



Analytical Methods

Development of a multiplex PCR–ELISA method for the genetic authentication of *Thunnus* species and *Katsuwonus pelamis* in food products

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ABSTRACT

In the present work a PCR–ELISA technique for the authentication of *Thunnus* species was developed. This method is composed by four systems that can be used in a hierarchical way allowing the identification of several scombroids species; or each individual system independently. The hierarchical strategy, proposes a first step, to assign one sample to the *Thunnus* genus. Next, if the result is positive, several tests can be applied to assign the sample to some particular species of the *Thunnus* genus. In the case that the result is negative (absence of *Thunnus* species), it is possible to verify if *Katsuwonus pelamis* is included in the sample. The method even allows the detection of mixtures of these species in relatively low amounts (up to 1%). Finally, this method was applied to 11 commercial samples to verify the labelling status of tuna products in the market, detecting that 18% were mislabelling.

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1. Introduction

The *Thunnus* genus belonging to the Scombridae family, includes 8 species which should be generally denominated by commercial labels as tuna. The economic importance of this taxonomic group at global scale is unquestionable. For instance, the mean total world catch of tuna species of 2006–2008 was of 4,387,909 tons (FAO, 2008), and this data reflects the importance of this taxonomic group as a food resource for humans, in fact due to this extensive consumption tuna are also known as “chicken of the sea”.

Other important aspect is the great diversity of species included in the family Scombridae, since contains 15 genera and 54 species (Collette, 2001). The great similarity amongst all the Scombridae species in processed products make possible that, in some cases, species which do not belong to *Thunnus* can be fraudulently used as substitutes of tunas. To control the fraudulent use of non-tuna species as tuna there has been different labelling regulations. For

instance, in Europe the Regulation 1536/92 (EEC, 1992) details labelling issues of canned tuna and bonito, with the aim of prosecuting mislabelling and fraud.

In order to verify the correct labelling of food products, a lot of analytical methods have been developed up to date. Most of them are based on molecular techniques that allow the assignation of biological samples to level species (Bartlett & Davidson, 1991; Blanco, Perez-Martin, & Sotelo, 2008; Chapela et al., 2002; Santaclara, Cabado, & Vieites, 2006; Sotelo et al., 2001). However, some regulatory legislation allows the assignation of commercial labels at genus level, and this approach has not been dealt with up to date in the case of fish.

Also, the molecular techniques generally used are based on amplification of DNA by PCR and subsequent characterisation of PCR product by means of different techniques such as DNA sequencing, RFLP (Restriction Fragment Length Polymorphism) or SSCP (Single Strand Conformation Polymorphism) analyses. One alternative to these techniques is ELISA (Enzyme-Linked ImmunoSorbent Assay) associated to PCR. This approach has been used in some previous works such as the detection of allergenic substances (hazelnut) (Foetisch et al., 2011), detection of virus (Milne et al., 2007; Milne, Gallacher, Cash, & Porter, 2006) and even quantification

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(Musiani, Gallinella, Venturoli, & Zerbini, 2007) showing comparable results to RT-PCR (Perelle et al., 2004). In the particular case of fish and seafood, PCR–ELISA has not been used very often, and only few works have been published (Asensio, Gonzalez, Pavon, Garcia, & Martin, 2008; Asensio et al., 2004). However, due to the current accessibility of conventional PCR, added to the wide use of ELISA in many laboratories for several applications/purposes (detection of allergenic substances, diseases in animals, etc.) may promote that PCR–ELISA could be extended to other fields and applications, like the identification and authentication of fish species and fishing products.

Therefore, the aim of the present work is to develop several systems based on PCR–ELISA technique, that when used in a sequential way allows to: firstly, determine if a particular sample belongs to the *Thunnus* genus or to other taxonomic group. When this first test is positive (the sample belongs to *Thunnus*) some of the proposed systems allow the assignment of the sample to *Thunnus albacares*, *Thunnus alalunga* – *Thunnus orientalis* and *Thunnus thynnus*; and when it is negative, another system allows to determine if the sample is *Katsuwonus pelamis*. The methodology developed is adequate to discriminate the mentioned *Thunnus* species and *K. pelamis* from other scombroids, including those closely related with *Thunnus*, such as *Scomber*, *Auxis* or *Sarda*, in different types of processed products.

2. Materials and methods

2.1. Sample collection, DNA extraction and storage

Samples belonging to *Thunnus* genus and other Scombridae species were collected from several locations around the world by collaborators belonging to different research centres (Table 1) and were included in the IIM (*Instituto de Investigaciones Marinas*, Vigo Spain) tissue bank. When it was possible, the individuals were identified attending to morphological characters according to *FAO Species Catalogue* (Collette & Nauen, 1983). In other cases, the samples were authenticated in origin by collaborators.

DNA was extracted from 30 mg of muscle in fresh and frozen samples, and 200 mg in the case of processed samples, according to the method described by Blanco et al. (2008). The concentration and purity of the extracted DNA was measured using a *Nanodrop 2000* spectrophotometer (*Thermo Scientific*) at 260 nm and the ratio 260/280, respectively. Samples and DNA extractions were appropriately labelled and stored at -80 and -20 °C, respectively for subsequent studies.

Table 1
Samples included in this work.

Genus	Species	Common name
<i>Thunnus</i>	<i>T. alalunga</i>	Albacore
	<i>T. albacares</i>	Yellowfin tuna
	<i>T. atlanticus</i>	Blackfin tuna
	<i>T. maccoyii</i>	Southern bluefin tuna
	<i>T. obesus</i>	Bigeye tuna
	<i>T. orientalis</i>	Pacific bluefin tuna
	<i>T. thynnus</i>	Bluefin tuna
	<i>T. tonggol</i>	Longtail tuna
<i>Auxis</i>	<i>A. rochei</i>	Bullet tuna
	<i>A. thazard</i>	Frigate tuna
<i>Euthynnus</i>	<i>E. alletteratus</i>	Little tunny
<i>Katsuwonus</i>	<i>K. pelamis</i>	Skipjack tuna
<i>Rastrelliger</i>	<i>R. kanagurta</i>	Indian mackerel
<i>Sarda</i>	<i>S. chiliensis</i>	Pacific bonito
	<i>S. sarda</i>	Atlantic bonito
<i>Scomber</i>	<i>S. colias</i>	Atlantic chub mackerel
<i>Scomberomorus</i>	<i>S. sierra</i>	Serra spanish mackerel

2.2. Primers and probe design

The primers and the probes used in this work were designed from a set of sequences from NCBI (*National Center for Biotechnology Information*) (Accession Number: EU935746 – EU935790) and sequences available in the database of the research group. A multiple alignment was constructed from the sequences using BioEdit versión 7.0.0 (Hall, 1999), allowing the location of diverging regions between the *Thunnus* species, as well as the other sequences of scombroid included in the alignment, and several outgroups (specimens of *Genypterus*, *Epinephelus*, *Merluccius* and *Sardina* genera). These diverging regions were tested to design the primers and probe using the software *Primer Express 3.0* (*Applied Biosystems*). HPLC purification grade primers and probe herein designed were obtained from *Sigma Genosys*.

2.3. PCR amplification and DNA sequencing

In all cases PCR reactions were carried out in a total volume of 50 μ L with the following composition: 50 ng of template DNA were added to PCR mix consisting of 5 μ L of dNTP mix (*PCR DIG Labeling Mix*, *Roche*), 5 μ L of $10\times$ buffer (*Bioline*), 1.5 mM $MgCl_2$ (*Bioline*), 0.75 units of *BIOTAQ™* DNA Polymerase (*Bioline*), 0.8 μ M of each primer (*Sigma Genosys*) and molecular biology grade water (5 Prime) needed to adjust the final volume. The *PCR DIG Labeling Mix* is a mixture of the sodium salts of dATP, dCTP, dGTP, dTTP and digoxigenin-11-dUTP lithium salt.

Once verified the correct functioning of each PCR individually for the four designed systems, these ones were grouped in two duplex PCR. Several primer concentrations were tried, in order to obtain the same amplicon concentration in each duplex. The remaining conditions were the same used in simplex PCR.

PCR were carried out in a *GeneAmp®* PCR System 9700 (*Applied Biosystems*), in the following conditions: an initial step at 95 °C for 4 min, 35 cycles of amplification (95 °C for 15 s, 50–62 °C for 30 s, 72 °C for 45 s) and a final extension step of 72 °C for 3 min.

A positive control was also carried out in the same conditions, using the primers *FISH C+ F* and *FISH C+ R* (Santaclara, Espiñeira, Cabado, & Vieites, 2007) (Table 2).

PCR amplicons were visualised on 2% agarose gels (*Pronadisa*) in $0.5\times$ TBE buffer (*Sigma*) with *RedSafe™* 1 $\times$ (*iNtRON Biotechnology*) using the *Mini-Sub®* Cell GT system (*Bio-Rad*). DNA fragments were visualised using the *Gel Documentation System Gel Doc XR System* and the software *Quantity One®* v 4.5.2 (*Bio-Rad*). The *O'GeneRuler™* 100 bp DNA Ladder (*Fermentas*) was used to estimate the size of the amplicons.

The PCR products were purified using the *Nucleospin Extract II* kit (*Macherey-Nagel*) according to the manufacturer's instructions. The concentration and purity were estimated by means of a *Nano-Drop 2000 Spectrophotometer* (*Thermo Scientific*).

In order to verify the correct assignation of the specimens used in the set-up of the method, a new PCR product was amplified and sequenced on an *ABI Prism 310 Genetic Analyzer* (*Applied Biosystems*). This task was carried out using the primers L14735 (Burgener & Hübner, 1998) and *CytBI-3R* (Sevilla et al., 2007) and the *BigDye Terminator Cycle Sequencing Ready Reaction Kit v 1.1* (*Applied Biosystems*) following the supplier's recommendations. The electropherograms obtained were analysed using the *DNA Sequencing Analysis Software v 3.4* (*Applied Biosystems*) and manually corrected with *Chromas v 1.45* (McCarthy, 1996). The nucleotide sequences were compared with those present in the NCBI database using the tool *Megablast*.

2.4. Development of PCR–ELISA systems

DIG labelled PCR products previously amplified and purified were used in the ELISA assay. This step was carried out using the

Table 2

Primers and PCR–ELISA probes used in this work.

Name	Position in mitochondrial genome			PCR amplicon	Molecular marker	Sequence 5'–3'	Size (bp)	Annealing/Hybridisation temperature (°C)	Described
	Start	End	Strand						
<i>ThunSpp F</i>	15216	15234	+	<i>THUN 1</i>	<i>Cyt b</i>	CAC CGT TGT TAT TCA ACT A	121	58	In this work
<i>ThunSpp R</i>	15315	15336	–			ATC ATG CAG ARA TAT TAG AGG G			
<i>ThunSpp probe</i>	15263	15280	+			[Biotin] – AAC TCA CCC GCT ACT AAA			
<i>Thun2 F</i>	15541	15553	+	<i>THUN 2</i>	<i>Cyt b</i>	GCC GAG GAC TTT ACT ACG	145	58	In this work
<i>Thun2 R</i>	15668	15685	–			GTA AGG ACG GTA GCTCCT			
<i>ThunAlb probe</i>	15561	15581	+			[Biotin] – TCT TAC CTA TAC AAG GAA ACA			
<i>KatPel probe</i>	15581	15602	+			[Biotin] – TGT AGT CCT ACT TCT CCT AGT A	22	55	
<i>Thun3 F</i>	6760	6777	+	<i>THUN 3</i>	<i>COXI</i>	GGC ATC AGT TGA CCT AAC	152	58	In this work
<i>Thun3 R</i>	6888	6911	–			TAA TTA GTA CAG CTC ATA CAA ACA			
<i>ThunAla probe</i>	6863	6884	–			[Biotin] – GTG TTT GAT ATT GAG AGA TAG C			
<i>Thun4 F</i>	15340	15359	+	<i>THUN 4</i>	<i>Cyt b</i>	ACT TTG GCT CAC TAC TTG GC	78	58	In this work
<i>Thun4 R</i>	15396	15417	–			CAG GGG TAT AGT GTA TTG CGA G			
<i>ThunThy probe</i>	15366	15386	+			[Biotin] – CTT ATT TCT CAG ATC CTT ACA G			
<i>FISH C+ F</i>			+	<i>AMP-CONT.</i>	<i>12S rDNA</i>	CCY AGG GAT AAC AGC GCA ATC	153	63	Santaclara et al. (2007)
<i>FISH C+ R</i>			–			TCC GGT CTG AAC TCA GAT CAC			

kit PCR ELISA (DIG Detection) (Roche) that includes all the components needed, except the biotinylated probe: microplate coated with streptavidin, conjugate (antibody linked to the peroxidase enzyme), hybridisation buffer, wash buffer, denaturation solution, conjugate dilution buffer, ABTS buffer, amongst others.

ELISA was performed following the supplier's recommendations and it is described here: 50 ng of the DIG labelled amplicon (simplex PCR) or 100 ng (in the case of duplex PCR) were mixed with 20 µL of the denaturation solution in a 1.5 mL tube; this mixture was incubated in a *Thermomixer compact* (Eppendorf) at 25 °C for 10 min. Subsequently, 170 µL of the hybridisation solution containing the pertinent probe at 0.03 µM was added, and this solution was pipetted in the streptavidin coated well. The microplate was sealed with adhesive film to prevent the evaporation and concentration of the wells contents. The samples were incubated in a *VorTemp* (UniEquip) at different temperatures (Table 2), to regulate the stringency and 300 rpm of agitation for 3 h.

In the next step, the solution was removed from the wells by aspiration, and these ones were washed with 250 µL of *Wash solution* 5 times. Furthermore 200 µL of the conjugated *Anti-DIG-POD working solution* was added per well and incubated at 37 °C for 30 min in the dark on the *VorTemp* (UniEquip).

After this period, the contents of the wells were removed again and these ones were washed 5 times with *Wash solution*. Then, 200 µL of the *ABTS substrate solution* were added per well. The plate was incubated 30 min at 37 °C in the dark in the *VorTemp* (UniEquip), with 300 rpm shaker. When this time finished, a photometric measurement was carried out in a *SynergyMx* spectrophotometer (BioTek Instruments), at a wavelength of 405 nm.

2.5. Methodological validation of the PCR–ELISA

Individuals of the different species assigned on the basis of their morphological traits or previous genetic analyses were used to carry out the validation of the PCR–ELISA method. These reference individuals were used to prepare products with different thermal treatments to simulate the transformation process of commercial products. The most extreme treatment applied to the samples was the sterilisation in a steel retort at 115 °C for 50 min, with 1.2 bars of overpressure.

Products were analysed with the method developed in the present work in order to establish the exclusivity and inclusivity. The average number of samples analysed with each system was 26 for exclusivity and 15 for inclusivity. Results of the previous

species assignment on the basis of morphology were compared with those obtained by PCR–ELISA method.

Moreover, five samples containing diverse mixtures of the studied species were elaborated, in order to determine the detection limit and the ability of the method to detect mixtures (data not shown). The total amount of tissue included in these samples was 300 mg, and the complete method herein described was applied.

2.6. Application to commercial samples: market study

Once the methods were validated, they were applied to 11 commercial products labelled as tuna, bonito, mackerel and frigate tuna. These ones included salted, refrigerated, and canned products, and were purchased in supermarkets and shops from Spain, with the aim of evaluating their correct labelling.

3. Results and discussion

Most genetic identification techniques used for fish authentication in seafood products aim to identify fish at species level, since different species have different market value. In the particular case of the species of *Thunnus* genus, there has been a great variety of works dealing with the identification of these species (Bartlett & Davidson, 1991; Bottero, Dalmasso, Cappelletti, Secchi, & Civera, 2007; Espiñeira, Gonzalez-Lavin, Vieites, & Santaclara, 2009; Pardo & Perez-Villareal, 2004; Quinteiro et al., 1998; Terol, Mascarell, Fernandez-Pedrosa, & Perez-Alonso, 2002), that denotes the relevance of this taxonomic group. Amongst the techniques applied, the PCR technique being the most important, followed by different types of techniques: sequencing of amplicons (Vinas & Tudela, 2009), digestion with restriction enzymes (Lin & Hwang, 2007), real time PCR and melting curves (Terio et al., 2010), amongst others.

However, there has not been any published methodology focused on the identification at genus level to date, although different regulations consider the possibility of labelling the fish at genus level, adding the commercial name to the associated genera on the product label, such as *Thunnus* spp. Moreover, these regulations also provide specific commercial denominations for particular species, as for instance the Council Regulation (EEC) N° 1536/92 of 9 June 1992 (EEC, 1992). These matters mentioned above have been solved in this study, where a method for the authentication of *Thunnus* genus and several particular species is proposed, using a PCR–ELISA strategy.

3.1. DNA extraction

The methodology herein proposed starts with the DNA extraction from the sample. The method evaluated for this purpose worked correctly, both in fresh and in processed products. The quality and quantity of DNA obtained (evaluated by means of optical measurements) were optimal to successfully amplify the DNA by PCR in all cases. The spectrophotometric values obtained at 260 nm were higher than 30 ng/μL, and the 260/280 ratio was between 1.8 and 2.0. These values and ratios show a low amount of proteins and RNA in the DNA extracts.

3.2. Primers and probe design

The primer sets and probes herein designed (Table 2) amplify diverse mitochondrial regions: the 3' end of glutamine tRNA, several regions of the cytochrome *b* gene, and a fragment of the cytochrome oxidase subunit I (COXI). The position of these regions is shown in Fig. 1. The five biotinylated probes (*ThunSpp*, *ThunAlb*, *KatPel*, *ThunAla* and *ThunThy* probe) are internal to their corresponding amplicons (Fig. 1).

The selection of these mtDNA locations as target for the development of a PCR–ELISA methodology is based on the existence of an extensive number of published sequences, not only for this taxonomic group but also for other taxa. These mitochondrial regions have been reported as appropriate for identification of many different kind of organisms (Akasaki, Yanagimoto, Yamakami, Tomonaga, & Sato, 2006; Blanco et al., 2008; Kartavtsev et al., 2007; Lin, Shiau, & Hwang, 2005; McVeigh, Bartlett, & Davidson, 1991) due to its mutation rate, and it being a mitochondrial DNA marker, it is expected to a high number of copies per cell, making the PCR amplification easier.

The design of the primers took into consideration size restrictions, and all the amplicons herein designed are shorter than 155 bp (Table 2). Several authors have established that the maximum size of PCR products that can be amplified in canned products is around of 170 bp (Quinteiro et al., 1998; Santaclara, Espiñeira, et al., 2006; Santaclara, Espiñeira, & Vieites, 2007). With this design, the methodology proposed must be suitable to any kind

of seafood product, from thermally unprocessed fresh fish up to sterilised products like canned fish.

It was verified that the amplified fragments contain enough nucleotide information to allow the authentication of the *Thunnus* genus and particular tuna species regarding the rest of Scombridae species (data not shown).

3.3. PCR amplification and sequencing

The PCR–ELISA methodology presents as an important step the incorporation of a triphosphate nucleotide DIG labelled (*digoxigenin-11-dUTP*) during the PCR process, resulting in the labelling of amplicons which renders them suitable for subsequent ELISA detection.

The amplification conditions were adjusted in order to allow the correct amplification of scombroid species, while avoiding the amplification of other taxonomic groups. The parameter adjusted was the annealing temperature. Finally, this parameter was adjusted to 58 °C, since this allows the efficient amplification of the *Thunnus* species and *K. pelamis* in all cases. Each system was tested and optimised first independently.

With the aim of reducing the number of steps of the methodology, PCR amplification of the four designed systems were grouped in two duplex PCRs (THUN 1 – THUN 2 and THUN 3 – THUN 4). The conditions were the same used in simplex PCR, with the exception of the primer concentrations that were mixed in the ratio of 1:3 for THUN 1:THUN 2; and 3:1 for THUN 3:THUN 4, (1 meaning 10 pmol primer/25 μL PCR solution). In these conditions, similar concentrations of PCR products were obtained in both duplex PCR.

In the case of THUN 1-THUN 2 duplex PCR, a non-specific PCR product of ≈500 bp was obtained with fresh or frozen products, even with mild thermal treatments such as cooking. This unspecific PCR product originates from the primers *ThunSpp* F (position 15216 in complete mitochondrial genome) and *Thun* 2R (position 15668) (Table 2 and Fig. 1). These non-specific bands were eliminated by means of direct purification from agarose gels, cutting the target bands and rejecting the non-specific with the *Nucleospin Extract II* kit (Macherey-Nagel). In canned products this problem was not present, due to the fragmentation of DNA template previously mentioned that prevented this non desired amplification.

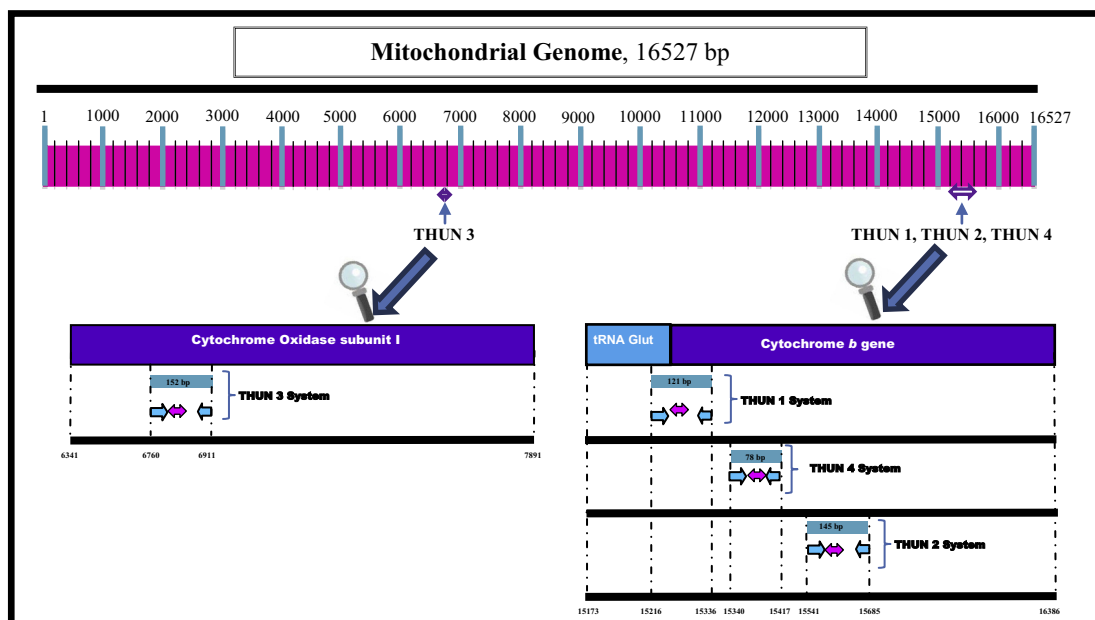


Fig. 1. Location and position of primers and probes designed in this work.

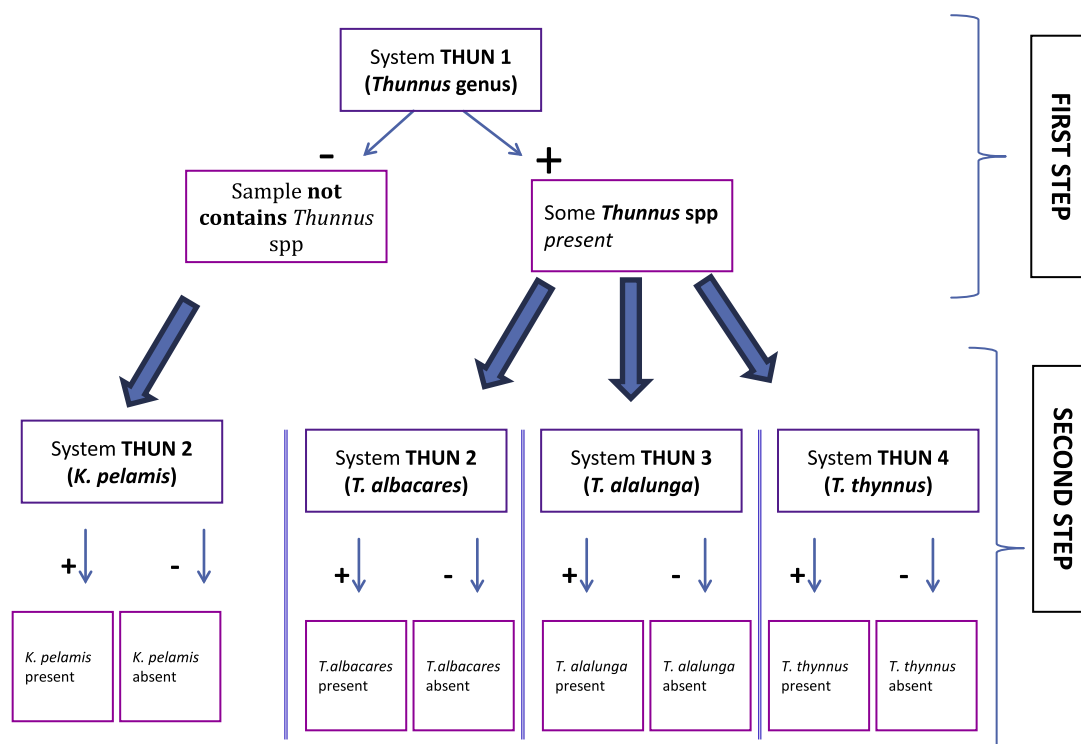


Fig. 2. Flowchart of the proposed method.

In the amplification conditions previously described, some of the Scombroids species evaluated in the set up process did not show amplification. For this reason the primer set *FISH C+* was used as amplification control in PCR, to avoid the occurrence of false negative amplifications due to problems associated with the samples such as presence of inhibitors, poor quality or fragmented DNA associated to transformation process. Primers for this control PCR are located in two highly conserved regions of the 12S rDNA, thus allowing amplification with all fish species in the established amplification conditions.

The main advantage of the duplex PCR strategy is the reduction of the number of PCRs that must be carried out, and it is especially justified when more than one sample or the complete scheme should be applied. The THUN 1 – THUN 2 duplex allows to detect the presence of the *Thunnus* genus; and in the case of a positive result, it also allows to verify if *T. albacares* (light tuna) is present; if negative (no *Thunnus* genus present) it is possible determine the presence of *K. pelamis* (tuna). So, this first duplex could be applicable to a large number of tuna samples, since the mentioned species are the most abundant in the market. Besides, the THUN 3-THUN 4 duplex allows to verify the presence of *T. alalunga* and *T. thynnus*, being these species of great added value and having their own commercial denomination in Spanish Legislation (“Atún Blanco” and “Atún Rojo”, respectively) (see Fig. 2).

Alternatively, the individual use of each particular system is also possible, allowing it to determine the presence of the genus and the four species studied.

Another option for the DIG labelling step is to use a DIG labelling primer, as the triphosphate nucleotide DIG label (*digoxigenin-11-dUTP*) is very expensive. For this alternative, it is only necessary to label the primer that extends the complementary strand of the probe, as the rest of the protocol is not variable. This option was not evaluated in this study.

The *Thunnus* specimens used in the set-up of the proposed method were sequenced to verify the specific assignation based on morphological traits, specifically a PCR product of 808 bp

(760 bp without primers). These sequences were submitted to NCBI database (Accession numbers: KC522352 to KC522359). Moreover a *Megablast* search was carried out in order to substantiate that these were correctly assigned to a particular species.

3.4. Development of PCR–ELISA methodology

Amplicons obtained from PCR can be characterised by means of multiple techniques, each of them with advantages and drawbacks. The technique with the highest informative power is nucleotide sequencing, since it allows the obtaining of the previously amplified complete sequence. However, this technique presents some important drawbacks such as the high cost of the equipment and reagents. Therefore, a wide variety of alternative techniques are used to characterise and determine the identity of PCR products, for instance RFLP, LP (Length Polymorphism), or SSCP.

The ELISA technique uses the capacity of the antibodies to recognise particular antigens, and these antibodies are used in some applications to detect for instance bacteria, virus and allergenic substances (Gutierrez et al., 1997; Holzhauser, Stephan, & Vieths, 2002; Musiani, Venturoli, Gallinella, & Zerbini, 2007). However, also it is possible to combine PCR and ELISA such as shown in previous works (Milne et al., 2007; Musiani, Gallinella, et al., 2007; Perelle, Dilasser, Grout, & Fach, 2007; Perelle et al., 2004), although few of them are focused on the field of fish and fishing products (Asensio et al., 2004), despite that the capacity of this technique seems adequate.

When the DIG-labelled PCR product is amplified and purified, this one can be used in the ELISA assay, which comprises the following steps: (1) hybridisation of the DIG labelled PCR product and the biotinylated probe; (2) binding of the hybridisation product to streptavidin coated microplate; (3) washing; (4) binding of antibody anti-DIG linked to enzyme labelled; (5) washing; (6) addition of specific substrate to the revealing enzyme; (7) evaluation of results.

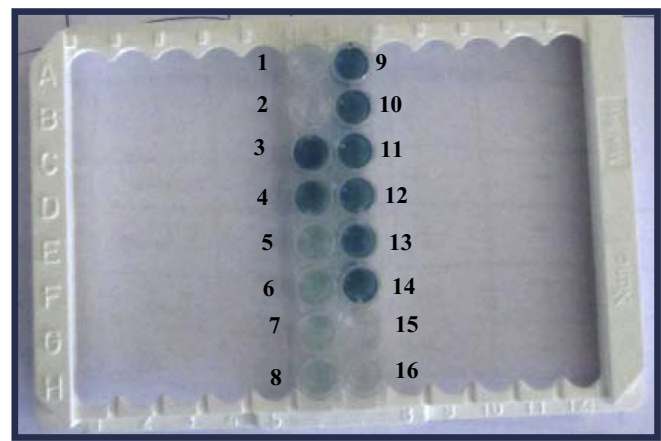


Fig. 3. ELISA microplate shown the results obtained for several *Scombridae* species previously amplified by PCR. In the enumerated wells DNA from the following species are present: (1) *Scomber colias*; (2) *Scomber scombrus*; (3) *Thunnus obesus*; (4) *T. albacares*; (5) *Katsuwonus pelamis*; (6) *Auxis rochei*; (7) *Auxis thazard*; (8) *Sarda chiliensis*; (9) *T. alalunga*; (10) *T. tonggol*; (11) *T. orientalis*; (12) *T. thynnus*; (13) *T. maccoyii*; (14) *T. atlanticus*; (15) PCR blank; (16) ELISA blank.

Probe hybridisation is the critical phase of ELISA. For this reason, several conditions were evaluated to establish those optimal that allow to obtain the highest signal while maintaining the specificity of the different designed probes. The remaining steps of the test were carried out following the supplier's recommendations. Regarding the hybridisation step, we have evaluated temperatures comprised between 50 and 58 °C to regulate the stringency and enhance the specificity of the designed probes with the amplicons of *Scombridae* species. The optimal hybridisation temperature is shown in Table 2. In these conditions, the specificity is complete: negative samples showed values lower than 0.1 AU and signal of positive samples is highest (values of absorbance higher than 1 AU) (Fig. 3). Higher temperatures produce the same specificity results, but there is an evident loss of absorbance measure. Another

parameter adjusted was the duration of hybridisation step, initially was set up for 3 h, but it was found that 2 h allowed to obtain practically the same signal of absorbance as with 3 h.

Moreover, once the hybridisation ends, it is very important to carry on with the assay without delay, removing the contents of the wells and starting the wash step quickly, since the contrary allows the decrease of the temperature thus facilitating the formation of non-specific hybridisations, and this fact generate certain signal intensity at the end of the assay, and in the worst scenario may generate doubtful results, or even false positives. For this reason, we recommend to analyse samples in batches of 3 ELISA strips (24 wells).

The signal intensities obtained with the positive samples analysed were in all cases at least sixfold higher compared with the negative samples. As a result of the former, the results reading of the proposed method can be carried out with the naked eye, without use the spectrophotometer (Fig. 3), since positive wells have a strong green colour, compared with the negatives, which have no colour or a hardly perceptible light green colour.

The only exception to the previous is the result obtained with *T. orientalis* and the system THUN 3. We have observed that the ThunAla probe generates almost half of the signal intensity of that obtained for the target species (*T. alalunga*), and therefore could generate doubtful results, which must be confirmed by means of alternative methods. The advantage is that the system works perfectly with *T. alalunga*, and the presence in the market of *T. orientalis* is low compared with the former species.

3.5. Quality controls of the assay

It is recommended to carry out some minimum quality controls with each assay to avoid incorrect results. We suggest analysing all samples in duplicate in the ELISA plate, as this allows detecting problems with the sample handled during the assay, using the standard deviation of the duplicates and the average standard deviation of the assay, as this allows the samples with incoherent duplicates to be rejected.

Moreover, the following controls must be carried out:

Table 3
All possible results obtained applying the complete methodological method herein proposed. Green colour represent positive result and red negative result in the PCR–ELISA test herein proposed.

PCR-ELISA RESULTS					ASSAY RESULT*
THUN 1	THUN 2		THUN 3	THUN 4	
	KatPel	ThuAlb			
					<i>Thunnus spp</i>
					<i>T. albacares</i>
					<i>T. alalunga</i>
					<i>T. thynnus</i>
					<i>Katsuwonus pelamis</i>
					<i>T. albacares</i> + <i>T. alalunga</i>
					<i>T. albacares</i> + <i>T. thynnus</i>
					<i>T. alalunga</i> + <i>T. thynnus</i>
					<i>T. albacares</i> + <i>T. alalunga</i> + <i>T. thynnus</i>
					<i>K. pelamis</i> + <i>T. albacares</i>
					<i>K. pelamis</i> + <i>T. alalunga</i>
					<i>K. pelamis</i> + <i>T. thynnus</i>
					<i>K. pelamis</i> + <i>T. albacares</i> + <i>T. alalunga</i>
					<i>K. pelamis</i> + <i>T. albacares</i> + <i>T. thynnus</i>
					<i>K. pelamis</i> + <i>T. alalunga</i> + <i>T. thynnus</i>
					<i>K. pelamis</i> + <i>T. albacares</i> + <i>T. alalunga</i> + <i>T. thynnus</i>

*When complex samples or unstructured products are analysed, it is not possible to discard the presence of the *Thunnus* species not included in the analytical scheme (*T. atlanticus*, *T. maccoyii*, *T. obesus*, and *T. tonggol*).

- (a) In the PCR: amplification control, positive control amplification of the potentially present species, and reagents blank.
- (b) In ELISA: blank of PCR, positive control of the potentially present species, and negative control with DNA from a closely related of that species.

3.6. Methodological validation of the PCR–ELISA

The results obtained from the PCR–ELISA assay applied to the samples manufactured in the *Marine Research Institute* were in accordance with the initial assignation. The assay showed 100% inclusivity and 100% exclusivity for the samples analysed from the reference individuals (data not shown). At the same time, it was checked that cooking and canning processes do not affect the correct performance of the developed method.

The fish mixtures elaborated in the laboratory were analysed and the results have confirmed the applicability of the method to complex samples and shows that target species can be detected when are present in very low quantities (down to 1%). Furthermore, in this validation experiment it was verified that the systems that integrate the method could be used independently with total reliability. Nevertheless, it is advisable that the complete protocol is applied to complex or unstructured products (i.e. those products which do not contain muscle structure), since they could contain more than one tuna species. In these cases, the positive result to a particular system does not imply that other species are present in a sample as it is shown in Table 3. For instance, when a slice of tuna is analysed the complete scheme is not necessary, but in canned tuna the complete method should be applied in order to discard the possibility of the presence of species mixture.

3.7. Application to commercial samples: market study

Once the methods overcame the validation step, these were applied to 11 commercial samples, allowing us to evaluate and determine a broad outline, the labelling situation of these products in the Spanish market. All of the samples analysed were identified as some species of those included in this work. Two analysed samples contained a different species of those indicated in the label, meaning an 18% of wrong labelling (one sample that contains *T. alalunga* and the label indicate *Sarda chiliensis*; and other that contains *T. obesus* and is labelled as *T. thynnus*). Moreover, fish mixtures were not detected in these samples, containing only one species in each product.

In conclusion, the technique described in this paper is a useful tool to authenticate the species belonging to the *Thunnus* genus and *K. pelamis* in all kind of products, from fresh fish to processed products. It can be useful to verify the correct labelling of fishing products elaborated with the species labelled as tuna, since these species must belong to the *Thunnus* genus. Other remarkable advantages of the proposed method are that the analysis can be carried out in one working day, and it allows to be used as a high throughput screening for all kind of samples with total reliability. Moreover, amongst the results of this work stand out the applicability of the PCR–ELISA in the identification and authentication of fish species, and shows this technique as a powerful methodological alternative, with great potential of utilisation in many laboratories, since most of them have the equipment and qualified personal needed to carry out the ELISA test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2014.11.076>.

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