #2)	10 %	90 %
Mix 7 (92 % maize, 5 % Chicken PAPs, 3 % Cattle blood)	0 %	80 %
Mix 8 (92 % maize, 5 % Chicken PAPs, 3 % Pig blood)	0 %	60 %
Mix 9 (92 % soybean, 5 % Cattle PAPs, 3 % Pig blood)	0 %	75 %

DNA extraction from a single particle and Real Time PCR analysis

The extraction of DNA from single particles was tested with volumes from 3 to 30 µl of Direct PCR® Lysis Reagent. In parallel, different volumes of extract (from 1 to 13.5 µl) were also put in the PCR. The difficulty was to find a good compromise between the efficiency of the lysis, the final DNA concentration of the extract and the amount of DNA in the PCR. A volume of 7 µl seems the best compromise for an efficient lysis. The extract is then diluted (1 volume of extract/4 volumes of MilliQ Water) and a maximum of 5 µl of the diluted extract are put in the PCR to avoid any PCR inhibition.

Using these parameters, 5 PCR can be performed with the DNA extract of almost every particles. Five different targets corresponding to 5 species can be tested or the result with one target can be confirmed on replicates. Nevertheless, the heat treatment has an impact on the PCR detection; the rate of detection decreases when the temperature of thermal treatment increases.

13.4 DISCUSSION

Two new procedures based on the combination of methods have been presented for identification of the animal origin of PAPs. The first procedure has shown the combination of optical microscopic identification of muscle fibres with immunochemical detection of species specific muscle proteins. This requires several steps : development and characterisation of (species specific) antibodies, concentration of muscle fibres from a feed, optimization of antibody incubation and detection. The availability of sensitive species-specific antibodies is crucial for this method. From the commercially available ELISA kit and from protein database searches it was known that species-specific amino acid sequences were present in Troponin I, a muscle protein. Therefore, monoclonal antibodies were raised against bovine Troponin I. Only one species-specific monoclonal antibody (MAb) was obtained from two fusions. Seven MAbs that reacted with two or more species were obtained in total. The Chinese antibody and the RIKILT MAb were unfortunately less sensitive than the MAb applied in the commercial kit. If this antibody would become available, it would greatly improve the detection limit of the assay. Using our method 25 grams of feed was sedimented as opposed to 10 grams of feed used in the official microscopy procedure. This improves the limit of detection, which will especially be useful for PAPs in which only low amounts of muscle fibres are present. The preliminary results clearly show that the method has potential although the limit of detection still has to be established. At the moment, analysis time is rather long, but can easily be shortened when the primary antibody is labelled (incubation with a second antibody is then redundant) and when higher concentrations of antibody are available herewith reducing incubation times by hours. Furthermore, several samples can be analysed simultaneously. Its major advantages are that it is an easy procedure that delivers information on the group of species present in the material and for the labs which are running the current control programmes, there is no investment of expensive equipment, and no extra trained lab personnel needed. Additional studies have to be performed with feed spiked with lower concentrations of PAPs , to establish detection limits and validate the method.

The second procedure combining NIR microscopy with Real Time PCR is still under development. The weak point of the procedure is the transfer of the suspicious animal particles to the PCR technology after NIRM analysis, which remains manual and tedious. The actual transfer of particles limits drastically the number of particles analysed and is therefore not routinely applicable. The solution could come from the use of a laser microdissection platform coupled with a capture system. Nevertheless, the results obtained with the PCR analysis allowed to build certified species specific spectral databases, which can be useful to detect species specific NIR markers necessary to develop discriminant models. Recent studies based on such discriminant models have established some key parameters to develop also a quantitative NIRM approach in order to fulfil the new requirements of the European legislation, making an additional advantage of the technique.

Among the several steps required in this method combination, the extraction from a single particle of DNA fit for PCR is no more a problem. The remaining challenge is the optimisation of the cleaning treatment of the particles. The action of DNA Erase and water seems to be a good compromise for the removal of external DNA while keeping an efficient amplification of the DNA from the particle. Nevertheless, for the moment, the performance of the whole protocol (cleaning of the particle, DNA extraction and PCR) remains dependent on the ingredients present in the sample. The cost of authorised ingredients like egg and milk powder is nevertheless a limitation for their use. In real world samples, their presence is limited to low amounts (< 5%) and the strategy of combining NIRM with PCR allows to authenticate the origin of a majority of the particles.

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