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Evaluation of a Real-Time Polymerase Chain Reaction (PCR) Assay for Detection of *Anisakis simplex* Parasite as a Food-Borne Allergen Source in Seafood Products

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Anisakis simplex has been recognized as an important cause of disease in humans and as a food-borne allergen source. Actually, this food-borne parasite was recently identified as an emerging food safety risk. An *A. simplex*-specific primer-probe system based on a real-time polymerase chain reaction (PCR) detection assay has been successfully optimized and validated with seafood samples. In addition, a DNA extraction procedure has been optimized to detect the presence of the nematode in food samples. The assay is a very reliable, specific, and sensitive methodology to detect the presence of traces of this parasite in seafood products, including highly processed samples. As a result, 13 sequences of cytochrome *c* oxidase II gene were obtained and scrutinized to calculate intra- and interspecific variabilities of 0 and 35−67%, respectively. Finally, an efficiency of 2.07 ± 0.14 of the assay was calculated, and a limit of detection of 40 ppm parasite in 25 g of sample was also optimized. Actually, the presence of this parasite in several seafood products has been demonstrated, enforcing the necessity of a design for a good manufacturing practice protocol for the processing industry to minimize the presence of this parasite as a food-borne allergen source in seafood products.

KEYWORDS: *Anisakis simplex*; real-time PCR; safety; allergens; seafood

INTRODUCTION

Anisakis simplex is a parasite of marine mammals, fish, and cephalopods at its different larvae stages. This parasite is a worldwide distributed nematode located almost everywhere, and its infection is indeed frequent in many fish species, including scombriforms, gadiforms, perciforms, clupeiforms, etc. Taking into account that important commercial fish species are included in these groups (tuna, cod, anchovy, herring, hake, and others), its prevalence in seafood products is very common (*1*). In fact, over the past few years, *A. simplex* has been recognized as an important cause of disease in humans as well as an infective pathogen and a food-borne allergen source (*2*). Actually, this food-borne parasite was recently identified as an emerging food safety risk, and an increase in the number of notifications has been detected over the past few years (*3*). When humans eat infected fish, either raw or inadequately cooked, the nematode may enter the tissue of the gastrointestinal tract and then cause different problems associated with gastric and abdominal infections and allergic reactions (*1*). In addition, some studies have recently reported allergenic reactions in highly sensitized patients after eating chicken meat (from chicken fed with fishmeal), which stresses the allergenic capacity of some *Anisakis* allergens after crossing the digestive barrier and having undergone a cooking process (*4*). Other works reported that some allergens from *A. simplex* are highly resistant to heat and pepsin treatments (*5*−*7*).

Food allergy is an emerging public health problem, especially in developed countries. Protein detection methods are largely employed to detect the presence of plant/animal food allergens [sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), immunoblotting, enzyme-linked immunosorbent assay (ELISA), and dipstick assays] (*8*−*11*).

Polymerase chain reaction (PCR)-based methods are well-established and comprehensive tools for species differentiation and detection of transgenic foods. However, there is little information about whether PCR technology is suitable for the detection of allergens in processed foods and the correlation between protein and DNA-based assays. Stephan and Vieths (*11*) indicate that ELISA and PCR technologies yield comparable results concerning the detection of peanuts in processed foods as sensitive and specific tools.

The major advantages of DNA-based methodologies are from the high specificity and the relatively high stability against environmental and technological influences. The use of high-temperature and/or high-pressure industrial practices could allow for the partial (or complete) denaturation of food proteins. In fact, the protein-based selection method for food allergen detection is the so-called ELISA technology. ELISA assays are sensitive and specific. However, in some instances, the sensitivity

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and specificity of this immunological test may not be satisfactory. Thus, one new allergen of *A. simplex* has recently been discovered (*12*), which has increased to nine, the number of described allergens of this parasite.

However, the employment of DNA analysis in allergen detection is controversially discussed, because proteins are the allergenic component and PCR results cannot be linked to any allergen/protein content.

DNA analyses, in particular, the PCR, are possible alternatives or synergic approaches to evaluate the safety of a certain food (*13*, *14*). In fact, the use of PCR and some PCR-correlated techniques (random amplified polymorphic DNA, nested PCR, multiplex PCR, and amplified fragment length polymorphism) have recently revolutionized the scene of analytical food biotechnology (*15*−*17*). Just a few copies of DNA can be specifically amplified and detected in complex processed food. Also, in this case, some challenges may appear, such as substances commonly present in foods that are polymerase inhibitors, fatty acids, phenolic compounds, and other specific molecules (*18*).

The use of the real-time PCR technique, largely used for the detection and quantification of genetically modified organisms (GMOs) (*19*, *20*) and recently suggested for peanut detection in food (*21*), allows for the rapid and sensitive detection of a specific genomic DNA sequence in food.

With regard to *A. simplex* detection by means of DNA techniques, we have previously characterized the polymorphic DNA region on the mitochondrial cytochrome *c* oxidase II gene and designed an innovative primer-probe detection system based on real-time PCR technology (*22*). However, this detection system should be methodologically validated to optimize the more adequate DNA extraction method and establish its specificity, accuracy, and precision. For this purpose, a comparison of several genomic DNA extraction methods was assessed to determine the higher yield and a better capacity for PCR amplification, including the absence of co-extraction of PCR inhibitors from seafood products. Moreover, we have successfully evaluated the real-time PCR assay, calculating its limit of detection and repeatability with commercial seafood samples.

At present, the food industry is quite complex and the possibility of coming into contact with allergenic food or with an allergenic ingredient is very common. Seafood is usually present in many different products, being a potential food-borne allergen source. In addition, seafood is recognized as a healthy product, and its inclusion in human diets is recommended (*23*).

The development and evaluation of analytical methods for allergen detection is necessary to monitor the implementation of such guidelines, to protect consumers from hidden allergens, and to help manufacturers establish concepts for hazard analysis and critical control points (HACCPs) to control the risk of contaminants.

MATERIALS AND METHODS

Sample Preparation. Commercial fish samples of different species (*Coryphaenoides rupestris*, *Engraulis encrasicolus*, *Engraulis anchoita*, *Gadus macrocephalus*, *Gadus morhua*, *Katsuwonus pelamis*, *Merlangius merlangius*, *Merluccius capensis*, *Molva molva*, *Pollachius pollachius*, *Sarda sarda*, *Scomber japonicus*, *Theragra chalcogramma*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus thynnus*, and *Trisopterus lusus*) were purchased at local markets. Concerning *A. simplex*-free tissue samples, one specimen of farmed turbot (*Psetta maxima*) was bred in the laboratory under controlled conditions and some samples of terrestrial species (such as pork, chicken, turkey, and rabbit) were also collected. The third-stage larvae of *A. simplex* were extracted with forceps from the abdominal cavity of fresh blue whiting (*Micromesistius potassou*). Nematodes were characterized following taxonomic keys (*24*, *25*). Residual proteins from the host fish were removed by washing in 1% (w/v) NaCl. Following this process, the larvae were stored at −80 °C until they were used. Fish samples were well-characterized by PCR-sequencing their respective mitochondrial cytochrome *b* gene fragments as previously described (*26*).

For assay repeatability, 25 and 50 g of turbot *A. simplex*-free tissue samples were spiked with one *A. simplex* larvae of 1 mg and subsequently minced and homogenated.

For evaluation of food matrix effects, we purchased 22 different commercial seafood products at local markets. These samples were industrially manufactured as salted and smoked fish, pâté, surimi, croquette, canned, baby food, and ready-to-eat presentations.

Before we proceeded toward the DNA extraction from commercial seafood products, salt and oil excess was removed by washing and blotting with a filter paper, respectively. The defatted and unsalted tissue was stored at −20 °C until it was used.

DNA Extraction and Clean up. For DNA isolation of *A. simplex* larvae, six different protocols were performed. In all cases, except for the commercial kits with a specific lysis buffer, the larvae were previously digested with 300 μL of extraction buffer [1% (w/v) SDS, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and Tris-HCl at pH 8.0] supplemented with 50 μL of 5 M guanidine tiocianate and 30 μL of proteinase K and subsequently incubated at 56 °C overnight. After centrifugation for 5 min at 16000*g*, the supernatant was purified with the following methods: NaCl method (*27*) and phenol chloroform method (*28*). DNA isolation was also performed by means of the following commercial kits: Wizard DNA Clean-Up System (Promega), Wizard Genomic DNA Purification Kit (Promega), and QIAmp DNA Blood Mini Kit (QIagen). Eventually, a Modified Wizard Genomic DNA Purification Kit was also performed after substituting its lysis buffer by means of the digestion procedure stated above.

Binary tissue mixtures were prepared by homogenizing in a blender (Kinematica Polytron PT 10/35) 25 g of fish tissue samples with one parasite (1 mg) in the presence of 25 mL of sterile Milli-Q H_2O and 40 μL of proteinase K for 5 min. Then, 300−400 mg of the mixture was mixed with 300 μL of extraction buffer [1% (w/v) SDS, 150 mM NaCl, 2 mM EDTA, and Tris-HCl at pH 8.0] supplemented with 10 μL of 5 M guanidine tiocianate and 10 μL of proteinase K and subsequently incubated at 56 °C for 1 h. After centrifugation for 5 min at 16000*g*, the supernatant was purified using the Wizard DNA Clean-Up System (Promega) and finally eluted with 50 μL of sterile Milli-Q H_2O. The final DNA concentration was measured by absorbance at 260 nm and stored at −20 °C until used. DNA quality was estimated measuring the absorbance at 280 nm, whereas the presence of undesirable RNA was evaluated on 0.6% (w/v) agarose gels.

TaqMan Probe and Primer Design. All of the primers and fluorogenic probes were designed using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). The probes were labeled on the 5′ end with the fluorescent reporter dye 6-carboxyfluorescein (FAM), whereas the 3′ end was labeled with a nonfluorescent quencher and a minor groove binder (MGB). All of the primers and probes were provided by Applied Biosystems. Multiple alignments were carried out using the Clustal W program (*29*).

Mitochondrial Cytochrome *c* Oxidase II Gene Fragment Amplification. The amplification of the 260 bp fragment of mitochondrial cytochrome *c* oxidase II gene (COII) was carried out with primers CYTCII-F (5′-TTTCTAGTTATATGGATTGATTCCATAA-3′) and CYTCII-R (5′-GACCAGTGACTTTCACAGTCAAATTAC-3′) as follows: 10 mM Tris-HCl at pH 9.0, 50 mM KCl, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 μM primer, and 1−100 ng of template DNA (*22*). The reaction was started by adding 1 unit/reaction Taq DNA polymerase (Amersham Biosciences AB, Buckinghamshire, U.K.). Amplification reactions were developed in a Mastercycler Personal (Eppendorf) for 40 cycles with an annealing temperature of 58 °C.

Sequencing. The sequencing of amplified fragments was carried out directly on the purified fragments with a 3700 DNA Analyzer ABI PRISM, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0 (Applied Biosystems, Foster City, CA).

Real-Time PCR Analysis. Real-time PCR amplification was performed in a MicroAmp Optical 96-well reaction plate. Amplification reactions were carried out with TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) containing primers QCYTCII-F (5′-AGTAAGAAGATTGAATATCAGTTTGGTGA-3), QCYTCII-R (5′-AAGTAAACTCAAAGAAGGCACCATC- 3′), and the specific probe CYTCII

(5′-FAM-TTCCTACTTTAATTTTGGTTGCTC-MGB-3′) (*22*). Reactions were run on the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) 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.

The PCR amplification efficiency (E) was calculated from linear regression of standard curves of four independent experiments from the equation $Ct = (1/\log E) \log N + \log N_C$, where N is the initial amount of DNA template and N_C is the amount of DNA at the Ct cycle (*30*). In the linearity and cross-reactivity studies carried out, each Ct value was obtained from the mean of three replicates, with a standard deviation under 0.4. Alternatively, for detection limit optimization and sample determination, each Ct value was obtained from the mean of duplicates in three independent DNA extractions.

The specificity of the detection system was evaluated *in silico* after calculating the inter- and intraspecific variability of the DNA fragment (V^{inter} and V^{intra}, respectively) as follows:

$$V^{inter} = [1 - (((AN) - B)/(AN))] \times 100$$

$$V^{intra} = [1 - (((AN) - C)/(AN))] \times 100$$

where N is the number of individuals, A is the number of polymorphisms checked, B is the number of polymorphic variations among individuals belonging to different species, and C is the number of polymorphic variations among individuals belonging to the same species

The *in silico* multiple alignments were carried out using the Clustal W program (*29*) from DNA sequences obtained from the GenBank Database (www.ncbi.nlm.nih.gov).

The methodological validation of the real-time PCR detection system was demonstrated by evaluating the precision and accuracy of the procedure after calculating its repeatability and detection limit. The repeatability (r) was calculated as the number of "true positive" results obtained (TR) after analyzing the same positive sample "n" times ($r = (TR/n) \times 100$), whereas the limit of detection (LD) was calculated as the average of Ct (y) obtained from analyzing at least 10 times a negative template control plus 3 times its standard deviation ($LD = y - 3SB_{n-1B}$) (*31*).

RESULTS AND DISCUSSION

Comparison of DNA Extraction Methods. The aim of an extraction procedure is to isolate high-quality DNA from the sample to be analyzed for identification. It means that DNA should contain as little proteins, RNA, or any other PCR inhibitor as possible. Actually, the presence of PCR inhibitors in food samples is a major concern that could negatively affect the efficiency of the amplification reaction (*18*). The quantity and quality of DNA were evaluated by considering the 260 nm absorbance of the DNA isolated (A_{260}) and the ratio of absorbance 260/absorbance 280 to evaluate the quality (A_{260}/A_{280}). DNA fragmentation is another concern that should be considered, but it can be minimized using a detection system based on a very short DNA probe (< 100 bp), which was actually performed for this study, as described later. Six different DNA extraction methods were finally tested, and the results are summarized in **Table 1**. The highest yield was obtained with NaCl and phenol chloroform methods; however, this result was overestimated by the presence of RNA in the sample, which absorbs at 260 nm. With regard to the commercial kits, the highest yields were obtained with the Wizard Genomic DNA Purification Kit (91−236 ng/μL), but these good results were negatively affected by the ratio A_{260}/A_{280}, which indicates worst quality than others as far as isolated DNA is concerned. In fact, the highest quality DNA (ratio of A_{260}/A_{280} of 1.9 and absence of RNA) was obtained with the Wizard DNA Clean-Up System, which was eventually the method of choice. Actually, this commercial kit is frequently used in highly processed food samples (*15*, *32*), and some authors have also used it to isolate DNA from *Anisakis* nematodes (*33*−*35*).

Table 1. Comparison of DNA Extraction Methods from 1 mg of *A. simplex*[a]

extraction method	yield (ng/μL)	ratio of A_{260}/A_{280}	DNA (μg)	presence of RNA
NaCl	256.0	1.7	12.8	yes
Wizard Genomic DNA Purification Kit	91.4	1.5	9.1	yes
QIAmp DNA Blood Mini Kit	22.0	1.5	4.4	no
Wizard DNA Clean-Up System	66.8	1.9	3.3	no
phenol chloroform	400.0	1.7	20.0	yes
Modified Wizard Genomic DNA Purification Kit	236.2	1.4	23.6	no

[a] Data show one representative experiment performed of three experiments.

Primer-Probe Design and Cross-Reactivity Studies. To design a sensitive method based on DNA technology to identify the presence of food-borne allergic ingredients in high processed food products, it is very important to choose the most adequate genetic marker. In animal eukaryotic organisms, there are actually two possibilities: nuclear or mitochondrial DNA fragments. The nuclear genome is very adequate for quantification approaches, because in quantitative analysis, it is essential to determine the number of copies in the samples and, consequently, desirable to select one-copy nuclear genes as markers. Nevertheless, the use of nuclear markers significantly affects the sensitivity of the procedure. Conversely, the use of mitochondrial genes as markers improves the amplification yield because eukaryotic cells have hundreds or thousands of mitochondrial genome copies; thus, the initial number of copies as the template in the reaction is higher. In addition, the mitochondrial genome is more stable under thermal treatment than the nuclear genome in the processing of food products (*36*). As a result, a real-time PCR detection system based on mitochondrial genes is much more sensitive and eventually more adequate to identify the presence of food-borne allergic ingredients in highly processed food.

DNA sequence information for *A. simplex* is extremely scarce. In fact, GenBank provides information about three fragments of the cytochrome *c* oxidase II gene and one fragment of the cytochrome *c* oxidase I gene. Although, the complete mitochondrial genome of *A. simplex* has recently been published (*37*), the shortage of sequences makes it difficult to evaluate the intraspecific variability between individuals. This variability is critical to devise a reliable and specific primer-probe system. After the alignment of the available COII sequences, a pair of primers was designed to amplify a 260 bp fragment. Thus, 13 COII fragments were successfully amplified and subsequently sequenced from different *A. simplex* individual larvae. The alignment analysis of these sequences pointed out five nucleotide variations, which are detailed in **Figures 1**−**3**. As a result, a consensus 96 bp region into the COII fragment was selected as a template to design the detection system after calculating an intraspecific variability (V^{intra}) of 0%. Moreover, after evaluating 31, 37, and 61 polymorphisms (A) by comparing *A. simplex* consensus COII sequence with sequences from species of *Anisakis* ($N = 6$; **Figure 1**), parasites ($N = 4$; **Figure 2**), and commercial fish species ($N = 11$; **Figure 3**), we detected 65, 67, and 544 nucleotide variations (B) calculating an interspecific variability (V^{inter}) of 35, 45, and 87%, respectively. These results indicate that this region is specific of *A. simplex*, which is adequate to design a real-time PCR detection system based on TaqMan MGB probes design. Thus, a primer-probe detection system (90 bp), as short as possible, was designed in the consensus region by forcing the Primer Express program. In fact, the size of the system is decisive because the DNA extracted from high processed seafood products is strongly degraded, most likely because of the thermal treatment applied. As previously reported from seafood products, the size of most of the DNA

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A.simplex consensus*  -TTTCTAGTTATATGGATTGATTCCATAANTTTAATTGTAGTTTGCTTTTNGGTGTTCTT 59
A.typica              T.............A.....G..T.....T...................T.....CT.G 60
A.physeteris          T.............A.....G..T.....T...........G.....A..T.....AT.G 60
A.brevispiculata      T.............A.....G..T.....GC..T.......G..A.....T.....GT.A 60
A.paggiae             T.............A.....G..T....GT...........G.......T..G..GT.G 60
A.ziphidarum          T.............A.....G..T.....T...................T......T.A 60

A.simplex consensus*  TCTTTTGTNTCTGTTATGTTTGGNTATCTTCTTTTTAGAAATTTTTANTTTAAGAGTAAG 119
A.typica              ........C.............TA..CT.G........C.G......T........G... 120
A.physeteris          G.......TA.G.....A....TT...T.A.......AT.G......T...........A 120
A.brevispiculata      G.......TA.A.....A....TT...T.A.......AT.GG.....T.....A.....A 120
A.paggiae             G.......T........A....TT...T.A.......AT.G......T.....A.....A 120
A.ziphidarum          ........T........A....TT...T.A........G.G......T........A... 120

A.simplex consensus*  AAGATTGAATATCAGTTTGGTGAACTTTTATGTAGTATTTTTCCTACTTTAATTTTGGTT 180
A.typica              ........G........C..G........G.........................C....G 180
A.physeteris          ..A.....G.............................G.G...........G........A 180
A.brevispiculata      ........G..............G...C.T......G.C...........G..C..A... 180
A.paggiae             ..............................G..CA.........................G 180
A.ziphidarum          ..............................G...........C......C.T........G 180

A.simplex consensus*  GCTCAGATGGTGCCTTCTTTGAGTTTACTTTATTATTATGGTTTGATGAATCTTGATAG 238
A.typica              ..C.....A.....C........A..G....................A........... 239
A.physeteris          ....................A..AC.TT.G............C.T.............. 239
A.brevispiculata      .....A..A..A........A..AC.T....AC......................... 239
A.paggiae             TT.........T........A...C.T....AC...........A..A...T.G..... 239
A.ziphidarum          ..C..A.....G........A.....G....A...............A...T.G..... 239

A.simplex consensus*  TAATTTGACTGTGAAAGTCAC 260
A.typica              ......A.....C..G..TA. 261
A.physeteris          ....C.T.....T..G..TA. 261
A.brevispiculata      G..C..G.....T.....AA. 261
A.paggiae             A..C..A.....T.....TA. 261
A.ziphidarum          ............T.....TA. 261
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Figure 1. *In silico* alignment of the 260 bp mitochondrial CII fragment of *A. simplex* with other *Anisakis* species: *A. ziphidarum* (DQ116430.1), *A. physeteris* (DQ116432.1), *A. typica* (DQ116427.1), *A. paggiae* (DQ116434.1), and *A. brevispiculata* (DQ116433.1). Species sequences were obtained from GenBank (accession number). Underlined sequences indicate the 96 bp consensus sequence, and the 31 polymorphisms are highlighted in bold. (∗) *A. simplex* COII consensus sequence was obtained from 13 larvae of *A. simplex*. Boxes indicate the polymorphism detected in *A. simplex* consensus sequence (N).

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A.simplex consensus*  TTTCTAGTTATATGGATTGATTCCATAANTTTAATTGTAGTTTGCTTTTNGGTGTTCTTT 60
P.ceticola            .............A.....G..TT....T...........G........T.....GT.G. 60
C.multipapillatum     --------------------------..T..............A.....T..G...T.A. 34
H.pelagicum           --------------------------..T..........AAC.T.....C..G....... 34

A.simplex consensus*  CTTTTGTNTCTGTTATGTTTGGNTATCTTCTTTTTAGAAATTT--TTANTTTTAAGAGTA 118
P.ceticola            .......T......A......TT.....GC.......T.G...--TT.T...-..A.... 117
C.multipapillatum     .......TA.....A.....TTT...T.GT.G..A...CG...--TT.T...-....... 91
H.pelagicum           .C.....G...ACGG......CC...C..A.GC.G..GC.C..--TT.C..C-..A.... 91

A.simplex consensus*  AGAAGATTGAATATCAGTTTGGTGAACTTTTATGTAGTATTTTTCCTACTTTAATTTTGG 175
P.ceticola            .A..A.....G..C..A........GT.G..G...........CC.GA....G....... 174
C.multipapillatum     ..........G.....A.....A..G.....G.....GG........A....G.....A. 148
H.pelagicum           ....A..C..G..C..A.........T.G..G......GTA..C...A.AC.T...C.T. 148

A.simplex consensus*  TTGCTCAGATGGTGCCTTCTTTGAGTTTACTTTATTATTATGGTTTGATGAATCTTGATA 235
P.ceticola            .G........A...........A...C.T.....C................C..G..C. 234
C.multipapillatum     ..ATG.....AA.T........A.....GT.G..............A......T.A.... 208
H.pelagicum           .AATA......A.T.....C..A...........C.....C........A.......... 208

A.simplex consensus*  GTAATTTGACTGTGAAAGTCAC 260
P.ceticola            .A..CC.A.....T..G..A.. 294
C.multipapillatum     .G.G......AA.T.....T.. 268
H.pelagicum           .......A.....T..G..T.. 268
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Figure 2. *In silico* alignment of the 260 bp mitochondrial CII fragment of *A. simplex* with some parasites: *Pseudoterranova ceticola* (DQ116435), *Contracecum multipapillatum* (AF179910), and *Hysterothylacium pelagicum* (AF179915). Underlined sequences indicate the 96 bp consensus sequence, and the 37 polymorphisms are highlighted in bold. (∗) *A. simplex* COII consensus sequence was obtained from 13 larvae of *A. simplex*. Boxes indicate the polymorphism detected in *A. simplex* consensus sequence (N).

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A.simplex consensus*   TTTCTAGTTATATGGATTGATTCCATAANTTTAATTGTAGTTTGCTTTTNGGTGTTCTTT 60
A.rochei               ----------------------------------A.GACCACGCC..GA.AATC..CT..C 27
K.pelamis              C...ACC.GT...A..AGA.C.T.T.C.C...C..GACCACGCC..GA.AATC..CT..C 60
T.thynnus              C...ACC.GT...A..AGA.C.T.T.C.C..CC..GACCACGCC..GA.AATC..CT..C 60
S.sarda                C...ACC.GTC..A..AGA.C.T.T.C.C...C..GACCACGCC..GA.AATC..CT..C 60
S.scombrus             C...ACCCCTA..A..AGA.C.T.TAC.C..CC.CGACCA.GCC..AA.AATCC.GT.CC 60
T.chalcogramma         ---------------------------------..GA.CACGCC..AA.AATC..AT... 27
G.morhua               ---------------------------------..GA.CACGCC..AA.AATC..AT... 27
M.merlangus            ---------------------------------..GA.CACGCC..AA.AAT...AT... 27
P.virens               CA..ACCCGTA..A..AGAGT.A.TAC.C..CC.CGA.CACGCC..AA.AAT...AT... 60
E.encrasicolus         ---------------------------------.CGACCA.GCA..AA.AATC..AT... 27

A.simplex consensus*   CTTTTGTNTCTGTTATGTTTGGNTATCTTCTTTTTAGAAATTT--TTANTTTTAAGAGTA 118
A.rochei               TGA.CAGCA.GC.GG.AC..TACAT.A..G.GGCG.T.GTC.CAACC.AAC.A.CT.AC. 87
K.pelamis              TAA.CAGCA.GC.GG.AC..TACAT.A..G.GGCG.T.GTC.CAACC.AAC.C.CT.AC. 120
T.thynnus              TGA.CAGCA.AC.GG.AC..TACAT.A..G.GGCG.T.GTC.CAACC.AAC.C.CT.AC. 120
S.sarda                TAA.CAGCA.AC.GG.AC..TACAT.A..G.GGCG.T.GTC.CAACC.AAC...CT.AC. 120
S.scombrus             TAA..AGC..AT..G.AC..TACAT.A..G.GG...T..TT.CAAC.TCCC...CC.AC. 120
T.chalcogramma         TAA..AGTA..C..G.AC..TACAT.A..G.CGCA.T.GTC.CCAC..AA..A.CC.AC. 87
G.morhua               TAA..AGTA..C..G.AC..TACAT.A..G.CGCA.T.GTC.CCAC..AA..A.CC.AC. 87
M.merlangus            TAA..AGTA.CC..G.AC..TACAT.A..G.CGCA.T.GTC.CCAC..AA..A.CC.AC. 87
P.virens               TAA..AGTA..C..G.AC..TACAT.A..G.CGCG.T.GTA.CCAC..AA..A.CC.AC. 120
E.encrasicolus         TAA..AGTA..T.AG.TC..TACGTCA.CG.AGC..TGGTC.CTA.C.AGC...C..AC. 87

A.simplex consensus*   AGAAGATT---GAATATCAGTTTGGTGAACTTTTATGTAGTATTTTTCCTACTTTAATTT 175
A.rochei               .AT.C..CCTA..T.CC..AGAAATC...A..A.C..A.CA...C.C..AG..A.T..CC 147
K.pelamis              .AT.T..CCTA..T.CC..AGAAATC...A.CA.C..A.CA...C.C..AG..A.T..CC 180
T.thynnus              .AT.T..CCTA..T.CC..AGAAATC...A.CA.C..A.CA...C.C..AG..A.T...C 180
S.sarda                ..T.T...TTA..T.CC..AGAAATC...A..A.C..A.CA...C....AG..A.T...C 180
S.scombrus             .AT.TG.GCTA..C.CC..AGAGATC..GA..A.C..A.CGG.GC.C..CG.CG.C...C 180
T.chalcogramma         .AT.T...CTA..T.C...AGAAAT...GA..A.C..A.CAG..C.C..AG..G.T...C 147
G.morhua               .AT.T...TTA..T.C...AGAAAT...GA..A.C..A.CAG..C.C..AG..G.T...C 147
M.merlangus            .AT.T...TTA..T.C...AGAAAT....A..A.C..A.CAG.CC.C...G..G.T...C 147
P.virens               ..T.T...CTA..T.C...AGAAAT...GA..A.C..A.CAG..C.C..AG..G.T...C 180
E.encrasicolus         .AT.C...CTA..T.CA..AGAGATC...A.CG.G..A.C.A..C.C...G..G.C...C 147

A.simplex consensus*   TGGTTGCTCAGATGGTGCCTTCTTTGAGTTTACTTTATTATTATGGTTTGATGAATCTTG 235
A.rochei               .TA..CTCATTGCCC.T..C..CC.AC.AA.T..C..CCT.ATG.ACGAA..T..C---. 204
K.pelamis              .TA..CTCATTGCCC.T..C...C.AC.AA.T..C..CCT.ATG.ACGAA..C..C---. 237
T.thynnus              .TA..CT.ATTGCCC.T..C..CC.AC.CA.T..C..CCT.ATG.ACGAA..T..C---. 237
S.sarda                .TA.CCT.ATTGCTC.C..C..CC.AC..A.T.....CCT.ATG.ACGAA..C...---. 237
S.scombrus             .TA..CTCATCGCCC.C..C..CC.TC.GA.C.....CCTAATG.ACGAG..C...---. 237
T.chalcogramma         .TA..TTAATTGCAC.C..A..A..AC.AA.T......CT.ATA.ACGAA..T...---. 204
G.morhua               .TA..TTAATTGCCC.T..A..A..AC.AA.T......CTCATA.ACGAA..T...---. 204
M.merlangus            .CA.CTTAATTGCAC.T..A..CA.T.CGAATTC....CT.ATA.A.GAA..T...---. 204
P.virens               .AA..CTAATTGCAC.T..A..A..AC.AA.T......CT.ATA.A.GAA..C...---. 237
E.encrasicolus         .TA..ATGATCGCAC.A..G...C.AC.AA.C..C..CCTCATG.A.GAA..C..C---. 204

A.simplex consensus*   ATAGTAATTTGACTGTGAAAGTCAC 260
A.rochei               ..CCAC.CC.A..AA.T....CTGT 264
K.pelamis              .CCCAC.CC.A..AATC....CTGT 297
T.thynnus              .CCCCC..C.A..AA.T....CTGT 297
S.sarda                .CCCCC.CC.A..AA.T....CTGT 297
S.scombrus             .CCCAC.CC....AA.T....C.GT 297
T.chalcogramma         ..CCAC..C.T..CA.T....CA.T 264
G.morhua               .CCCGC..C.T...A.T....CA.T 264
M.merlangus            .CCCGC..C.T...A.T....CA.T 264
P.virens               .CCCGC..C.T...A.T....CG.T 297
E.encrasicolus         .CCC.C....A..CA.T....CTGT 264
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Figure 3. *In silico* alignment of the 260 bp mitochondrial CII fragment of *A. simplex* with some commercial fish species: *Auxis rochei* (AY971774) *Katsuwonus pelamis* (AY971773), *Thunnus thynnus* (AY971770), *Sarda sarda* (AY971771), *Scomberomorus cavalla* (NC_008109), *Scomber scombrus* (NC_006398), *Theragra chalcogramma* (DQ356946), *Gadus morhua* (DQ356939), *Merlangius merlangus* (NC_007395), *Pollachius virens* (DQ356945), and *Engraulis encrasicolus* (NC_009581). Species sequences were obtained from GenBank (accession number). Underlined sequences indicate the 96 bp consensus sequence, and the 61 polymorphisms are highlighted in bold. (∗) *A. simplex* COII consensus sequence was obtained from 13 larvae of *A. simplex*. Boxes indicate the polymorphism detected in *A. simplex* consensus sequence (N).

fragments obtained was smaller than 200 bp (*15*). As a result, the *A. simplex*-specific primer probe system devised is consistent with the expected fragment size.

To determine the cross-reactivity of the assay, the primer-probe detection system was tested with the species listed in the Materials and Methods. However, in a number of cases, the Ct values detected were significantly low (e.g., 30.9 for cod; **Table 2**), which can be explained as the presence of *A. simplex* in the fish sample. Even though all fish tissue samples were collected from the dorsal muscle area, in some cases, the individuals are so infected that larvae cross their digestive barrier and migrate to the tissues (*1*). In addition, *in silico* analysis (**Figure 1**) showed enough nucleotide differences between *Gadus* species and *A. simplex* to stress the specificity of the detection system. Besides, a turbot farmed sample (*P. maxima*) bred in the laboratory under controlled conditions and some terrestrial species were also successfully

analyzed as *A. simplex*-free tissue samples, with Ct values of 40 (**Table 2**).

Real-Time System Setup. The *A. simplex*-specific primer-probe system was optimized by establishing the reaction conditions, for which the concentration of the pair of primers and the probe in the reaction mix was set up. Balanced melting temperatures (T_m) of primers (forward and reverse) result in an efficient amplification. Although the T_m of every pair of designed primers was 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. An optimal reaction was achieved by selecting the primer concentrations that provide the lowest Ct and highest fluorescence. No imbalance was detected; thus, there was no need to adjust the forward and reverse primer concentrations of every system. The optimal concentration calculated for the pair of primers was 300 nM. With regard to the specific probe, different concentrations were assayed and the optimal probe concentration was 150−250 nM. Finally, we selected 250 nM as the optimal probe concentration because high fluorescence leads to high feasibility with a low number of gene copies.

Table 2. Selectivity of the Primer-Probe System (25 ng of DNA as Template)[a]

scientific name	Ct
Anisakis simplex	18.3
Fish Species	
Coryphaenoides rupestris	30.9
Engraulis anchoita	35.5
Engraulis encrasicolus	33.7
Gadus macrocephalus	40.0
Gadus morhua	30.9
Katsuwonus pelamis	40.0
Merlangius merlangius	40.0
Merluccius capensis	35.1
Molva molva	40.0
Pollachius pollachius	30.9
Sarda sarda	35.1
Scomber japonicus	40.0
Theragra chalcogramma	36.4
Thunnus albacares	38.3
Thunnus obesus	40.0
Thunnus thynnus	40.0
Trisopterus lusus	40.0
Psetta maxima	40.0
Terrestrial Species	
Sus scrofa	40.0
Gallus gallus	40.0
Meleagris gallopardo	40.0
Orictolagus cuniculus	40.0

[a] The Ct values shown are at least measured in duplicates.

Linearity, Sensitivity, and Repeatability of the Assay. In real-time PCR analysis, the threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus, to test the linearity of the system, Ct values were plotted versus the logarithm of the DNA concentration using 10-fold dilutions of DNA template. **Figure 4** shows good linear correlation between the Ct values and the concentration of DNA present in the amplification reaction and also indicates the sensitivity of the assay to detect *A. simplex*. In fact, as little as 0.1 pg of *A. simplex* DNA was consistently amplified with this assay, which is comparable to those previously described for other ingredients using a real-time PCR system (*16*, *38*−*40*). Thus, Brzezsinski (*16*) reported a limit of detection approximately between 1 and 5 pg of allergic cashew nut DNA. Additionally, López-Andreo et al. (*39*) described a sensitivity of 0.04 pg for porcine, horse, and wallaroo DNA and 0.2 pg for bovine DNA. Thus, we can conclude that the *A. simplex*-specific real-time PCR detection system developed in this study is especially sensitive. With regard to PCR efficiency (E), this value provides information about the deviation from the optimum to duplicate the number of copies per cycle for the primer-probe system. The theoretical value of 2 indicates 100% PCR efficiency. The E value is deduced from the equation described in the Materials and Methods. This way, the efficiency was calculated as $E = 2.07 \pm 0.14$, from the estimated slope of linear regression of four independent experiments. The efficiency obtained approached the optimum of 100%.

As a result, considering that the assay requires 200 ng of total DNA as the template (which will be discussed in the next section), an evaluation of the DNA excess effect upon the efficiency was performed. This way, we tested the linearity of the system again using 10-fold dilutions of *A. simplex* DNA as the template, supplemented with foreign DNA (from pork) until a final concentration of 200 ng in every dilution was reached. The curve obtained is similar (**Figure 4B**) to those calculated directly from the DNA of *A. simplex* as the template (**Figure 4A**). Thus, the range of linearity is maintained between 100 ng and 0.1 pg, and the efficiency of 2.12 (calculated from the slope) indeed approaches the optimum and positively stresses the sensitivity of the detection assay.

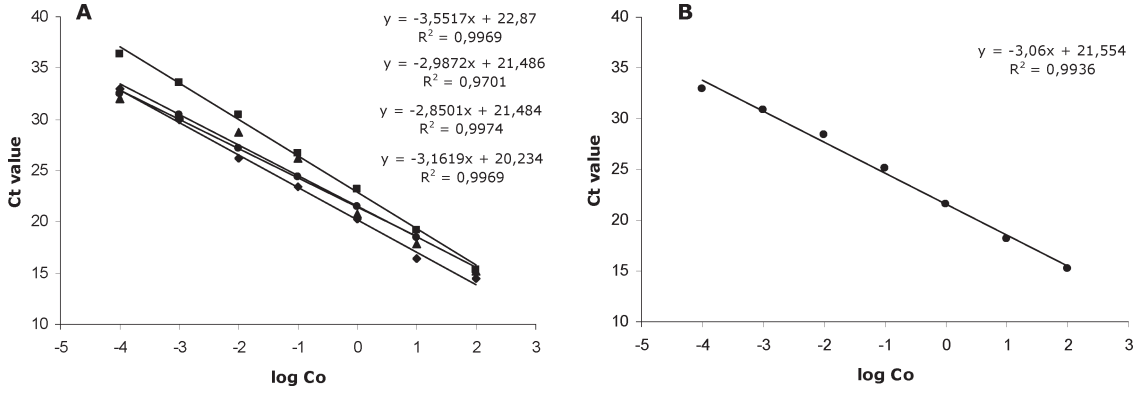

Figure 4. Linearity test with *A. simplex* DNA as template: (A) 10-fold dilutions series of DNA starting from 100 ng to 0.1 pg used in four independent experiments and (B) 10-fold dilution series of DNA supplemented with foreign DNA (pork) until a final concentration of 200 ng in every dilution was reached. Ct values are plotted versus the logarithm of the DNA concentration.

Table 3. Real-Time PCR Detection Assay Optimization for Seafood Products[a]

seafood sample	$Ct^{not\ spiked}$	presence of *A. simplex* DNA	Ct^{spiked}	presence of *A. simplex* DNA
baby food (hake)	31.7 ± 1.12	yes	28.6 ± 4.87	yes
canned 1 (cod)	37.5 ± 0.64	yes	30.5 ± 0.71	yes
canned 2 (cod)	39.5 ± 1.05	no	31.7 ± 0.67	yes
canned 3(cod)	36.7 ± 0.53	yes	30.6 ± 0.98	yes
canned 4 (cod)	40.0 ± 0.13	no	30.3 ± 3.57	yes
croquette 1 (cod)	31.6 ± 0.58	yes	21.1 ± 0.23	yes
croquette 2 (cod)	31.3 ± 0.55	yes	21.1 ± 0.23	yes
croquette 3 (cod)	29.3 ± 0.59	yes	24.6 ± 0.37	yes
croquette 4 (cod)	31.1 ± 1.05	yes	20.8 ± 0.45	yes
croquette 5 (cod)	27.3 ± 0.66	yes	23.6 ± 0.36	yes
croquette 6 (cod)	27.0 ± 1.36	yes	21.1 ± 0.21	yes
croquette 7 (cod)	28.0 ± 1.08	yes	20.3 ± 1.50	yes
croquette 8 (cod)	26.5 ± 0.13	yes	21.7 ± 0.43	yes
pâté 1 (cod)	36.4 ± 2.36	yes	28.5 ± 0.86	yes
pâté 2 (cod)	33.8 ± 1.11	yes	30.2 ± 1.23	yes
ready to eat 1 (cod)	38.1 ± 1.08	no	28.7 ± 0.06	yes
ready to eat 2 (cod)	37.1 ± 1.04	yes	30.8 ± 0.82	yes
ready to eat (hake)	33.76 ± 2.6	yes	28.5 ± 2.79	yes
salted (cod)	40.0 ± 0.23	no	34.7 ± 3.13	yes
smoked 1(cod)	39.8 ± 0.42	no	30.4 ± 0.79	yes
smoked 2 (cod)	38.7 ± 1.60	no	33.4 ± 0.84	yes
smoked 3 (cod)	37.6 ± 1.63	no	29.4 ± 1.03	yes

[a] Every sample was analyzed by duplicates in three independent DNA extractions ($n = 6$). The presence of *A. simplex* were evaluated comparing the Ct value obtained with the LD = 39.07.

Furthermore, the methodological validation of the real-time PCR detection system should also be demonstrated, evaluating the precision and accuracy of the procedure by calculating the repeatability (r) and limit of detection (LD), respectively. The former was calculated from two positive 25 and 50 g of turbot *Anisakis*-free tissue samples spiked with one *Anisakis* larvae, which were analyzed 6 times ($n = 6$) using the real-time PCR detection system, obtaining $r^{50\,g} = 50\%$ and $r^{25\,g} = 100\%$. As a result, the analytical system will be tested on 25 g of food samples, where its precision is optimal. The latter will be further discussed in the next section.

Limit of Detection and Assay Optimization in Seafood Samples. Nowadays, there is very little information to estimate the threshold dose for specific allergenic foods. Anecdotal reports indicate that allergenic reactions have occurred from incidental contact, such as inhaling food vapors while cooking (*41*). Furthermore, protein contact dermatitis caused by *Anisakis* species has been demonstrated (*4*). These data confirm that the ingestion of a very small amount of allergenic food may provoke allergenic reactions in some sensitive individuals. Therefore, allergists assumed that the threshold dose for the offending food was theoretically zero. However, zero tolerance provokes enormous practical problems in the food industry (*41*). Detection limits need to be at least in the range of the established allergic threshold levels determined by oral food challenge studies, or maybe, they should even be of a lower magnitude (*35*). Thus, it is generally agreed that the detection limits for different food products need to be somewhere between 1 and 100 ppm (milligrams of allergenic protein per kilogram of food) depending upon the type of food (*42*).

With regard to *A. simplex*, there is no information about the minimum allergen concentration that causes allergenicity. Actually, *A. simplex* contains multiple allergenic proteins that can vary in abundance and allergenicity. In general, we can conclude that a single larvae contains sufficient allergen to induce an antibody response in sensitive individuals (*43*). Considering this approach, we optimized an assay capable of detecting the presence of one parasite in 25 g of seafood. Preliminary studies revealed the necessity of increasing the amount of DNA used as the template (200 ng) in the real-time analysis to improve the detection limit of the assay eventually (data not shown). **Table 3** shows the Ct values obtained after the analysis of different seafood samples spiked with one individual larvae of *A. simplex*. As a conclusion, the method was capable of detecting one larvae of 1 mg in 25 g of different seafood samples (40 ppm), which is consistent with the general opinion that establishes a range of 1−100 ppm (*42*). The accuracy of the assay was calculated as the average of Ct (y) obtained from analyzing a negative template control 14 times ($n = 14$) plus 3 times its standard deviation ($LD = y - 3SB_{n-1B}$). As a result, a limit of detection of 39.07 was obtained. This Ct value corresponds to 40 ppm of parasite in 25 g of sample.

Other authors described similar results; thus, Hird et al. (*21*), published a real-time PCR protocol for the detection of peanut traces in a large variety of food matrices and showed a detection limit of 2 ppm in spiked cookies. Additionally, Stephan and Vieths (*11*) evaluated the sensitivity of the peanut detection real-time PCR method, clearly detecting a range of 10−200 ppm in industrially manufactured chocolate samples. Furthermore, no significant loss of sensitivity occurred as a result of processing. This means that the degraded DNA isolated for processed seafood is intact enough to obtain the same sensitivity in the real-time assay as long as small probes are used, as was performed for this study.

Besides, the described DNA extraction method yielded amplifiable DNA of high purity, without any inhibitors of the PCR reaction. Actually, after a variety of highly processed food samples were contaminated with one parasite, the quality of DNA isolated let us detect the presence of the parasite successfully, which stresses the good quality of the DNA extracted from different food matrices and the absence of PCR inhibitors (**Table 3**).

Moreover, the presence of *A. simplex* was positive in most of the non-spiked food samples analyzed (**Table 3**). This is explained by the fact that those seafood samples contained fish species susceptible of being infected by the nematode, such as cod. In fact, it has been reported that gadiform species are some of the most frequently infected by *A. simplex* (*1*). In addition, these food samples, with a significant presence of *A. simplex*, have been presumably

manufactured after fish tissue mincing (e.g., croquettes and pâté). Conversely, hardly processed samples as salted and smoked were negative.

To summarize, the assay developed in this study is a very reliable, specific, and sensitive methodology to detect the presence of this parasite in seafood products, including highly processed samples. Actually, the presence of this parasite in several seafood products has been demonstrated, enforcing the need to design a good manufacturing practice protocol for the processing industry to minimize the presence of this parasite as a food-borne allergen source in seafood products.

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