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Application of Relative Quantification TaqMan Real-Time Polymerase Chain Reaction Technology for the Identification and Quantification of *Thunnus alalunga* and *Thunnus albacares*

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A novel one-step methodology based on real-time Polymerase Chain Reaction (PCR) technology has been developed for the identification of two of the most valuable tuna species. Nowadays, species identification of seafood products has a major concern due to the importing to Europe of new species from other countries. To achieve this aim, two specific TaqMan systems were devised to identify *Thunnus alalunga* and *Thunnus albacares*. Another system specific to Scombroidei species was devised as a consensus system. In addition, a relative quantification methodology was carried out to quantify *T. alalunga* and *T. albacares* in mixtures after the relative amount of the target was compared with the consensus. This relative quantification methodology does not require a known amount of standard, allowing the analysis of many more samples together and saving costs and time. The utilization of real-time PCR does not require sample handling, preventing contamination and resulting in much faster and higher throughput results.

KEYWORDS: Species identification; real-time PCR; DNA; *Thunnus albacares*; *Thunnus alalunga*; canned tuna

INTRODUCTION

During the past years fish species identification has been an important concern in order to label correctly the seafood products. Moreover, an increase in the consumption of fish has facilitated the importation of new species from other countries. In the case of Spain, the commercialization of Scombroidei species from South American countries has currently increased considerably. The problem appears when these species are fraudulently labeled as the most valuable tuna species. According to the European Union labeling legislation (EU Regulation 1536/92) products labeled as *white tuna* can include only *Thunnus alalunga* (albacore), whereas those products labeled as *light tuna* must contain *Thunnus albacares* (yellowfin).

When the external morphological characteristics of the fish are removed due to filleting or processing such as canning, the only possibility to authenticate the food product is by means of using a molecular marker. There are two big groups of molecular markers that have been extensively used during recent years: proteins and deoxyribonucleic acid (DNA). Protein analysis techniques are based on its physicochemical differences (*1*), whereas DNA techniques consist in the detection of any nucleotide variation within sequence (*2*). Nonetheless, when the product to be authenticated is highly processed (like canned tuna), the adequacy of each marker is critical. The utilization of electrophoretic analyses of proteins extracted from canned samples is unsuitable because of the irreversible changes on water solubility during the thermal treatment (*3*). However, the DNA molecule appears to be much more stable to thermal treatment than others. In fact, the use of DNA as a molecular marker has shown itself to be the most powerful tool for species identification (*4*).

According to DNA analysis of canned tuna, many works have described different techniques based on restriction site polymorphic fragments (RFLP) (*5*−*7*), forensically informative nucleotide sequencing (FINS) (*6*, *8*), and others (*9*−*11*) with the aim of identifying Scombroidei species. However, these techniques are not able to detect reliably a specific species in a mixture, which is widespread in canning. Real-time Polymerase Chain Reaction (RT-PCR) technology is based on the detection and quantification by a high-quality optical detection instrument of a fluorescence reporter included within a specific fluorogenic probe. This methodology is routinely used for the quantification of genetically modified organisms (GMOs) in food (*12*). Moreover, recently RT-PCR has also been used for the identification of species such as beef (*13*), peanut (*14*), and gadoids (*4*, *15*) and even for the semiquantification of beef in food (*13*).

The main aim of this study was to achieve two relative quantification procedures to identify and quantify two of the most valuable commercial Scombroidei species from the canning industry. To our knowledge, this system is the first method capable of detecting and quantifying tuna species by RT-PCR.

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Table 1. Scombroidei Species Collected during This Study

scientific name	code	common name	source	N[a]
Euthynnus alleteratus	LTA	little tunny	IIM-CSIC	3
Katsuwonus pelamis	SKJ	skipjack	local market	5
Sarda sarda	BON	Atlantic bonito	IIM-CSIC	3
Scomber japonicus	MAS	chub mackerel	IIM-CSIC	3
Thunnus alalunga	ALB	albacore	local market	5
Thunnus albacares	YFT	yellowfin tuna	local market	5
Thunnus obesus	BET	bigeye tuna	local market	5
Thunnus thynnus	BFT	bluefin tuna	local market	1

[a] *N* = number of individuals.

Table 2. Primers and TaqMan Probes

name	sequence (5′–3′)
primers	
L-YFT	5′-CGAGGCCTTTACTACGGCTCTT-3′
H-YFT	5′-CGGTCATCATAACTAGGAGTAGGAGTAC-3′
L-ALB	5′-GCCTCTTTCTTCTTTATCTGCATCTAC-3′
H-ALB	5′-TACTCCGATGTTTCATGTTTCTTTG-3′
L-16SCOM	5′-CTCGATGTTGGATCAGGACATC-3′
H-16SCOM	5′-TCTGAACTCAGATCACGTAGGACTTTA-3′
TaqMan MGB probes	
YFT	5′-(FAM)-CCTATACAAGGAAACATGAAA-3′
16SCOM	5′-(FAM)-TTGAACAAACGAACCCTTAA-3′
TaqMan probe	
ALB	5′-(FAM)-TCCACATCGCCGAGGCCTTTACTA-(TAMRA)-3′

MATERIALS AND METHODS

Sample Preparation. The *Scombroidei* specimens were obtained from a local market or provided by the tissue collection belonging to the Seafood Biochemistry Group from Instituto de Investigaciones Marinas, CSIC (IIM-CSIC) (Vigo, Spain). Those individuals collected from the local market were morphologically identified by external characteristics. Aliquots of light muscle of each specimen were stored at −80 °C in 96% (v/v) ethanol. Thirty individuals of species from the Scombroidei suborder are listed in **Table 1**.

To prepare the canned samples, the fish was gutted, and the obtained chunks (diameter = 60 cm, height = 30 cm) were steam-cooked (102−103 °C) for 45 min in brine. Then, after placing the light muscle in the cans, vegetable oil was added. The cans were sterilized at 110 °C for 60 min. Tuna mixed samples were prepared by homogenizing 50 g of white muscle with a blender.

To validate the relative quantitative technique developed in this study, 20 different commercial canned tunas were purchased at the local market.

DNA Extraction. For DNA isolation two different methods were carried out. The reference fish species samples were treated as previously described (*7*). The DNA from tuna canned samples was extracted from 0.25 g of tissue with an ABI PRISM 6100 Nucleic Acid PrepStation following NucPrep Chemistry: isolation of genomic DNA from animal and plant tissue protocol (Applied Biosystems, Foster City, CA). DNA concentrations were measured by absorbance at 260 nm.

TaqMan Probes and Primer Design. All of the primers and fluorogenic probes were designed using the Primer Express v. 2.0 software (Applied Biosystems) following the information provided. Primers and TaqMan probes were chosen to be consistent with the alignment of mitochondrial sequences collected from GenBank and by Pardo and Pérez-Villareal (*7*). The sequences of primers and probes are listed in **Table 2**. The probes were labeled on the 5′-end with the fluorescent reporter dye 6-carboxyfluorescein (FAM). On the 3′-end, two probes (YFT and 16SCOM) were labeled with a nonfluorescent quencher and a minor groove binder (MGB), whereas the ALB probe was labeled with a fluorescent quencher, 6-carboxytetramethylrhodamine (TAMRA), at the 3′-end. All of the primers and probes were purchased from Applied Biosystems.

Multiple alignments were carried out using the Clustal X program (*16*).

Real-Time PCR Conditions. Amplification was performed using a total reaction volume of 25 μL in a MicroAmp Optical 96-well reaction plate (Applied Biosystems). Real-time reactions were carried out with TaqMan Universal Master Mix (Applied Biosystems) containing the primers and probes described in **Table 2**. Reactions were run on the ABI Prism 7000 sequence detection system (Applied Biosystems) with the following thermal conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

PCR efficiency was calculated, from each linear regression of standard curves, using the equation $[10^{1/\text{slope}} - 1] \times 100$.

Relative Quantification Method. This method uses an unknown amount of standard, but it compares the relative amount of the target sequence to any of the reference values. The target and endogenous control amplifications were carried out in separate tubes in triplicate. Finally, for each reaction, a C_t variation ($\Delta C_t = C_t^{\text{target}} - C_t^{\text{ref}}$) for each target was calculated from the C_t values. At the end, each ΔC_t value was transformed to a percentage ($\%^{\text{target}} = 2^{-\Delta C_t} \times 100$).

Statistical Analysis. Linearity tests were developed three times with different individuals. Each C_t value was obtained by the means of three replicates with a standard deviation of <0.2.

RESULTS AND DISCUSSION

Primer and Probe Design. DNA sequence information for the Scombroidei is focused on mitochondrial genome. Actually, most of the studies regarding authentication of tuna are based on the analysis of a genetic marker obtained by PCR from mitochondrial DNA (*6*−*8*). This genome is a robust tool to identify the presence of specific species in a mixed sample. Nevertheless, it is not useful for absolute quantification because it requires a feasible DNA extraction, as well as the assumption of a similar number of genomes per mass of muscle tissue for different species. Mitochondrial volume density varies depending on the muscle tissue and the species (*17*). As a result, in absolute quantification studies nuclear genes are preferred rather than mitochondrial genes. Due to the scarcity of nuclear sequence information available for Scombroidei, we had to develop another strategy to deal with our aim. A relative quantification strategy allows working with mitochondrial genes estimating the relative presence of a particular mitochondrial gene target with the consensus gene. In a similar way, Brodmann and Moor (*13*) devised a system to quantify beef content using a mammalian system as housekeeping gene. In addition, relative quantification eliminates the standard curves, facilitating the analysis of results and increasing the number of samples per plate.

Ribosomal RNA ($_r$RNA) genes are widely used for phylogenetic analysis (*18*). Along with mitochondrial $_r$RNA genes, 12S has been extensively used to study phylogenetic relationships in vertebrates (*19*, *20*). Regarding fish, several works have described different genetic techniques to differentiate flatfish (*21*, *22*), gadoids (*23*, *24*), and tunas (*25*). Among mitochondrial ribosomal genes, the 16S $_r$RNA gene is the most conserved (*24*). For that reason, published mitochondrial DNA sequences of the 16S $_r$RNA gene of species that belong to the suborder Scombroidei were aligned to design a specific system. The aligned sequences were *Auxis rochei* (NC_005313, AB105165, AB103467, and AB103468), *Auxis thazard* (AB105447 and NC_005318), *Euthynnus alleteratus* (AB099716 and NC_004530), *Katsuwonus pelamis* (NC_005316 and AB101290), *Lepidopus caudatus* (AF100917, AF100918, AF100919, and AF100920), *Lepturacanthus* sp. (AB125749), *Rexea solandri* (AF221898), *Scomber australasicus* (AB032522), *Scomber japonicus* (AB032521), *Scomber scombrus* (AF055615 and AY048303), *Scomberomorus tritor* (AF231539), *Thunnus alalunga* (NC_005317 and AB101291), *Thunnus thynnus* (NC_004901, AY302574, and AB097669), *Trichiurus japoncusi* (AB112550),

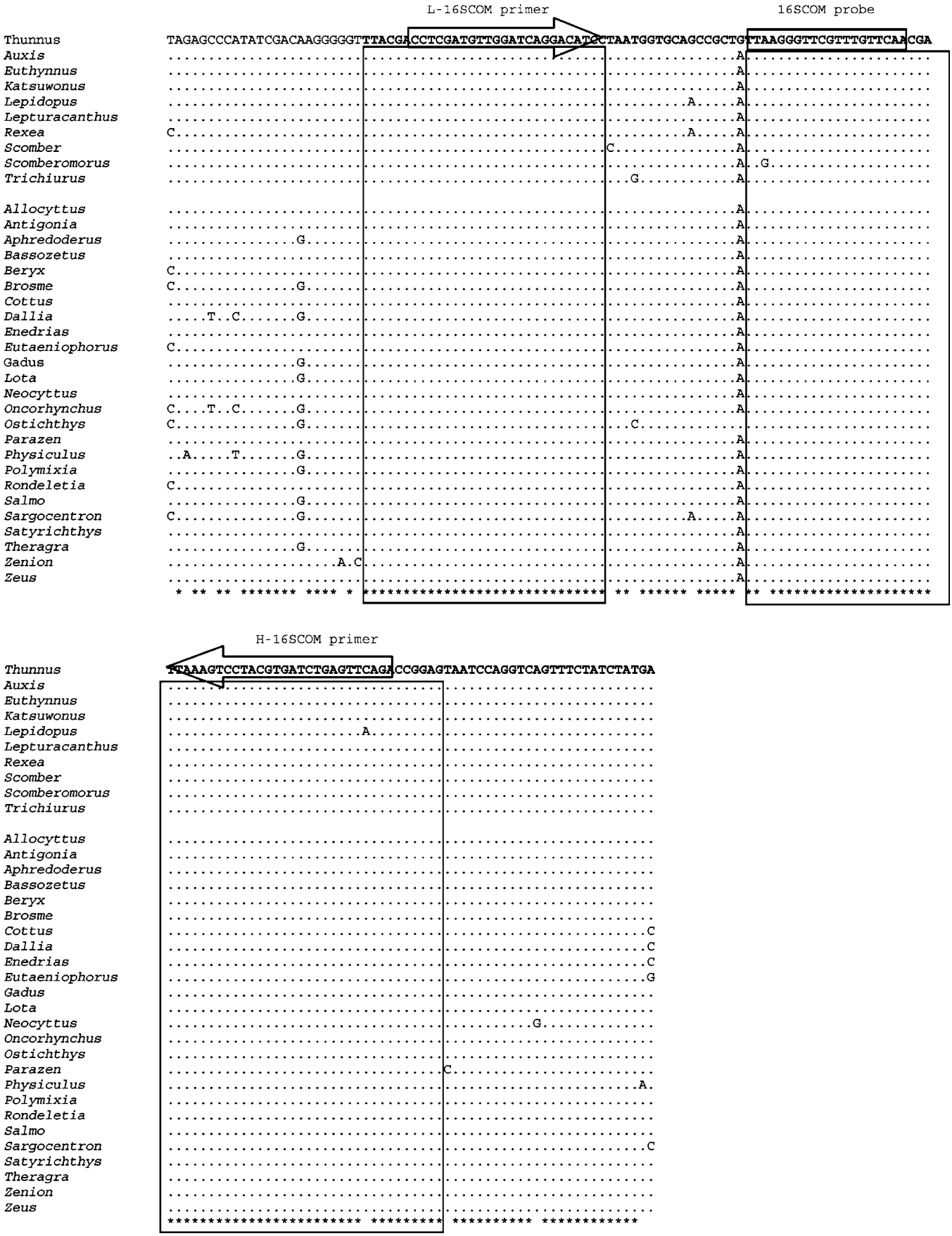

Figure 1. Comparison of primers and probe of the Scombroidei detection TaqMan system (16SCOM) with different fish genera including the alignment with the genus of Scombroidei (first block). The 130 bp consensus region is in bold.

and *Trichiurus lepturus* (AY216492, AY216493, and AY216494). As a consequence of this alignment, a highly conserved region of 130 basepairs was found at position 1520, and two conserved primers and a specific probe were devised (**Figure 1**). The

Table 3. Published Mitochondrial DNA Sequences of 16S $_r$RNA Gene of Species Belonging to 10 Fish Orders

order	species	GenBank accession no.
Berciformes	*Beryx decadactylus*	NC_004393
Berciformes	*Beryx splendens*	NC_003188
Berciformes	*Ostichthys japonicus*	NC_004394
Berciformes	*Sargocentron rubrum*	NC_004395
Escociformes	*Dallia pectoralis*	NC_004592
Gadiformes	*Brosme brosme*	NC_004404
Gadiformes	*Caelorinchus kishinouyei*	NC_003169
Gadiformes	*Gadus morhua*	NC_002081
Gadiformes	*Lota lota*	NC_004379
Gadiformes	*Melanonus zugmayeri*	NC_004378
Gadiformes	*Physiculus japonicus*	NC_004377
Gadiformes	*Theragra chalcogramma*	NC_004449
Ophidiiforrmes	*Bassozetus zenkevitchi*	NC_004374
Perciformes	*Antigonia capros*	NC_003191
Perciformes	*Enedrias crassispina*	NC_004410
Percopsiformes	*Aphredoderus sayanus*	NC_004372
Polymixiiformes	*Polymixia japonica*	NC_002648
Salmoniformes	*Oncorhynchus mykiss*	NC_001717
Salmoniformes	*Salmo salar*	NC_001960
Salmoniformes	*Salvelinus alpinus*	NC_004395
Scorpaeniformes	*Cottus reinii*	NC_004404
Scorpaeniformes	*Satyrichthys amiscus*	NC_004403
Stephanoberyciformes	*Eutaeniophorus* sp.	NC_004390
Stephanoberyciformes	*Rondeletia loricata*	NC_003186
Zeiformes	*Allocyttus niger*	NC_004398
Zeiformes	*Neocyttus rhomboidalis*	NC_004399
Zeiformes	*Parazen pacificus*	NC_004396
Zeiformes	*Zenion japonicum*	NC_004397
Zeiformes	*Zeus faber*	NC_003190

calculated homologies of the chosen sequences were 100% except for *Scomberomorus* and *Lepidopus* genera at 98.8%.

Although this study was focused on tuna species, the Scombroidei conserved region was aligned with other fish sequences belonging to other taxonomic orders (**Table 3**) to investigate the possibility of applying this system in other fish (**Figure 1**). Wang et al. (*20*) reported a pair of primers based on conserved regions of $_t$RNA^{PHE} and 16S $_r$RNA of the mitochondrial genome to amplify a fragment from major lineages of vertebrates. Further investigations could confirm the role of the Scombroidei consensus region described in this work as a target to design universal primers. Actually, the consensus region is maintained constant after alignment of 27 different species of 10 fish orders.

On the other hand, this consensus system could be also used to determine the mitochondrial copy number in a specific tissue. To date, this estimation has been performed following a complex methodology based on a quantitative competitive reaction (*17*).

As stated above, mitochondrial genes have been extensively used to identify tuna and another type of fish (*6*−*11*). Mitochondrial sequences from the cytochrome *b* gene compiled by Pardo and Pérez-Villareal (*7*) were realigned to design primers and specific probes of *T. alalunga* (17 sequences were analyzed) and *T. albacares* (44 sequences were analyzed). To obtain a specific and reliable TaqMan methodology, each polymorphic site was placed for each tuna in the middle of the probe (*7*). The primers that flank each target were located close to the probe by means of forcing the Primer Express program. In this way, we have developed two specific detection systems to detect *T. alalunga* and *T. albacares*.

Real-Time System Setup. Three specific TaqMan systems were developed to identify and quantify two tuna species. To optimize them, it was absolutely necessary to establish the reaction conditions by setting up the concentration of the pair of primers for each probe in the reaction mix. Balanced melting temperatures (T_m) of primers (forward and reverse) give an efficient amplification. Although T_m values of every pair of designed primers were balanced between 58 and 60 °C, an optimization for every system was achieved because the theoretical T_m is not always accurate. Primer optimization leads to imbalance between the two primers, allowing us to compensate for this difference. For this reason, a primer matrix (from 100 to 900 nM) was built using a fixed amount of target template and probe. Optimal reaction was achieved by selecting the primer concentrations that provide the lowest C_t and highest fluorescence. No imbalance was detected, so it was not necessary to adjust the forward and reverse primer concentrations of every system. The optimal concentrations calculated for ALB, YFT, and 16SCOM pairs of primers were 300, 900, and 300 nM, respectively.

On the other hand, different probe concentrations were assayed, and the optimal probe concentration resulting was between 150 and 250 nM. Finally, we decided to work at 250 nM because a high fluorescence leads to a high feasibility, in particular, with a low number of gene copies.

Selectivity of Systems in Scombroidei Species. Detection systems were tested for their selectivity and cross-reactions with those Scombroidei species listed in **Table 1**.

The Scombroidei system used as endogenous gene was applicable to every species listed in this work, whereby the detected C_t values were between 35 and 40 cycles.

Neither the ALB system nor the YFT system has detected cross-reactivity with other related species, although a slight signal was revealed by some individuals from *Thunnus* genus species. This fact was negligible compared to the strong signals measured for ALB and YFT individuals.

We concluded that the systems are specific to their targets. Moreover, in accordance with the alignment described above and some preliminary tests with other fish species from gadoid species (data not shown), the Scombroidei system could be used as mitochondrial reference gene to carry out relative quantification methods with other fish species.

Relative Quantification. Sensitivity and Linearity. The relative quantification method does not use a known amount of standard but compares the relative amount of the target sequence to any of the reference values. Thus, we have designed two relative quantification methods (ALB and YFT methods) to detect and quantify two tuna species by comparing the relative amount of the target sequence (ALB and YFT systems) with a reference (16SCOM system). At the end of each reaction, we obtained two C_t values for each method: one for the target and another one for the reference. After the relative quantification equations, described under Materials and Methods, had been applied, a C_t variation that could be transformed to a percentage was obtained. To apply correctly these equations, it was necessary to validate the detection methods through testing of the linearity and efficiency of every TaqMan system involved in the methodology.

To test the linearity, the resulting C_t values were plotted versus the logarithmic of the DNA concentration. **Figures 2** and **3** show the linearity test with intact DNA obtained from frozen samples and degraded DNA obtained from sterilized samples, respectively. The sensitivity range of systems at every method was determined by using 10-fold dilutions of DNA template, and the linearity was maintained from 250 to 0.25 ng of template (3 orders of magnitude). Brodmann and Moor (*13*) described the detection limit in canned meat as being ∼10 times

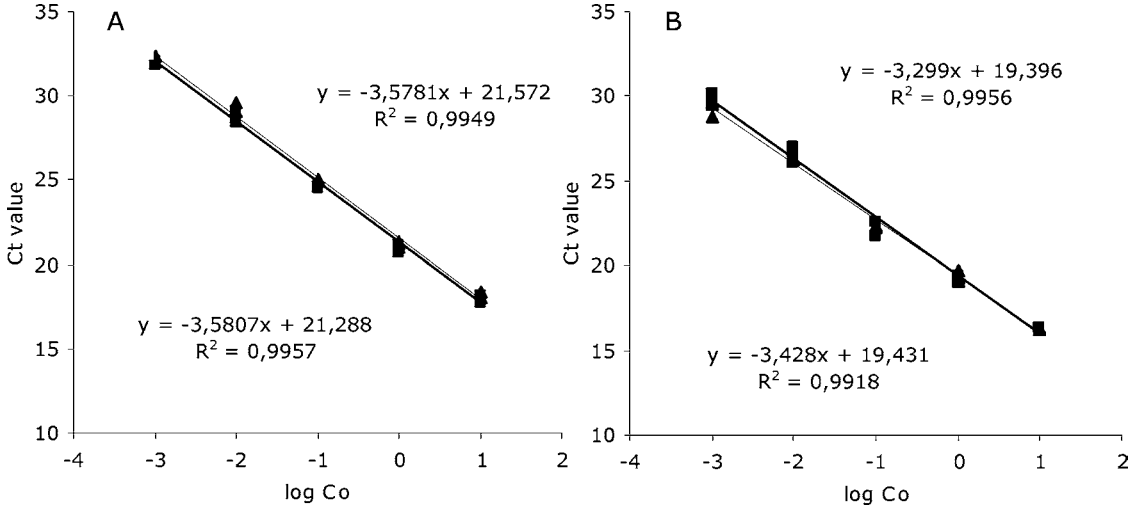

Figure 2. Linearity test with intact DNA as template: (**A**) ALB (▲) and 16SCOM (■) specific TaqMan systems belong to the *T. alalunga* specific quantification method using DNA from *T. alalunga* as template; (**B**) YFT (▲) and 16SCOM (■) specific TaqMan systems belong to the *T. albacares* specific quantification method using DNA from *T. albacares* as template. C_t values are plotted versus the logarithm of the DNA concentration.

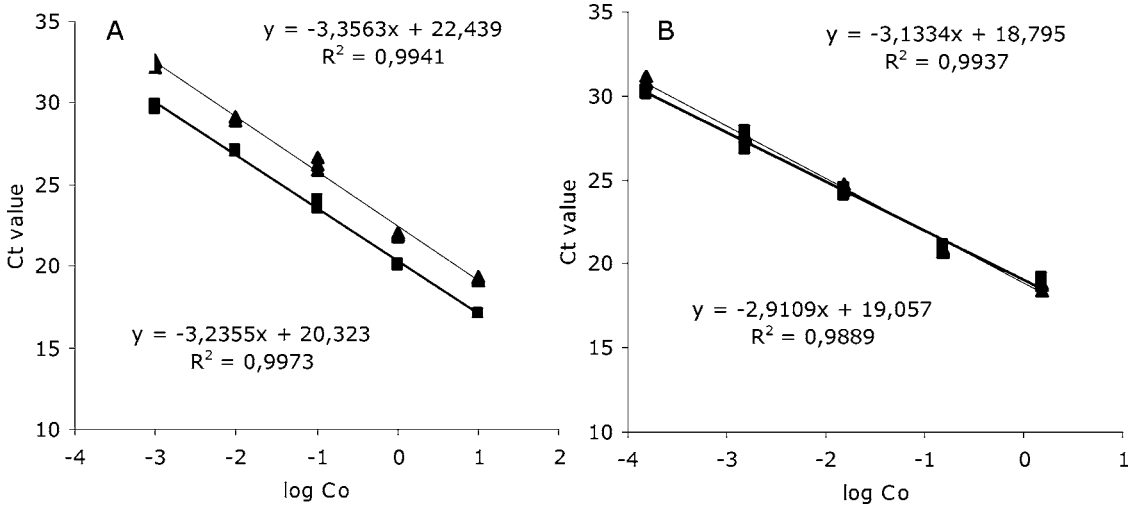

Figure 3. Linearity test with degraded DNA as template: (**A**) ALB (▲) and 16SCOM (■) specific TaqMan systems belong to the *T. alalunga* specific quantification method using DNA from *T. alalunga* as template; (**B**) YFT (▲) and 16SCOM (■) specific TaqMan systems belong to the *T. albacares* specific quantification method using DNA from *T. albacares* as template. C_t values are plotted versus the logarithm of the DNA concentration.

lower than that in raw meat. No significant variation in the detection limit between canned and frozen fish was detected.

A PCR efficiency of 100% is ideally achieved when the slopes are close to the theoretical value of −3.32. According to the slopes shown in **Figures 2** and **3**, an efficiency of nearly 100% was estimated for the YFT and ALB detection methods. In fact, and according to the equation $[10^{1/\text{slope}} - 1] \times 100$, the calculated efficiency ranged from 90 to 100%. Furthermore, the slopes from the target and endogenous genes of the detection method should be identical. Otherwise, a normalization factor should be added to the equations to work in reliable conditions. According to **Figures 2** and **3**, both methods presented very similar target and endogenous slopes, so it was not necessary to apply for normalization.

Nevertheless, some differences were detected in the *y*-axis of the regression lines calculated for different DNA origins with the 16SCOM system. This different behavior of the 16SCOM system in relation to the DNA origin (*T. alalunga* or *T. albacares*) is caused by the different number of mitochondrial genomes per mass of tissue to different species (*17*). This behavior introduces a deviation factor that cannot be minimized because it is inherent to the origin of the template. In addition, the standard curves with degraded template showed the same behavior. Additionally, another effect on the regression lines obtained with degraded DNA was also observed. **Figure 3** shows that the regression lines cross the *y*-axis at different C_t values, indicating different behaviors of both specific TaqMan systems at the same template concentration. This means that the degradation rate of DNA affects the C_t value. After several experiments with three individual samples of albacore and yellowfin, we estimated variation ratios of the C_t value of 1.57 ± 0.46 and 0.63 ± 0.52, respectively, introducing an error factor in the relative quantification estimation that will be discussed in the next section.

On the other hand, the presence of inhibitors of PCR in seafood products is very common (*5*). As a matter of fact, it is uncertain whether the lack of amplication was caused by inhibition or by the absence of target in the sample. In the case of no amplification, the 16SCOM system plays a role as internal control to detect the presence of inhibitors. In addition, the real-time methodology was validated using 20 commercial samples with optimal results in all cases.

Relative Quantification Assays with Binary Mixtures. One of the main problems to be solved was the fact that canned

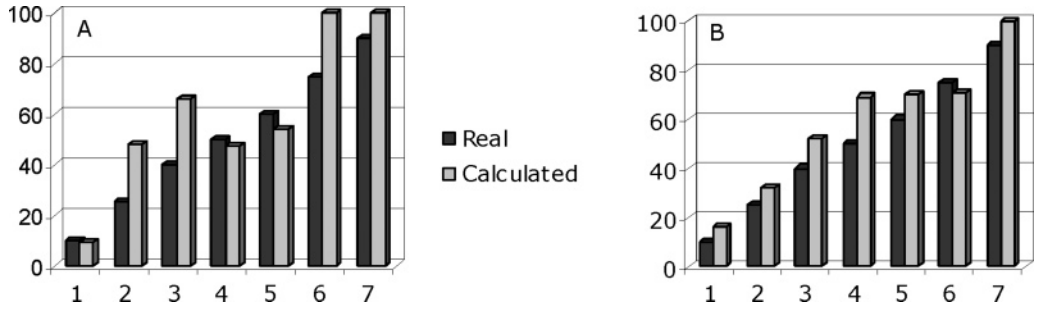

Figure 4. Relative quantification (percent) of binary mixtures of albacore and yellowfin frozen samples: (**A**) percentage of yellowfin calculated with the YFT method; (**B**) percentage of albacore calculated with the ALB method. 1, 10%; 2, 25%; 3, 40%; 4, 50%; 5, 60%; 6, 75%; 7, 90%.

samples presented high levels of DNA degradation because sterilization of the cans reduces the PCR signal due to DNA fragmentation. To solve this problem, different authors have developed canned tuna identification methods by shortening the amplified genetic marker (*6*, *8*) or increasing the efficiency by using nested primers (*7*). However, to our knowledge, there are no methodologies to quantify tuna species in mixtures.

According to quantification methodology, the results are strongly conditioned by the DNA fragmentation. In fact, the estimation of gene copy number in processed food may be lower due to degradation of template. With regard to canned tuna, the size of most of the fragments obtained from canned tuna was smaller than 200 bp (*6*). For that reason, the three TaqMan systems developed in this study had targets no longer than 100 bp. In this way, we tried to minimize the fragmentation influence of the target sequences in the quantification results. Moreover, the employment of MGB probes allowed the use of shorter probes by increasing the T_m, which simplifies the system design in the short target sequences usual in canned tuna samples as previously remarked.

On the other hand, the extractability of DNA plays a critical role in the absolute quantification because it is dependent on the mixture composition (*26*). However, this extractability factor has no effect on relative quantification strategy because it does not require standard curves.

Both methods (ALB and YFT) were tested in binary mixtures of *T. albacares* and *T. alalunga*. **Figure 4** shows the correlation between the real and calculated percentages from binary mixtures. A deviation was detected (ranging from 0 to 25%) because of the variation factor introduced by the different numbers of mitochondrial genomes per mass of tissue for different species as we previously discussed. In any case, taking into account that the correlation was made from mixtures of tissue, these results can be considered as promising as they clearly show a significant improvement compared to previous work (*4*). Indeed, Sotelo et al. (*4*) obtained an error ranging up to 50% in binary mixtures of DNA from cod and hake by using DNA absolute standard curves.

In addition to this, binary mixtures of sterilized tissue of albacore and yellowfin (simulating the canning process) were tested with both methods. The error obtained after these experiments ranged up to 50% (data not shown). This error was due to the degradation of DNA that exerts an influence in the calculation of C_t values and therefore in the relative estimation in a mixture. These results are in good agreement with those previously achieved in the linearity test of degraded DNA. However, when a mixture of sterilized tissue of albacore was assayed, a cross-reaction with yellowfin was not detected and vice versa. Thus, the real-time methodology described here is suitable to detect the fraudulent presence of yellowfin or even to identify the absence of albacore in cans labeled as white tuna.

Conclusions. Real-time PCR does not require post-PCR sample handling, thus preventing contamination and resulting in much faster and higher throughput assays. The methodology described here is a one-step protocol because the analysis is carried out during the amplification reaction. The relative quantification does not require precise quantification of DNA due to the absence of standard curves, allowing more samples to be analyzed per plate, saving costs.

Both methods allow the detection and quantification of albacore and yellowfin in frozen mixtures. In addition, they are also suitable to detect the presence or absence of these species in canned samples labeled as white tuna or light tuna, respectively.

Future research should be focused on testing these methods with not highly processed food samples, where the degradation of DNA is not so critical. New specific probes to less valuable tuna species that could be fraudulently used in the canning industry should also be devised.

The one-step methodology presented here allows the analysis of a considerable number of samples together. This is indeed a step forward in the identification of tuna species for the canning industry and food control laboratories.

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