Development of a real time PCR protocol for the species origin confirmation of isolated animal particles detected by NIRM

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2010-063.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	26-Mar-2010
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Methods/Techniques:	Authenticity, Molecular biology - PCR
Additives/Contaminants:	Animal products
Food Types:	Animal feedingstuffs, Animal feed

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1 Development of a real-time PCR protocol for the species origin
2 confirmation of isolated animal particles detected by NIRM

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Abstract

At present, European legislation prohibits totally the use of processed animal proteins (PAPs) in feed for all farmed animals [Commission Regulation (EC) n°1234/2003 – extended feed ban]. A softening of the feed ban for non-ruminants would nevertheless be considered if alternative methods could be used to gain more information concerning the species origin of PAPs than that which can be provided by classical optical microscopy. This would allow control provisions such as the ban of feeding animals with proteins from the same species or intra-species recycling [Regulation (EC) n°1774/2002]. Two promising alternative methods, Near Infrared Microscopy (NIRM) and real-time Polymerase Chain Reaction (PCR) were combined to authenticate, at the species level, the presence of animal particles. The present paper describes the improvements of the real-time PCR method which were made to the DNA extraction protocol, allowing 5 PCR analyses to be performed with the DNA extracted from a single particle.

Keywords: PAP, animal proteins, BSE, feed ban, real-time PCR, NIRM.

Introduction

Since the outbreak of bovine spongiform encephalopathy (BSE), the use of processed animal proteins (PAPs) including meat and bone meal as feed ingredients for farmed animals is drastically controlled within the European Union through several regulations (Regulation (EC) 999/2001, Regulation (EC) 1774/2002 and Regulation (EC) 1234/2003). Presently, classical optical microscopy is the only official method for the detection of PAPs in compound feed in the European Union [Commission Regulation (EC) 152/2009] but the method offers very limited species discrimination perspectives. The Regulation (EC) 152/2009, however, stated that alternative methods can be used to gain more information about the origin of the PAPs. The capabilities of the different analytical methods for the detection, the species identification and the quantification of PAPs in feedingstuffs were recently reviewed by Fumière et al. (2009). Among the developed alternative methods, NIRM (Near Infrared Microscopy) and PCR (Polymerase Chain reaction) are in-house validated and routinely used under accreditation (ISO 17025 standard) at the Walloon Agricultural Research Centre. Moreover, they have been successfully assessed in international inter-laboratory studies describing their performance profile and their specific pros and cons (e.g. Prado et al., 2007). Other validation studies were conducted under the framework of the European SAFEED-PAP project. The results of these studies will be published soon.

Since 1998, the NIRM method that combines a NIR spectrometer and a microscope is in development at CRA-W for the detection of animal by-products in feed (Piriaux and Dardenne 2000). Through different Belgian and European projects e.g. STRATFEED and SAFEED-PAP, the method was continuously improved and the discrimination between terrestrial PAPs and fish by-products on particles from the whole sample (raw fraction) and the particles having a density > 1.62 obtained through a sedimentation process with tetrachloroethylene (sediment fraction) has been demonstrated (Baeten et al. 2004; Baeten et al. 2005; De la Haba et al. 2007; von Holst et al. 2008).

Looking at the discrimination between PAPs particles from terrestrial animal species, the results obtained in previous studies tend to indicate that it might be possible. Nevertheless, such information is only considered as an indication and could not be used as a forensic result. An interesting aspect of the NIRM is the non-destructive property of the method towards the analysed samples thereby allowing to isolate the suspicious particles and to perform additional analyses.

PCR is currently the only technique able to determine the origin of animal by-products present in a feed at the species level. Thanks to the good stability of the DNA to high temperatures and rendering processes, different real-time PCR methods using small sized multi-copy targets already proved their efficiency for the detection of PAPs in animal feed at low level (Aarts et al. 2006; Fumière et al. 2006; Prado et al. 2007; Cawthraw et al. 2009). Nevertheless, PCR alone is not sufficient to evidence non compliance with the feed ban as it cannot distinguish authorised animal DNA sources (e.g. blood or milk products) from forbidden ones. Due to the high sensitivity of some PCR methods, the species origin of by-products containing only traces of DNA such as fat (e.g. tallow) can be determined (Bellorini et al. 2005) and could even lead to false positive results from a legal point of view (Prado et al. 2007).

The combination of both techniques allowing the detection of individual PAPs particles by NIRM and their authentication at a species level by real-time PCR was therefore investigated. This paper describes the last developments achieved in the PCR protocol to determine the origin of a PAPs particle at a species level with 5 PCR.

Materials and methods

Samples. The raw materials used in this study are all representative of the real world feed ingredients: the meat and bone meals were obtained from plants members of the European Fat Processors and Renderers Association (EFPRA) and were produced as required by European

legislation (heat treatment at a temperature of ≥ 133 °C and 3 bars for 20 min). Concerning the authorised ingredients, the blood powders were also provided by the EFPIA. The egg powder came from a Belgian feed producer. The milk powders were obtained from the dairy industry.

NIRM analysis. The raw materials were ground with a Retsch ZM200 grinding mill (Retsch GmbH & Co., Haan, Germany) to obtain particles with a diameter < 2 mm. Prior to the NIRM analysis, the samples were sieved to select the fraction of the particles with a size with a diameter > 250 μm . Sixty particles were then manually spread on a spectralon plate. Using the microscope pointer of an auto image microscope connected to a Fourier transform near infrared spectrometer (FT-NIR; Perkin Elmer, Waltham, MA, USA), the infrared beam was focused on each particle and their NIR-spectrum (1100-2500 nm) was collected. The particles were arranged to be able to track the link between the spectrum and the corresponding particle. After the NIRM analysis, each particle was individually and carefully transferred in a 200 μl vial for PCR analysis and its origin (animal or vegetal) was predicted by existing discrimination models (Baeten et al. 2004).

Cleaning of the particles. After NIRM analysis, the particles detected as animal are isolated and put in a 200 μl vial. A cleaning procedure is then required to remove unspecific traces of DNA stuck to the particle before the DNA extraction (see the result section). Different reagents degrading the DNA were tested alone or in combination to clean the particles: DNA Erase (MP Biomedicals, Solon, Ohio, USA), RBS (Chemical Products R. Borghgraef s.a., Brussels, Belgium), hydrogen peroxide, acetone and isopropanol. Different incubation times and/or concentrations were tested. The protocols described here are the final ones giving the best results.

Cleaning with DNA Erase. The DNA Erase is classically used in laboratories to clean and decontaminate from DNA traces small equipments (e.g. pipets) or working surfaces. The cleaning is realised by adding 100 μl of DNA Erase to the particle. The vial is gently shaken during 4 min and

briefly centrifuged in a centrifuge 2K15 (SIGMA Laborzentrifugen GmbH, Osterode, Germany) before to remove the DNA Erase. The particle is then rinsed with 100 µl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA) and dried in a concentrator 5301 (Eppendorf AG, Hamburg, Germany).

Cleaning with RBS. The RBS is a detergent used in our lab for the cleaning of the mill used to grind the samples. The particle is incubated in 100 µl of RBS diluted 1 vol:19 vol in water for 2 min and briefly centrifuged in a centrifuge 2K15 (SIGMA Laborzentrifugen GmbH, Osterode, Germany) before to remove the RBS. The particle is then rinsed with 100 µl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA) and dried in a concentrator 5301 (Eppendorf AG, Hamburg, Germany).

Cleaning with hydrogen peroxide. The particle incubates in 100 µl of a solution of hydrogen peroxide at 30 % during 2 min. The vial is then briefly centrifuged in a centrifuge 2K15 (SIGMA Laborzentrifugen GmbH, Osterode, Germany) and the hydrogen peroxide is removed. The particle is then rinsed with 100 µl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA) and dried in a concentrator 5301 (Eppendorf AG, Hamburg, Germany).

Cleaning with acetone and isopropanol. A first cleaning is realised by adding 100 µl of acetone (ICN Biomedicals Inc., Aurora, OH, USA) to the particle. The vial is gently shaken by inverting four times and briefly centrifuged in a centrifuge 2K15 (SIGMA Laborzentrifugen GmbH, Osterode, Germany) before to remove the acetone. The cleaning process is managed so that the particle is in contact with the reagent during 2 min. A second cleaning with 100 µl of isopropanol (Sigma Aldrich Chemie GmbH, Steinheim, Germany) is immediately performed using the same way and timing. The particle is then rinsed with 100 µl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA) and dried in a concentrator 5301 (Eppendorf AG, Hamburg, Germany).

DNA extraction from a single particle. Several kits dedicated to DNA extraction from small amount of starting material were identified and tested: Invisorb® Spin Tissue Mini Kit and Invisorb® Spin

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Forensic Kit (Invitex GmbH, Berlin, Germany) are two kits in which a lysis step with a buffer is followed by a purification step on a spin column. Invisorb® Forensic Kit I (Invitex GmbH, Berlin, Germany) is a kit using the nanoparticles technology to recover the DNA extracted after the lysis step. Two other DNA extraction kits, PicoPure™ DNA Extraction Kit (Arcturus Bioscience, Mountain View, CA, USA) and Direct PCR® (tail) Lysis Reagent for Genotyping using Crude Lysates (Viagen Biotech Inc., Los Angeles, CA, USA), were also tested. These latter 2 kits allow to get a ready-to-use DNA extract directly after the lysis in a buffer.

The protocols presented here are the ones adapted to our specific application according to the constraints developed in the Results and discussion part of the paper from the original guidelines provided by the suppliers.

Invisorb® Spin Tissue Mini Kit is used to extract and purify DNA from small amounts (0.5 – 40 mg) of animal tissue. The lysis step of the particle is performed with 400 µl of Lysis Buffer G containing 40 µl of a Proteinase K solution provided with the kit. The lysate is centrifuged at 12000 rpm during 2 min. Forty µl of RNase A (10 mg/ml) are added to the supernatant recovered and incubated at room temperature during 5 min. After the addition of 200 µl of Binding Buffer T, the lysate is dropped on a spin column. After 1 minute, the spin column is centrifuged at 12000 rpm for 2 min. The spin column is then cleaned twice by the addition of 550 µl of Wash Buffer followed by a centrifugation at 12000 rpm during 1 min. The remaining ethanol is removed by a centrifugation at 12000 rpm during 2 min. Finally, the DNA is eluted by a centrifugation at 10000 rpm during 2 min after an incubation during 3 min in 60 µl of Elution buffer warmed at 52 °C.

Invisorb® Spin Forensic Kit is optimised to recover small amounts of DNA from forensic samples (e.g. blood, sperm or saliva stains, stamps, cigarette butts,...). The lysis step of the particle is performed at 56 °C for 2 hours in 500 µl of Lysis Buffer G containing 25 µl of a Proteinase K solution provided with

the kit. The lysate is centrifuged at 10000 rpm during 2 min. After the addition of 250 µl of Binding Buffer T to the supernatant recovered, the lysate is dropped on a spin column. After 1 min, the spin column is centrifuged at 10000 rpm for 1 min. The spin column is then cleaned twice by the addition of 800 µl of Wash Buffer followed by a centrifugation at 10000 rpm during 1 min. The remaining ethanol is removed by a centrifugation at 12000 rpm during 3 min. Finally, the DNA is eluted by a centrifugation at 8000 rpm during 1 min after an incubation during 1 min in 50 µl of Elution buffer warmed at 52 °C.

Invisorb® Forensic Kit I is optimised to extract DNA from the same forensic samples as the *Invisorb® Forensic Kit* but can also be used with bones (the following protocol is directly derived from the one describing DNA isolation from bone or ancient bone material in the manual of the kit). The lysis step of the particle is performed at temperature between 50 °C and 60 °C for 15-20 hours in 20 µl of Lysis Buffer G containing 4 µl of Bone Lysis Enhancer solution. The lysate is centrifuged at 12000 rpm during 2 min and the supernatant is recovered. Two µl of Carrier Suspension buffer made of nanoparticles are then added for an incubation at room temperature for 5 min. The mix is centrifuged at 10000 rpm for 1 second to pellet down the carrier and discard the supernatant. The nanoparticles are then cleaned twice with 20 µl of Wash Buffer. After a centrifugation, the pellet of nanoparticles is dried into a vacuum dessicator at 60 °C to completely evaporate the residual ethanol. The pellet is resuspended in 20 µl of Elution Buffer D and incubated at 60 °C for 5 min to release the DNA from the nanoparticles. A last centrifugation allows the removing of the nanoparticles and the recovering of the DNA.

PicoPure™ DNA Extraction Kit is developed for processing small samples of few as ten cells or several milligrams of tissue. It enables to recover high amounts of DNA ready for use in PCR assays. For optimal enzymatic activity, the lyophilised Proteinase K is freshly reconstituted in 155 µl of

Reconstitution Buffer. Thirty μl of the prepared buffer are added to the particle in a 200 μl vial and are incubated at 65 °C for 3 hours. Proteinase K is then inactivated at 95 °C for 10 min.

Direct PCR® (tail) Lysis Reagent for Genotyping using Crude Lysates is dedicated to mouse tail cells lysis and gives a DNA extract ready-to-use for the PCR. The extraction buffer is freshly prepared by adding Proteinase K (Invitrogen, Carlsbad, CA, USA) at a concentration of 0.4 $\mu\text{g}/\mu\text{l}$ to the Direct PCR® Lysis Reagent. Seven μl of the prepared buffer are added to the particle in a 200 μl vial and are incubated at 85 °C for 45 min. The extract with the particle is then centrifuged. Six μl of supernatant are recovered and are diluted with 24 μl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA).

Real-time PCR analysis. Cattle and pig primers and probes as well as real-time PCR conditions are the same as the ones presented by Fumière et al. (2006). The PCR amplifications are performed on a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) without any modification of the thermal program optimised on a GeneAmp 5700 real-time PCR device (Applied Biosystems, Foster City, CA, USA) composed of an initial decontamination step by uracyl DNA glycosylase at 50 °C for 2 min, a DNA denaturation combined to the activation of the *Taq* DNA polymerase performed at 95 °C for 10 min and 50 cycles including a melting step at 95 °C for 15 s, an annealing/elongation step at 50 °C for 60 s. The PCR is performed in a total volume of 35 μl . The reaction mix contains 5 μl of DNA extract, 17.5 μl of Real Time PCR Mastermix (Diagenode, Seraing, Belgium), 1.25 μl of each primer at 3 μM (Eurogentec, Seraing, Belgium), 1.25 μl of double dye probe at 10 μM (Eurogentec, Seraing, Belgium) and 8.75 μl of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA).

Chicken specific primers and probe were designed using the Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA) from the chicken mitochondrial sequence of the Genbank database. The sequences are: for the forward primer 5'-AGC ACT AGC CTT TTA AG-3', for the reverse primer 5'-GGT TTA ATT GGG GCA T-3' and for double dye probe 5'-ACA CCC TCC CCC TTA ATG AC-3'.

The thermal protocol is the same as for the cattle and pig probes. The PCR is performed in a total volume of 35 μ l. The reaction mix contains 5 μ l of DNA extract, 17.5 μ l of Real Time PCR Mastermix (Diagenode, Seraing, Belgium), 1.25 μ l of each primer at 10 μ M (Eurogentec, Seraing, Belgium), 1.25 μ l of double dye probe at 10 μ M (Eurogentec, Seraing, Belgium) and 8.75 μ l of PCR-grade water (ICN Biomedicals Inc., Aurora, OH, USA).

Maize and soja DNA detection were performed according to the protocols described by Kuribara et al. (2002) and Shindo et al. (2002).

Results and discussion

The first tests aiming to evaluate the feasibility to amplify the DNA from a single particle were performed using a very basic protocol: the particle was soaked in a solution of proteinase K at the concentration of 1 μ g/ μ l in the well of the PCR plate and submitted to an incubation at 60 °C (nearby the optimum t° of proteinase K) during 20 min before the inactivation of the enzyme at 95 °C during 10 min. Only after that, the PCR mix is added to perform the PCR thermal program. The results obtained were very encouraging but it rapidly appeared that a separate DNA extraction step would be needed to work with a purified DNA template and avoid PCR troubles such as inhibition or probe degradation leading to unspecific noisy signals (Figure 1).

The origin of these unspecific signals is not totally explained but the action of nucleases degrading the probe is a credible hypothesis: particles were analysed with a mix containing all the PCR reagents with the exception of the primers so that no amplification was possible. Similar signals were observed that substantiated the presence of molecules in the mix able to hydrolyse the double dye probe as measured by the emitted fluorescence.

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As presented in the Figure 2, the pre-PCR analysis of particles is composed of a cleaning step followed by a DNA extraction. Experimentally, this last step was however developed before the cleaning tests. Indeed it was mandatory to first obtain the best PCR usable DNA before to evaluate the effect of a cleaning process on the PCR results. That is why the developments of the protocols are described in this order.

Development of a DNA extraction protocol from a single particle. Starting from the general guidelines indicated in the manuals of the kits tested, the protocols were adapted to this particular application. As our aim was to obtain enough DNA to perform more than one PCR, the modifications took mainly into account the very limited amount of starting material (1 animal particle between 250 and 500 μm weighting around 0.03 mg – Abbas et al., submitted in 2010) and consequently, the limited amounts of the DNA extracted by reducing the volumes of the different buffers used to extract and purify the DNA. The influence of a second parameter, the incubation lysis time, was also studied. Nevertheless, the modifications of the protocols were sometimes limited by the design of the kits: the Invisorb® Spin Tissue Mini Kit and the Invisorb® Forensic Kit involve the use of a spin column for the purification of the DNA with the requirement to use minimum volumes of buffers (e.g. for the elution, a minimum volume of 50 μl is recommended by the manufacturer of the Invisorb® Spin Tissue Mini Kit).

Ten particles coming from the same three pure MBMs heat treated at 133 °C were analysed with optimised protocols. The performances obtained with the five kits are compared in Table 1.

Beside the cost of the DNA extraction per particle analysed and the time needed for extraction process, different criteria having a direct impact on the reliability of the method were examined: the risks of contamination due to manipulation, the number of available PCR replicates, the number of

successful PCR and the number of particles successfully detected by at least one of the PCR replicates.

The numerous centrifugations to perform with the spin column after the lysis step (Invisorb® Spin Tissue Mini Kit and the Invisorb® Forensic Kit) to purify the DNA appeared to us not convenient and a possible source of contamination. Moreover, it forced us to use minimum volumes of buffers probably inducing dilutions and loss of the DNA extracts. The rates of successful PCR amplification were closely dependent of the sample and the species origin of a significant part of the particles cannot be determined. In the case of the Invisorb® Forensic Kit, additional tests were performed: a longer lysis time (e.g. overnight at 42 °C) and the use of a Carrier RNA can be also performed in the case of extremely low amounts of DNA. Longer lysis time did not improve the successful PCR rates neither the number of particles successfully detected (data not shown). Concerning the use of a Carrier RNA, we tested a Poly(A) RNA from Roche Diagnostics as recommended by the manufacturer. Unfortunately, traces of bovine DNA were detected in the product making its use impossible for our purpose. The use of a home-made vegetal DNA coming from soyabean was also unsuccessfully tested. With the Invisorb® Forensic Kit I, the absence of spin column in the protocol helps us to adapt the volumes of the different buffers to use. Nevertheless, it was not possible to obtain any DNA extract usable for the PCR and the investigations were not carried on with that kit. With the PicoPure™ DNA Extraction Kit (Arcturus Bioscience, Mountain View, CA, USA) and the Direct PCR® (tail) Lysis Reagent for Genotyping using Crude Lysates, manipulations are limited to an incubation of the particle in the lysis buffer to obtain a PCR ready to use DNA extract. Therefore contaminations risks are reduced. As with the Invisorb® Forensic Kit I, it was easier to modify the initial provider's protocol of these two latter kits and to adapt it to our very particular application. The successful PCR rates and the number of particles successfully detected were significantly higher than with the three Invitex kits. Nevertheless the variability of the results with respect to the type of particles (muscle or

bone) submitted to the analysis is clearly higher with the PicoPure™ DNA Extraction Kit than with the Direct PCR® Lysis Reagent. Even an increase of the lysis incubation time from 3 hours to 18 hours (overnight) had a limited impact on the DNA extraction efficiency. Finally, the Direct PCR® Lysis Reagent was selected to continue the development of the method as it allowed to perform more successful PCR replicates than with the PicoPure™ DNA Extraction Kit, 5 and 2 PCR respectively, to authenticate the species origin of nearly all the particles. It must be stressed that with the DNA extraction protocol using the Direct PCR® Lysis Reagent no more unspecific signal as described above (Figure 1) was observed.

Development of a particle cleaning protocol. The PCR analysis of a single PAPs particle is only interesting if the DNA comes from the core of this particle and not from any external source especially from possible authorised ingredients present in a mix such as a compound feed. The possible presence of DNA molecules stuck to the surface of the particle was checked by preparing 8 mixes containing ingredients from two different animal species. The DNAs of the 8 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 composition of the 8 mixes and the Ct values obtained with the different PCR tests are presented in the Table 2.

Twenty MBM particles detected as from animal origin by NIRM analysis (MBM particles) and coming from 5 mixes containing one MBM and one authorised ingredient were analysed by PCR without any cleaning protocol before the extraction with the Direct PCR Lysis Reagent.

The results presented in the Table 3 show that in most of the cases a large majority of the MBM particles analysed gives a positive signal only with target corresponding to their species. These results show that the impact of the authorised ingredients can be limited when the particles are analysed according the protocol described in this paper. Nevertheless, the third mix (92 % of soja + 5 % of

chicken MBM + 3 % of milk powder) shows, that in some cases, the percentage of particles giving ambiguous results (positive result with more than one target) can increase and confirm that a cleaning would be useful.

The first cleaning protocol tested used a solution of bleach at 0.5 % in volume as recommended by Toyoda et al. (2004) for the cleaning of sedimented bone particles. Despite modified incubation times and bleach dilutions, the DNA denaturing action of bleach was too efficient and reduced drastically the number of successful PCR. Various other degrading reagents were also tested alone or in combination as described in the material and method section: DNA Erase, RBS, hydrogen peroxide (H_2O_2), acetone and isopropanol. Even if the NIRM analysis is able to distinguish an animal particle from a vegetal one, 20 particles of each ingredient from a mix containing 92 % of Maize, 5 % of a chicken MBM and 3 % of cattle blood were analysed by PCR after being submitted to the different cleaning protocols. The results obtained are presented in the Table 4.

The DNA Erase cleaning protocol gives clearly the more reliable results for the analysis of MBM particles: all the chicken MBM particles remains detectable with the chicken probe whereas no trace of DNA external to the particles was detected. For the other types of particles (blood and maize), the cleaning with the DNA Erase allows high levels of detection, 100 % and 90 %, for the blood particles and the maize particles respectively. With these two last types of particles, the frequencies of detection with the other probes are however higher. A maximum of 30 % of cattle blood particles gave a signal with the chicken probe. It must be stress that MBM particles can be easily distinguished from blood and vegetal ones by a NIRM analysis. So in a global analysis process combining NIRM and PCR analysis, these types of particles would not be analysed. Compared to the other cleaning protocols, the performances obtained with the cleaning with DNA Erase were considered most promising to test it with 3 other mixes. The results of the PCR analysis of 20 particles of MBM and of the vegetal ingredient are presented in the Table 5.

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With the MBM particles, the DNA Erase cleaning protocol allows an important decreasing of the simultaneous detection with the other animal probes. A notable exception must nevertheless be pointed out in the case of the mix containing blood powder. Seventy-five percent of the chicken MBM particles still give a positive detection with the pig probe. This result must however be attenuated by the figures presented in the Table 6. Looking at the Ct values obtained with chicken MBM particles analysed without and with the DNA Erase cleaning protocol, it clearly appears that the signals obtained with the chicken probe are significantly earlier than with the cattle probe. These results 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 MBM particle. Moreover, the cleaning protocol does not have the same influence on both signals: whereas the signals with the chicken probe (the probe for the species the MBM particle comes from) is slightly affected (no change or a delay of less than 2 cycles), the signals obtained with the other probes are strongly delayed (+/- 6 cycles) or completely eliminated (second mix tested in the Table 6).

Conclusion

The full application of the European legislation on the animal by-products requires reliable specific methods. Up to now, PCR is the only method able to detect the presence of animal ingredients up to the species level. Unfortunately, the impact of the PCR is limited by the use of authorised animal ingredients that are also sources of animal DNA and can interfere with the results from a legal point of view. In this paper, we demonstrate that the combination of NIRM with real-time PCR is feasible and could be integrated in a global analytical strategy as the one proposed by Fumière et al. (2009) to determine if the presence of animal constituents in feed is conform or not to the European legislation. Using a special buffer, the Direct PCR® Lysis Reagent, a rapid extraction protocol allowing the analysis of the DNA coming from a single PAPs particle with up to five different PCR targets has been developed. A protocol for the cleaning of the particles was also developed to eliminate possible DNA molecules stuck to the MBM particles surface. The protocol using the DNA Erase is the most efficient but its efficiency remains largely dependent on the sample and on the particle tested. No universal conditions could be found to eliminate totally the presence of DNA coming from authorised ingredients keeping in the same time enough PCR usable DNA from the core of the MBM particles. Nevertheless, due to their costs, the use of authorised ingredients such as egg and milk powder in feed is limited. The results obtained with real world samples containing limited amounts (< 5%) of authorised ingredients are quite encouraging and allow to authenticate the origin of a majority of the particles. Moreover when a signal is observed with more than one probe, the Ct values obtained reflecting the copy numbers of each species target can be used to determine the species origin of the MBM particle with a high level of confidence.

When possible, the results of previous analyses (presence of PAPs particles by classical microscopy, presence of animal species in the sample by PCR and NIRM) could even be used to focus the particle PCR analysis on a limited number of species and to use rationally the available PCR replicates for a

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reliable species authentication. Nevertheless, its use for routine analysis would be very tedious. The limitation of the method is essentially the number of particles that are manually analysed by NIRM and isolated previously to the analysis of their DNA. The overall process will be assessed soon with more complex samples.

It must be stressed that the combination of the real-time PCR could be also done with the classical microscopy following a similar protocol. Here again the analysis of a representative amount of particles is a critical point. An attractive solution to automate the identification of the suspicious particles and their isolation for the subsequent PCR analysis could be the use of a microscope coupled with a laser microdissection and pressure catapulting system.

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Acknowledgements

The research presented in this paper was supported by the Belgian Federal Public Service for Health, Food Chain Safety and Environment (Contract Nr S-6168) and the European Community within the framework of the SAFEED-PAP project (FOOD-CT-2006-036221) of the 6th EC Framework Program.

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Table 1. Comparative performances of Invisorb® Spin Tissue Mini Kit, Invisorb® Forensic Kit, Invisorb® Forensic Kit I, PicoPure™ DNA Extraction Kit and Direct PCR® (tail) Lysis Reagent for Genotyping using Crude Lysates.

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Figure 1. Real time PCR signals obtained with a pig MBM particle using the cattle and pig probes. The grey line is a normal amplification obtained with the pig probe. The black line is an unspecific signal obtained with the cattle probe.

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Figure 2. Overall analytical process of PAPs particles combining NIRM and Real Time PCR analysis.

The present paper is focussing on steps # 3, 4, 5 and 6 .

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Figure 1. Real time PCR signals obtained with a pig MBM particle using the cattle and pig probes.

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Table 1. Comparative performances of Invisorb® Spin Tissue Mini Kit, Invisorb® Forensic Kit, Invisorb® Forensic Kit I, PicoPure™ DNA Extraction Kit and Direct PCR® (tail) Lysis Reagent for Genotyping using Crude Lysates.

	Invisorb® Spin Tissue Mini Kit	Invisorb® Forensic Kit	Invisorb® Forensic Kit I	PicoPure™ DNA Extraction kit	Direct PCR® Lysis Reagent
Cost /DNA extraction	2.58 €	3.17 €	0.80 €	2.56 €	0.01 €
Extraction time	1.5 hour	2.5 hours	15 – 20 hours	3 hours	45 minutes
Contamination risk	High	High	Medium	Low	Low
Number of PCR	5	4	2	2	5
Successful PCR rates	10-65 %	70-85 %	0 %	75-100 %	96-100 %
Particles successfully detected	10-70 %	70-90 %	0 %	80-100 %	100 %

Table 2. PCR analysis of DNAs from 8 mixes containing 2 animal ingredients: Ct values (n = 2 extracts) obtained for the detection of chicken, cattle, pig, sojabean and maize DNA.

Mixes (% in weight of ingredients)	Ct Chicken	Ct Cattle	Ct pig	Ct Sojabean	Ct Maize
Sojabean (92 %) + Chicken MBM #1 (5 %) + Milk powder #1 (3 %)	25.90	38.47	NT ^a NT ^a		NT ^a NT ^a
Sojabean (92 %) + Chicken MBM #1 (5 %) + Milk powder #2 (3 %)	24.90	32.09	NT ^a NT ^a	28.90	NT ^a 29.16
Sojabean (92 %) + Chicken MBM #2 (5 %) + Milk powder #2 (3 %)	27.52	32.49	NT ^a NT ^a	28.05	28.81
Maize (92 %) + Cattle MBM (5 %) + Egg powder (3 %)	29.74	25.41	32.56	28.50	30.37
Maize (92 %) + Pig MBM (5 %) + Egg powder (3 %)	29.73	NT ^a	NT ^a	NT ^a NT ^a NT ^a	29.08
Maize (92 %) + Chicken MBM #1 (5 %) + Cattle blood (3 %)	23.09	34.79	25.79	NT ^a NT ^a	NT ^a
Maize (92 %) + Chicken MBM #3 (5 %) + Pig blood (3 %)	23.03	NT ^a 25.98	NT ^a		
Chicken MBM #1 (50 %) + Milk powder #2 (50 %)	19.08				

^a NT : not tested**Table 3.** PCR analysis of 20 MBM particles detected as from animal origin after NIRM analysis. Percentages of detection with the Chicken probe, the Cattle probe and the Pig probe.

Mixes (% in weight of ingredients)	Chicken	Cattle	Pig
Sojabean (92 %) + Chicken MBM #1 (5 %) + Milk powder #1 (3 %)	100 %	0 %	NT ^a
Sojabean (92 %) + Chicken MBM #1 (5 %) + Milk powder #2 (3 %)	100 %	5 %	NT ^a
Sojabean (92 %) + Chicken MBM #2 (5 %) + Milk powder #2 (3 %)	95 %	20 %	NT ^a
Maize (92 %) + Cattle MBM (5 %) + Egg powder (3 %)	0 %	100 %	NT ^a
Maize (92 %) + Pig MBM (5 %) + Egg powder (3 %)	5 %	NT ^a	95 %
Maize (92 %) + Chicken MBM #1 (5 %) + Cattle blood (3 %)	100 %	40 %	90 %
Maize (92 %) + Chicken MBM #3 (5 %) + Pig blood (3 %)	100 %	NT ^a	100 %
Chicken MBM #1 (50 %) + Milk powder #2 (50 %)	100 %	55 %	NT ^a

^a NT : not tested**Table 4.** PCR analysis of 20 particles of each ingredient from a mix containing 92 % of Maize, 5 % of a chicken MBM and 3 % of cattle blood. Percentages of detection with the Chicken probe, the Cattle probe and the Maize probe.

Type of particle	Without cleaning protocol			With RBS cleaning protocol			With DNA Erase cleaning protocol			With H ₂ O ₂ cleaning protocol			With acetone-isopropanol cleaning protocol		
	Chicken	Cattle	Maize	Chicken	Cattle	Maize	Chicken	Cattle	Maize	Chicken	Cattle	Maize	Chicken	Cattle	Maize
Chicken MBM	100 %	60 %	90 %	100 %	5 %	5 %	100 %	0 %	0 %	100 %	5 %	40 %	100 %	40 %	60 %
Cattle blood	100 %	100 %	100 %	90 %	100 %	45 %	30 %	100 %	15 %	35 %	95 %	60 %	100 %	100 %	95 %
Maize	100 %	45 %	95 %	15 %	0 %	95 %	5 %	10 %	90 %	100 %	20 %	95 %	95 %	10 %	90 %

Table 5. PCR analysis of 20 particles of MBM and of vegetal ingredient from three mixes. Percentages of detection with the Chicken probe, the Cattle probe, the Pig probe, the Maize probe and the Sojabean probe.

Mixes (% in weight of ingredients)	Particles analysed	Without cleaning protocol					With DNA Erase cleaning protocol				
		Chicken	Cattle	Pig	Maize	Sojabean	Chicken	Cattle	Pig	Maize	Sojabean
Chicken MBM #1 (50 %) + Milk powder #2 (50 %)	Chicken MBM	100 %	70 %	NT ^a	NT ^a	NT ^a	95 %	5 %	NT ^a	NT ^a	NT ^a
Soja (92 %) + Chicken MBM #1 (5 %) + Milk powder #2 (3 %)	Chicken MBM	95 %	20 %	NT ^a	NT ^a	85 %	100 %	0 %	NT ^a	NT ^a	0 %
	Sojabean	80 %	5 %	NT ^a	NT ^a	95 %	5 %	0 %	NT ^a	NT ^a	95 %
Maize (92 %) + Chicken MBM #3 (5 %) + Pig blood (3 %)	Chicken MBM	100 %	NT ^a	100 %	95 %	NT ^a	100 %	NT ^a	75 %	5 %	NT ^a
	Maize	100 %	NT ^a	100 %	95 %	NT ^a	10 %	NT ^a	15 %	85 %	NT ^a

Table 6. PCR analysis of MBM and blood particles of 2 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.

Mixes (% in weight of ingredients)	Particles analysed	Without cleaning protocol			With DNA Erase cleaning protocol		
		Chicken	Cattle	Pig	Chicken	Cattle	Pig
Maize (92 %) + Chicken MBM #3 (5 %) + Pig blood (3 %)	Chicken MBM	21.64	NT ^a	31.90	21.52	NT ^a	37.55
Maize (92 %) + Chicken MBM #1 (5 %) + Cattle blood (3 %)	Chicken MBM	23.27	39.00	NT ^a	25.10	ND ^b	NT ^a
	Cattle blood	29.54	29.47	NT ^a	36.74	29.74	NT ^a

^a NT : not tested
^b ND : not detected