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# Detection of Land Animal Remains in Fish Meals by the Polymerase Chain Reaction−Restriction Fragment Length Polymorphism Technique

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In the present study a technique was developed with the aim of guaranteeing the composition and security of fish meals, since it allows verification of whether these meals contain land animal remains. The method is based on polymerase chain reaction (PCR) and length polymorphism, followed by a restriction fragment length polymorphism (RFLP). Specific primers for every species were designed and calibrated, generating exclusively a PCR product with a specific size when DNA for each species was present in the sample. This technique allows the detection of land animal remains in fish meals, specifically cow, chicken, pig, horse, sheep, and goat. The identity of the PCR products can be confirmed by RFLP analysis using only one restriction enzyme. The selected restrictase generated one characteristic restriction profile for every species included in this study. The detection limit of this method was calculated by using mixtures of fish meals in different proportions and meal that exclusively contained remains of one of these land species studied. The analytical strategy herein proposed was applied to fish and meat meals, giving good results, both in the analyzed standards and in commercial samples.

**KEYWORDS: Fish meal; meat meal; PCR-RFLP; BLAST; adulteration; cross-contamination; land animal detection**

## INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a progressive neurological disorder of cattle that results from an infection by an unconventional transmissible agent termed "prion". The origin of BSE is linked to the use of ruminant proteins in the preparation of animal feed. A new variant of BSE is the Creutzfeldt−Jacob disease (nvCJD), also known as human spongiform encephalopathy, related to consumption of products contaminated with this infectious agent.

All these events have a big impact on the economy and public health, due to the fact that the disease could be transmitted from animals to humans. To prevent the transmission of this disease between animals and humans, European Authorities prohibited the use of animal meal for feeding of ruminants (*1*). This measure includes fish meals, although fish are not affected by this disease. European Authorities admit this fact and argue that the objective of this measure is to prevent adulteration and cross-contamination between fish and land animal meals, because fish meals could make the detection of meat and bone meals more difficult. As a consequence of the ban, residues generated in fish processing cannot be destined to farm animal feeding. This measure generated accumulation of residues coming from the manufacturing process and closed a possible way to business for companies belonging to the fishing transformation sector.

European Authorities also established that it would be necessary to develop new analytical methods that are more accurate than the existing ones. Commission Directive 2003/126/EC (*2*) includes the analytical method for determination of constituents of animal origin for the official control of feeding stuffs, and it is based on a microscopic technique. This analytical method has several drawbacks; among them, detection of typical structures is a subjective task, it is conditioned by the analyst expertise and tiredness, it is time consumig and laborious, and it is only valid for identification at the order level. Moreover, this method is not applicable to fish meals, because some typical structures detected are common to both fish and land animals, and it only gives useful results when bones are present in the sample.

Since then, several molecular techniques have been developed in attempts to resolve this problem, because these methodologies are the most suitable to detect species-specific DNA in highly processed samples. Some of them used DNA fragments of around 300 bp (base pairs) (*3*−*8*). In an intercomparative assay organized by the European Commision (*9*) different methodological strategies were compared (microscopy, immunoassay, polymerase chain reaction (PCR), and liquid chromatography).

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**Table 1.** Primers Used for Land Animal Amplification and Some Features of Them

| species | accession number | begin | end | name | sequence (5′–3′) | length (bp) | $T_m$ (°C) | fragment size (bp) |
|---|---|---|---|---|---|---|---|---|
| *C. hircus* (goat) | 33285125 | 14305 | 14329 | CAB H | CAA TAC ACT ATA CAT CCG ACA CAA T | 25 | 67 | 192 |
| | | 14472 | 14496 | CAB L | CAA TGT TTC ATG TTT CTA GAA AGG T | 25 | | |
| *G. gallus* (chicken) | 5834843 | 14932 | 14958 | POLL H | ATA ATT AAC AAC TCC CTA ATC GAC CTC | 27 | 72 | 161 |
| | | 15071 | 15092 | POLL L | ACG GAG GAG AAG GCT AGG GAT G | 22 | | |
| *S. scrofa* (pig) | 5835862 | 15495 | 15519 | CER H | GCA ATA CAT TAC ACA TCA GAC ACA A | 25 | 64 | 135 |
| | | 15605 | 15623 | CER L | GAT GAA TAG GCA AAT AAA GAA TAT G | 25 | | |
| *E. caballus* (horse) | 47156680 | 14216 | 14242 | HOR H | TAA TTA AAA TCA TCA ATC ACT CTT TTA | 27 | 65 | 168 |
| | | 14363 | 14384 | HOR L | ACG GAT GAG AAG GCA GTT GTC G | 22 | | |
| *O. aries* (sheep) | 5835554 | 14311 | 14336 | OV H | AGC AAT ACA CTA TAC ACC TGA CAC AA | 26 | 71 | 132 |
| | | 14416 | 14442 | OV L | AAT AGG CAG ATA AAA AAT ATT GAT GCC | 27 | | |
| *B. taurus* (cow) | 5834939 | 15189 | 15213 | VAC 1 | ATT AAG GAC ATC TTA GGG GCC CTC T | 25 | 71 | 134 |
| | | 15299 | 15323 | VAC 2 | GGG TTT GAT GTG AGG GGG TGT GTT G | 25 | | |
| fish | data not shown | | | PEC H | CCY AGG GAT AAC AGC GCA ATC | 21 | 67 | 156 |
| | | | | PEC L | TCC GGT CTG AAC TCA GAT CAC | 21 | | |

The results of this work pointed out that PCR product sizes greater than 175 bp are innapropiate and could be the cause of false negative results. This fact is due to the thermal treatment received in the elaboration of fish meal, which causes DNA degradation and prevents recovery of DNA fragments larger than 200 bp.

Most of the studies used the mitochondrial *cytochrome b* gene for detection and identification of animal material in food (*5*, *6*). SINE (short interpressed repetitive element) sequences (*10*) or several mitochondrial regions (*8*) also were used to reach this goal.

Dalmasso et al. (*11*) developed a multiplex PCR for the rapid identification of ruminant, poultry, fish, and pork materials, but this study does not allow detection at the species level. Kremar et al. only focused their study on the detection of the meat and bone of cow in animal feed (*12*). Recently, these authors published a method that allows the detection of four land species using real time PCR (*13*).

The goal of this work was to develop a DNA-based technique suitable for detecting land animal traces in fish meal, allowing detection of adulteration or cross-contamination of fish meal with six land species. In this way, it is possible to certify that analyzed fish meals do not include in their composition these land animal remains. This fact could contribute to lifting of the ban established in the European Union (EU) that prohibits the use of fish meal in the feeding of any farm animal species.

## MATERIALS AND METHODS

**Sample Collection.** *Animal Tissue Standard*. Four individuals of the following species have been used to calibrate the method developed in this research: cow (*Bos taurus*), horse (*Equus caballus*), sheep (*Ovis aries*), pig (*Sus scrofa*), goat (*Capra hircus*), and chicken (*Gallus gallus*).

Tissues of turkey (*Meleagris gallopavo*) and rabbit (*Oryctolagus cuniculus*) were used to verify the specificity of the primers. Several seafood species were also used to calibrate the inhibitor control: mussels (*Mytilus* spp.), cod (*Gadus morhua*), anchovies (*Engraulis* spp.), tuna (*Tunnus* spp.), sardine (*Sardina pilchardus*), hake (*Merluccius* spp.), and salmon (*Salmo salar*).

All these samples were obtained in shops and markets from Pontevedra, Spain.

*Standard of Fish Meals and Meat Meals*. These standards were sent to our laboratory by the Laboratorio de Sanidade e Producción Animal de Galicia (Xunta de Galicia, Spain) and rendering plants. Other samples from earthly origin were elaborated in our laboratory, where different muscular portions of tissue were treated in an autoclave (133 °C, 20 min, and 3 bar) for feigning the manufacturing process of fish meals.

**Primer Design.** Two DNA regions with different properties were selected to design the primers used in this work. The first of them, the *cytochrome b* gene, was selected because its sequence shows high interspecific variation even in closely related species. Moreover, it presents the advantages of a mitochondrial marker (haploid genome, multicopy, known sequence for several markers, etc.). All these reasons lead it to being a very used marker for genetic identification of species in processed products, such as canned fish (*14*−*17*). For these reasons, primer sets to amplify land animals were designed on the basis of the *cytochrome b* gene. The second region selected was the *16S rDNA* gene. This marker is more conserved than the *cytochrome b* gene and was selected to be used as an inhibitor control due to its high degree of conservation, allowing amplification with fish and land animals. These characteristics made feasible the use of this marker to design one primer set that works as an inhibitor control, allowing assessment of the presence of inhibitory agents in DNA extractions. When only one fish meal sample contained polymerase inhibitor agents, the PCR with this primer set would give a negative result. This primer set was designed using the sequences of representative species belonging to different taxonomic fish groups (*18*).

Sequences of both markers (*cytochrome b* and *16S rDNA* genes) were obtained from the National Center for Biotechnology Information (NCBI) and were aligned with BioEdit version 7.0.0 (*19*), allowing the location of diverging regions between the land species included in this work and conserved regions of a great number of fish species, which were used for the primer design.

**DNA Extraction.** DNA extractions from land animals and fish (standards) were carried out from 30 mg of tissue using the method described by Roger and Bendich (*20*) with slight modifications. In the case of fish meal samples, it was necessary to eliminate the oil and fat content, because both components can interfere with the DNA extraction process. The degreased process was carried out by resuspending the meals in a solution of methanol−chloroform−water (2:1:0.8) for 2 h. After this time, the solution was removed, and the fish meals were washed with ultrapure water to eliminate the remains of the solution used previously.

Two different DNA extraction methods were evaluated in the case of meals, both with 300 mg of sample: (1) Chelex resin (BIO-RAD), carried out according to the manufacturer's instructions, and (2) a method based on silica gel columns (Macherey-Nagel NucleoSpin Tissue kit). The kit was used following the manufacturer's instructions with minor changes to adapt the procedure to a high quantity of sample.

The quantity and quality of extracted DNA were estimated with the Eppendorf Biophotometer spectrophotometer.

**Calibration of Primer Specificity and Development of a PCR Length Polymorphism for Detection of Land Animal Remains in Fish Meals.** This step was carried out with four individuals of each of the six land animal species included in this study. The parameter calibrated for every primer set was the melting temperature ($T_m$). The calibration process was started at the $T_m$ given by the primer manufacturer (Sigma-Genosys) for each primer set and was increased 0.5 °C in each position of the gradient. The maximum temperature that allows amplification for each primer set was determined, and these values are shown in **Table 1**.

**Table 2.** Fragments Generated after Digestion of the PCR Amplicons of Land Species Included in This Study with Tsp 509 I: Restriction Profile with Tsp 509 I (/aatt)

| species | PCR product length (bp) | fragment size (bp) |
|---|---|---|
| *C. hircus* (goat) | 192 | 65, 127 |
| *G. gallus* (chicken) | 161 | 57, 104 |
| *S. scrofa* (pig) | 135 | 66, 69 |
| *E. caballus* (horse) | 168 | 168 |
| *O. aries* (sheep) | 132 | 78, 54 |
| *B. taurus* (cow) | 134 | 28, 106 |

The calibration process and DNA amplifications were conducted in a gradient thermocycler, iCycler IQ (BIO-RAD), with puRE Taq Ready-to-Go PCR beads (Amersham Biosciences) in a final volume of 25 $\mu$L containing 100 ng of DNA, 2 mM $MgCl_2$, and a 2 $\mu$M concentration of every primer. PCR conditions were the following: a preheating step of 95 °C for 5 min, 50 cycles of 95 °C for 30 s, $T_m$ (**Table 1**) for 30 s, 72 °C for 3 min, followed by a final extension step of 72 °C for 3 min.

Cross-amplifications do not take place and only amplification takes place when primers and DNA of each species are present in the PCR. Moreover, to verify the specificity of these primer sets, they were proved with other species [turkey (*M. gallopavo*) and rabbit (*O. cuniculus*)].

The process followed to calibrate the fish primer set was similar to the one used to calibrate the land animal primer sets. In this case, standards used were mussels (*Mytilus* spp.), cod (*G. morhua*), anchovies (*Engraulis* spp.), tuna (*Tunnus* spp.), sardine (*S. pilchardus*), hake (*Merluccius* spp.), and salmon (*S. salar*).

PCR products were evaluated in 2% agarose gels (Sigma) with TBE buffer and ethidium bromide (Sigma) at 10 mg/mL. The size of the amplified fragments was estimated from a molecular marker 50 bp ladder (Amersham Biosciences), proving that PCR products had the expected size.

**Confirmation of the Identity of PCR Products by Sequencing and the Basic Local Alignment Search Tool (BLAST).** All PCR products were cleaned before the sequencing reaction, with the GFX PCR DNA and Gel Band purification kit (Amersham Biosciences) following the supplier's protocol. PCR products were directly sequenced in both directions with primers used for PCR amplification, as indicated in **Table 1**. The sequencing process was conducted in an ABI Prism 310 genetic analysis system using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction kit according to the manufacturer's recommendations (Applied Biosystems).

Sequences obtained in this way were uploaded to the data base of NCBI (accession numbers DQ519401−DQ519424). BLAST is a suitable technique to find regions of local similarity between sequences and even can be a suitable technique to identify different species. Specifically, a Megablast search available at NCBI was realized for evaluation of the similarity degree between sequences obtained and the ones included in this data base. These sequences were downloaded and used to evaluate the haplotypic diversity with DnaSP 4.0 (*21*) in every species (data not shown).

**Development of a Restriction Fragment Length Polymorphism (RFLP) Methodology To Verify the Identity of PCR Products.** Once verified, the homology level between sequences obtained in our laboratory and those available from NCBI for the same species was used to design restriction maps (*22*). One enzyme was selected on the basis of its restriction profile, which allowed identification of all animals included in this work (**Table 2**).

To verify the usefulness of this enzyme and the restriction profiles generated in the studied species, all PCR products obtained were digested. Previous to the enzymatic digestion, PCR products were purified with the GFX PCR DNA and Gel Band purification kit (Amersham Biosciences) to eliminate primer dimer and primer excess.

Digestions were carried out overnight with 2 units of the selected enzyme and 200 ng of PCR product in a final volume of 20 $\mu$L and at a specific temperature recommended by the supplier (65 °C). Restriction products were electrophoresed in low melting 3% agarose gels (Pronadisa) in TBE buffer with ethidium bromide (10 mg/mL) for 120 min at 80 V. Gels were observed in an image analyzer, Gel Doc XR (BIO-RAD), under ultraviolet light. The fragment size was estimated from a ladder of 50 bp's (Amersham Biosciences).

**Table 3.** Proportions of Meat Meal in Fish Meals Used for Detection of the Sensitivity of the Method

| proportion of meat meal in fish meals (%) | PCR result with 300 mg of meal for DNA extraction | PCR result with 1200 mg of meal for DNA extraction |
|---|---|---|
| 100 | + | + |
| 50 | + | + |
| 25 | + | + |
| 10 | + | + |
| 5 | + | + |
| 1 | − | + |
| 0.5 | − | + |
| 0.1 | − | + |
| 0.05 | − | + |

**Sensitivity of the Method Developed.** Sensitivity was calculated using dilutions of known amounts of land animal standard tissue treated in an autoclave and fish meals. Proportions of land animals were between 100% and 0.05% (**Table 3**). These mixtures were homogenized with a high-speed homogenizer for 2 min. The DNA extraction was carried out from these mixtures to evaluate the minimum quantity of meat meal necessary to detect land animal remains.

Applying the methodology herein developed, land animal remains were detected in mixtures of fish meals in different proportions.

**Application to Fish and Meat Meals.** The method developed was applied to 20 meals, 16 of which were fish meals and 4 of which were meat meals.

## RESULTS AND DISCUSSION

**DNA Extraction from Fish and Meat Meals.** Several authors have demonstrated the efficiency of different protocols for DNA extraction from meal and feed samples (*3*, *13*, *23*). In the present work two methods were tried for DNA extraction from meals, Chelex resin (BIO-RAD) and NucleoSpin kit (Macherey-Nagel). The NucleoSpin Tissue kit showed better results than the Chelex methodology, because more quantity and better quality of DNA were obtained. Therefore, in this work the NucleoSpin kit was chosen for DNA extraction from meals.

**Calibration of the Specificity of the Primers and Evaluation of Length Polymorphism.** Land animal primer sets were optimized so PCR products generated by every primer set were exclusively obtained when specific DNA of its corresponding species was present (**Figure 1**). PCR products belonging to all standards used in this work presented the expected size according to their species (calculated on the basis of the nucleotidic sequence published by NCBI for every organism).

DNA from turkey and rabbit was used as a template for PCR with all primer sets for land animals, and no cross-amplification was detected. Then the total specificity of the primer sets designed was determined.

Due to the similarity in size of PCR products obtained with land animal primer sets, it was not possible to associate one determinate PCR product with one species in a univocal form (**Table 2**).

The primer set of fish generated a PCR product, with standards of both land animals and fish. The function of these primers is as inhibitor controls. This is especially important in the method herein developed, because when a fish meal does not contain land animals in its composition, all PCR will be negative except the inhibitor control. Thus, it will allow the guarantee that there are no DNA polymerase inhibitors in the

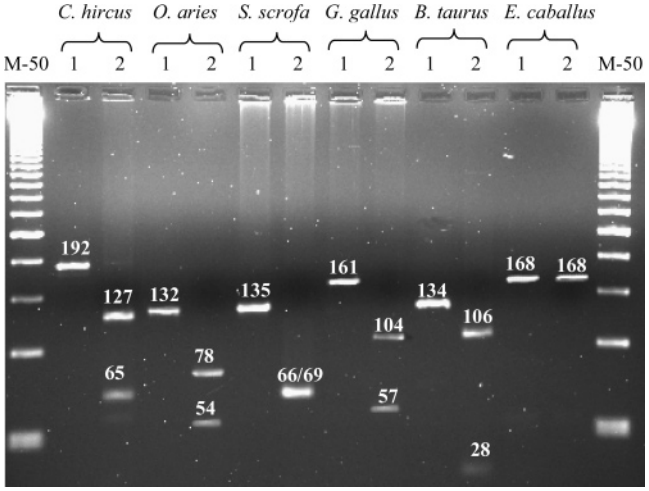


**Figure 1.** RFLP analysis of PCR products obtained by primers described in this work: lanes M-50, DNA size marker 50 bp ladder; lanes 1, PCR products; lanes 2, PCR products after digestion with Tsp 509 I.

DNA extraction. Moreover, any fish meal must contain at least one fish species, and therefore, PCR must generate a product.

**Confirmation of the Identity of PCR Products by Sequencing and BLAST.** Sequencing is a powerful technique very often used in laboratories devoted to food control, for instance, in the field of genetic identification of species, although it has the drawback of the high cost of the necessary material and equipment.

Land animal standards were sequenced to verify that PCR products obtained with specific primers had the expected size for the corresponding species.

All obtained sequences were identical to some of the same species available from NCBI, achieving a homology score of 100% with the Megablast algorithm. Therefore, this methodological approximation is suitable to confirm the identity of PCR products.

**Development of an RFLP Methodology To Verify the Identity of PCR Products.** RFLP is an alternative technique to sequencing, and it has been very used in the past for genetic identification of species (*14*, *17*, *24*). This technique has also been applied to detection of different species in feed (*25*), since it presents several advantages in comparison to sequencing. For instance, the necessary equipment has a very low cost, and it is a fast and easy methodology. However, some authors advise against using RFLP for forensic identification when there are moderate levels of intraspecific variability, because this fact could make the RFLP unstable and lead to misidentifications. This intraspecific variability can be due to the nature of the species or the studied markers. In this work intraspecific variability of the *cytochrome b* fragment from these six land species was studied. Available sequences from NCBI and those obtained in this work have been taken into account. Very low levels of intraspecific variability have been found in the studied species (data not shown), but they do not affect the restriction enzyme target. The restrictase selected was Tsp 509 I. Therefore, RFLP represents a suitable alternative technique to sequencing to assess the identity of a PCR product.

The PCR products of standards included in this work were digested with the enzyme Tsp 509 I, obtaining the characteristic restriction profiles for every species (**Figure 1**).

**Sensitivity of the Method Developed: Determination of Meal Quantity for DNA Extraction.** DNA extractions were carried out from different meal quantities to estimate the optimum quantity to obtain a high sensitivity. Specifically, DNA extractions were carried out from 300 and 1200 mg of meal standard mixtures in different proportions. The diagnostic method herein designed was applied to these mixtures. This allowed the minimum quantity of land animal remains which can be detected in fish meals using the PCR method developed in this work to be established. The detection limit is under 0.05% using 1.2 g of tissue remains for DNA extraction (**Table 3**). This result agrees with previous works where the detection limit is lower than that established in the microscopic technique (*7*, *10*).

**Application to Fish and Meat Meals.** In this study, 20 commercial samples were analyzed (16 fish meals and 4 meat

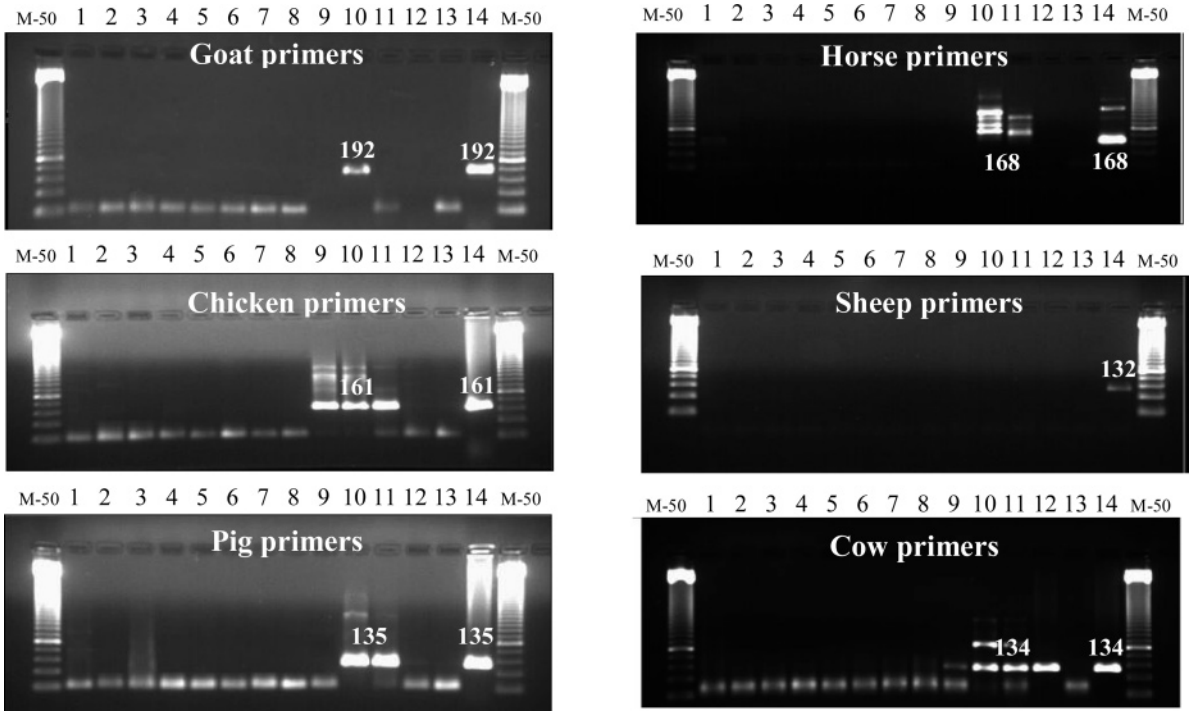


**Figure 2.** Application of the PCR method in commercial meals: lane M-50, DNA size marker 50 bp ladder; lanes 1–8, fish meals; lanes 9–12, meat meals; lane 13, negative control; lane 14, positive control.

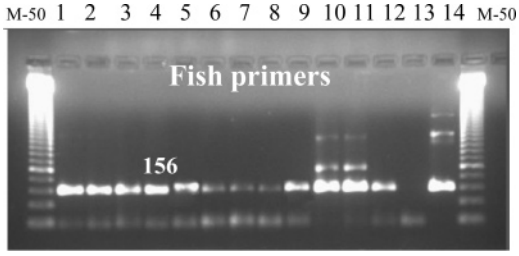


**Figure 3.** Inhibitor control test: lane M-50, DNA size marker 50 bp ladder; lanes 1–8, fish meals; lanes 9–12, meat meals; lane 13, negative control; lane 14, positive control.

meals) by the PCR−RFLP methodology proposed herein. All samples contained one of the species included in this work, determined by the PCR−RFLP technique developed.

None of the analyzed fish meals contained land animal remains, or they were under the detection limit of the developed method. All analyzed meat meals gave positive results for some land animal as shown in **Figure 2**. The first of them was positive for *G. gallus* and *B. taurus* (**Figure 2**, lane 9), the second was positive for *C. hircus*, *G. gallus*, *S. scrofa*, *E. caballus*, and *B. taurus* (**Figure 2**, lane 10), the third was positive for *G. gallus*, *S. scrofa*, *E. caballus*, and *B. taurus* (**Figure 2**, lane 11), and the last was positive for *B. taurus* (**Figure 2**, lane 12). These PCR products generated the expected RFLP and sequence according to the PCR product size. Sequencing followed by the homology analysis with Megablast confirmed the detection carried out by the PCR−RFLP technique.

All analyzed samples were accompanied by positive, negative, and inhibitor control (**Figures 2** and **3**). The results indicated the absence of polymerase inhibitors, since inhibitor control always presented amplification in both fish and meat meals (**Figure 3**). The inhibitor control was sequenced, and a mixed chromatogram was found. This can be due to several species being used in the elaboration of fish meals, making it not possible to determine the fish species included in the fish meals, although this was not the goal of the present work.

The technique proposed in this study can be considered a valid alternative or a complement to the microscopic method for the detection of land animal remains in fish meal. The main two advantages of this analytical proposal with regard to the microscopic technique are, first, it is not subjective, since it does not depend on the expertise of the analyst and, second, it allows determination of the nature of the land remains contained in a specific fish meal. In contrast, the microscopic method is only able to detect bone remains. Moreover, the detection limit of the methodology proposed is very low, improving the 0.1% limit of the official microscopic technique.

The method developed in the present paper allows detection of the six main land animal species utilized for human consumption independently. These species generate residues that can be used for elaboration of meat meal. Previous works detect fewer species and do not offer the possibility of confirming the identity of PCR products (*4*, *7*, *10*−*12*). In addition to that, this method presents a series of controls that allow verification of the obtained result.

This PCR-based method tries to meet the request of the European Authorities, who demanded new analytical methods more suitable and precise than the existing ones as a previous condition to lift the ban for use of fish meals in the feeding of farm animals. This methodology allows verification of the adulteration and cross-contamination of fish meals with the six land species studied.

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