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Fast Real-Time PCR for the Detection of Crustacean Allergen in Foods

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Supporting Information

ABSTRACT: Crustaceans are one of the most common allergens causing severe food reaction. These food allergens are a health problem, and they have become very important; there are various regulations that establish that labeling must be present regarding these allergens to warn consumers. In the present work a fast real-time PCR, by a LNA probe, was developed. This allows the detection of crustaceans in all kinds of products, including processed products in which very aggressive treatments of temperature and pressure during the manufacturing process are used. This methodology provides greater sensitivity and specificity and reduces the analysis time of real-time PCR to 40 min. This methodology was further validated by means of simulating products likely to contain this allergen. For this, products present on the market were spiked with crustacean cooking water. The assay is a potential tool in issues related to the labeling of products and food security to protect the allergic consumer.

KEYWORDS: *crustacean, allergy, fast real-time PCR, detection, LNA probe*

INTRODUCTION

Food allergies are nowadays recognized as a food safety issue. They are a set of adverse reactions to food (and to some food additives), due to ingestion, contact, or inhalation, that cause a series of chain reactions in the immunological system. These reactions affect only some individuals and may happen after ingestion of very small amounts of allergen. The prevalence of food allergies represents around 5−8% in children and 1−2% in adults, although self-reported food allergies are higher and run at around 25% of the population.[1]

Consumers intolerant or allergic to different foodstuffs can react to a wide range of quantities of allergenic foods. These can vary depending on the individual's personal tolerance, their health, and their current medication. Due to these reactions, sensitive individuals must avoid the consumption of any foods that could contain these allergens, so it is essential that all of the necessary information is included in the labeling of the products. There exist different regulations that force to reflect in the labeling of the food the presence of any potentially allergenic substances used in the production of that food and that remains in the final product (EU Directive 2003/89/EC and EU Directive 2007/68/EC, both amending 2000/13/EC).[2−4] However, allergens can be present in the final products although their ingredients are not allergens and, for this reason, these allergens were not included in the label. This is due to cross-contamination between raw materials, production lines, or equipment. Another cause is the fraud due to the substitution of a nonallergenic ingredient by another with minor commercial value that is allergenic or ingredients such as soy, used for the adulteration of products. The use of these substitutions makes these products more cost-effective, but also they are allergenic, which is not declared on the label.

Hypersensitivity reactions related to ingestion of seafood, including shellfish, are one of the most common food allergies in adults.[5] Crustaceans, including shrimp, prawns, crabs, lobster, and crayfish, are a frequent cause of anaphylaxis or hypersensitivity,[6,7] and shrimp and crab are the most common causes of allergy. Symptoms most often occur when the seafood is ingested, but can also appear when raw seafood is handled and even after inhaling steam while crustaceans such as shrimp are being cooked (may cause respiratory reactions).[8] Several studies have identified tropomyosin, a myofibrillar protein highly conserved, as the major allergen in mollusks and crustaceans.[9−12]

The most common methods for detecting allergens in shellfish are particularly enzyme-linked immunosorbent assay (ELISA) and the PCR technologies. There are some works that develop the ELISA method to detect the tropomyosin of crustaceans.[13−16] Nevertheless, genetic technologies based on DNA analysis present advantages such as that the DNA molecules are more thermostable than the proteins, allowing analysis of the highly processed products. In addition, these techniques are cheaper and faster and allow the detection of very small quantities.

It worth noting the work of Brzenzinki, who developed a PCR restriction fragment length polymorphism (PCR-RFLP) to detect and identify crustacean DNA in food products.[17]

Real-time PCR is new technique used increasingly in the detection of allergens.[18−20] Rapid detection of these allergens in the production chain is a topic of vital importance to prevent cross-contamination and ensure food security. It is very important to get these results in the shortest time possible to avoid economic losses produced by retention of products pending receipt of results. In this sense, the real-time PCR allows the detection of PCR amplification during the early phase of the reaction. This technique reduces significantly the analysis time unlike traditional PCR detection methods. On the other hand, it reduces the risk of contamination because it is no post-PCR processes are necessary. In addition to its high sensitivity and specificity, real-time PCR provides fast, precise, and accurate results. Numerous chemistries and instruments for

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Table 1. Species Included in the Present Work[a]

crustaceans	common name
Bathynectes maravigna	deep-sea crab
Callinectes sapidus	blue crab
Callinectes similis	lesser blue crab
Cancer borealis	Jonah crab
Cancer irroratus	Atlantic rock crab
Cancer novaezelandiae	pie crust crab
Cancer pagurus	edible crab
Carcinus maenas	green crab
Chionoecetes opilio	snow crab
Fenneropenaeus indicus	Indian white prawn
Fenneropenaeus merguiensis	banana prawn
Hyas coarctatus	Arctic lyre crab
Liocarcinus corrugatus	wrinkled swimcrab
Liocarcinus depurator	harbour crab
Litopenaeus vannamei	whiteleg shrimp
Macropipus tuberculatus	knobby swimcrab
Maja squinado	spider crab
Metapenaeopsis barbata	red rice prawn
Metapenaeus macleayi	white river prawn
Munida spp	squat lobster
Necora puber	velvet swimming crab
Ovalipes catharus	paddle crab
Pachygrapsus marmoratus	marbled rock crab
Panulirus argus	Caribbean spiny lobster
Parapenaeus longirostris	beep-water rose shrimp
Parapenaeopsis sculptilis	coral prawn
Penaeus esculentus	brown tiger prawn
Penaeus monodon	giant tiger prawn
Plagusia chabrus	red rock crab
Polybius henslowii	Henslow's swimming crab
Polybius holsatus	flying crab
Pollicipes pollicipes	goose neck barnacle
Pollicipes polymerus	leaf barnacle
Portunus gibbesii	iridescent swimming crab
Rimapenaeus faoe	indio shrimp
Rimapenaeus similis	roughback shrimp
Scyllarus demani	scyllarid lobster
Semibalanus balanoides	acorn barnacle
Solenocera vioscai	humpback shrimp
Trachysalambria curvirostris	southern rough shrimp
Xiphopenaeus kroyeri	Atlantic seabob
Zaops ostreum	oyster pea crab
bivalves	**common name**
Cerastoderma edule	common cockle
Chlamys varia	variegated scallop
Corbicula fluminea	Asian clam
Donax variabilis	variable coquina
Ensis ensis	pod razor shell
Paphia undulata	short-necked clam
Pecten jacobeus	pilgrim's scallop
Pecten maximus	great scallop
Tapes pullastra	carpetshell
Venerupis decussata	cross-cut carpet shell
Venerupis variegata	variegated carpet shell
fishes	**common name**
Coryphaena hippurus	mahi-mahi
Genypterus blacodes	pink cusk-eel
Lophius budegassa	European anglerfish
Merluccius bilineariz	Atlantic hake
Oreochromis aureus	blue tilapia
Sebastes marinus	golden redfish
Tetrapturus angustirostris	shortbill spearfish
Trachurus trachurus	horse mackerel
Xiphias gladius	swordfish
cephalopods	**common name**
Alloteuthis media	midsize squid
Eledone moschata	musky octopus
Illex coindetii	southern shortfin squid
Loligo forbesii	veined squid
Loligo gahi	Patagonian squid
Loligo vulgaris	European squid
Octopus defilippi	Atlantic longarm octopus
Octopus vulgaris	common octopus
Sepia pharaonis	pharaoh cuttlefish
Todarodes eblanae	lesser flying squid
gastropods	**common name**
Murex brandaris	baby-whelk

[a]Only one of the possible common names for each species is given.

detection are now available. There are two main types of fluorescent dyes, intercalating dyes such as SYBR Green I and specific probes labeled with fluorophores, but the probes are more specific than intercalating dyes because the latter bind nonspecifically to double-stranded DNA.

Fast real-time PCR is the most novel real-time PCR in which by optimizing the reaction and cycling conditions of existing assays, such as reducing the DNA polymerase activation time, the duration of each PCR cycle is shortened, and combining the annealing and extension steps allows faster run times. Also, a rapid hot start can be achieved by the use of a special buffer allowing a significant reduction of PCR cycling times. All of these things allow doing the analyses in 40 min. Another advantage over standard real-time PCR is its higher sensitivity, accuracy, dynamic range, and specificity.

In this work a fast real-time PCR for crustacean detection in all types of food products, regardless of the degree of processing that has been undergone, has been developed. To carry out the present work, a locked nucleic acids probe (LNA) has been selected. This probe is a novel type of nucleic acid analogue that contains a 2′-O,4′-C methylene bridge. The bridge is locked in 3′-endo conformation, restricting the flexibility of the ribofuranose ring and locking the structure into a rigid bicycled formation. Due to this structure, the higher Tm provided by LNA increases thermal stability and hybridization specificity. In the other hand, shorter LNA probes allow easier and more flexible probe designs for problematic target sequences.[21]

This methodology is a useful tool to ensure food safety and quality and, therefore, the safety of consumers. Due to its high sensitivity it is appropriate for the analysis of cross-contamination in production chains.

■ MATERIALS AND METHODS

Sample Collection, Storage, and DNA Extraction. Specimens of crustaceans obtained for the development of this methodology were morphologically characterized and authenticated by genetic analyses (sequencing) (Table 1). Also, different species of mollusks such as

bivalves, cephalopods, and fishes were obtained for the study of specificity of the method.

DNA extraction was performed using the SureFood PREP Allergen kit (R-Biopharm) according to the protocol described by the manufacturer. The quality and concentration were determined by measuring the absorbance at 260 nm and the 260/280 nm and 234/260 ratios using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).[22] DNA extractions were labeled and preserved at −80 °C.

Design of a Specific Fast Real-Time PCR Method To Detect Crustacean. The primers used in the present work were taken from a published single PCR system.[17] Sequences of the 16S rRNA of crustaceans were downloaded from the National Center for Biotechnology Information (NCBI) and subsequently were aligned with Clustal W[23] integrated into BioEdit 7.0.[24] A LNA TaqMan probe labeled as Crustaceous was designed inside the fragment of approximately 205 bp used by Brzezinski: 5′(6-FAM) TGC TAC CTT IGC ACG GTCA (BHQ1) 3′.

The PCR reactions were carried out in a total volume of 20 μL containing 125 ng of DNA template, 10 μL of TaqMan Fast Advanced Master Mix (Applied Biosystems), the amount of primers and probe that were optimized, and molecular biology grade water (Eppendorf) to adjust to the final volume. The optimal concentrations of primers and probe were evaluated by preparing dilution series. First, forward and reverse primer concentrations of 50, 300, 500, and 900 nM were used to determine the minimum primer concentrations giving the maximum ΔRn (the maximum fluorescence emission intensity and the lowest threshold cycle, C_t). All have been evaluated with a constant probe concentration of 50 nM. Following primer optimization, probe optimization was initiated, and concentrations of 50−250 nM were evaluated.[25]

All reactions included a negative control containing all reagents of the reaction and molecular grade water instead of DNA. The reactions were performed in triplicate on DNA samples in MicroAmp Optical 96-well reaction plates (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems) using the ViiA 7 Real-Time PCR System (Applied Biosystems). Amplification was carried out with the following conditions: 95 °C for 20 s and 40 cycles each of 95 °C for 1 s and 60 °C for 20s.

The specificity of the assay was tested with different species of mollusks, bivalves, cephalopods, and fishes among which are DNA extracted from several potential cross-reacting species; the species tested are included in Table 1.

A serial dilution of DNA of crustaceans was performed with DNA of other species of seafood in levels ranging from 125 ng to 1.25 pg, and the fluorescence signal was determined to calculate the limit of detection (LOD) of the method herein developed. The dilutions were prepared by adding crustacean DNA and DNA from different species until the final amount of 125 ng was completed. All measurements were performed in triplicate from five samples independently.

Methodological Validation. Species of crustaceans were authenticated on the basis of their morphological features and by means of genetic analyses. These individuals were cooked at 99 °C in water for 20 min, and the cooking water was used to spike different food matrices; elaborated products simulated products that can be found currently on the market such as canned fish, battered fish, and fishcakes. The amount of DNA in the cooking water added was the minimum quantity detected during the development of the present method. The presence of additives used in the alimentary industry as spices or sauces attenuates or inclusively inhibits the DNA amplification. Moreover, the different kinds of sauces added produce differences in the quantity and quality of the extracted DNA as this molecule is very sensitive to acid and alkaline agents. In this sense, it is worth highlighting the pickled products, in which the low pH produces higher DNA degradation.[26] The most extreme treatment that was applied to the samples was sterilization in a horizontal retort steel−air, at 115 °C for 50 min, with 1.2 bar of overpressure. These treatments applied to samples were performed in the pilot plant of ANFACO-CECOPESCA.

The products were analyzed according to the same method as described for the standard species. The objective of the methodological validation was to prove that the treatments to which processed products are subjected do not affect the detection of crustacean.

RESULTS AND DISCUSSION

Design of a Specific Fast Real-Time PCR Method To Detect Crustacean. In this study, a specific fast real-time PCR method for crustacean detection was designed.

Success in real-time PCR depends on the purity and integrity of the template and the primers and probe used. All samples used had a good quality of DNA with an A_{260}/A_{280} ratio between 1.7 and 2.0 because higher or lower ratios may indicate the presence of impurities or contaminants in the solution, typically either protein or phenol, that can affect the assay. It is very important to optimize the reaction to grant the highest level of sensitivity while maintaining the specificity of the technique. Optimal performance is achieved by selecting the primer and probe concentrations that provide the lowest C_T and highest ΔRn (normalized reporter, defined as emission intensity of reporter/emission intensity of passive reference). The conditions that allow one to obtain the best results in terms of specificity and sensitivity in the crustacean detection were established by means of mixtures of different concentrations of primers and probe. Finally, the optimal concentrations were 900 nM for both primers and 250 nm for probe.

The specificity of the primer/probe set was confirmed using pure genomic DNA from other taxonomic groups not belonging to crustacean. The specificity of primers was previously studied by Brzezinski. One of the advantages of the real-time PCR is that the use of a specific probe that recognizes only a specific amplicon increases the specificity of the assay. In the present work, the inclusion of the probe gives more specificity to the assay; this would never diminish. No cross-reactivity was detected with any of the tested samples (Table 1; Figure 1).

The optimal annealing temperature that allows the correct detection of crustacean was 60 °C. This temperature depends on the melting temperature (T_m) of the primers and probe and should be determined empirically. The mean values of C_t obtained were 18 ± 1 in fresh and frozen crustaceans with a concentration of 125 ng of DNA.

On the other hand, robust and precise assays are usually correlated with high PCR efficiency. The amplification plot of the experiment using the primer/probe crustacean set generates a slope of −3.392 or 97% of efficiency, with a correlation coefficient of 0.999 (Figure 2; Supporting Information, Figure 3).

These C_t and efficiency values demonstrate the utility of the fast real-time PCR system to detect crustacean in food.

The LOD is defined as the lowest concentration of crustacean DNA at which 95% of the positive samples are detected. The developed real-time PCR assay required 1.25 pg of crustacean for positive results.

Methodological Validation. The purpose of the methodological validation was to check whether the treatments that processed foods underwent had any influence on crustacean detection after application of the developed method.

The elaborated products in the pilot plant of ANFACO-CECOPESCA with the cooking water of crustaceans were analyzed according to the proposed methodology. One of the major problems of the DNA techniques is the quality of the extracted DNA. For this reason, this approach is useful to assess and optimize the conditions of the developed methodology because the quality and quantity of DNA in processed products can be affected due to thermal, mechanical, and chemical processes that can cause fragmentation or could reduce the

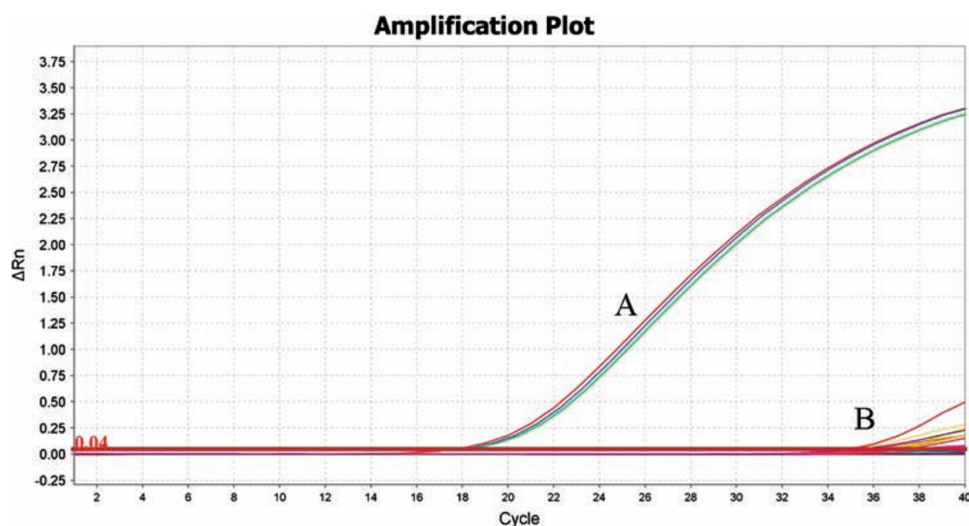

Figure 1. Specificity of the real-time PCR assay: (A) amplification pattern shown by a crustacean; (B) amplification pattern shown by other taxonomic groups.

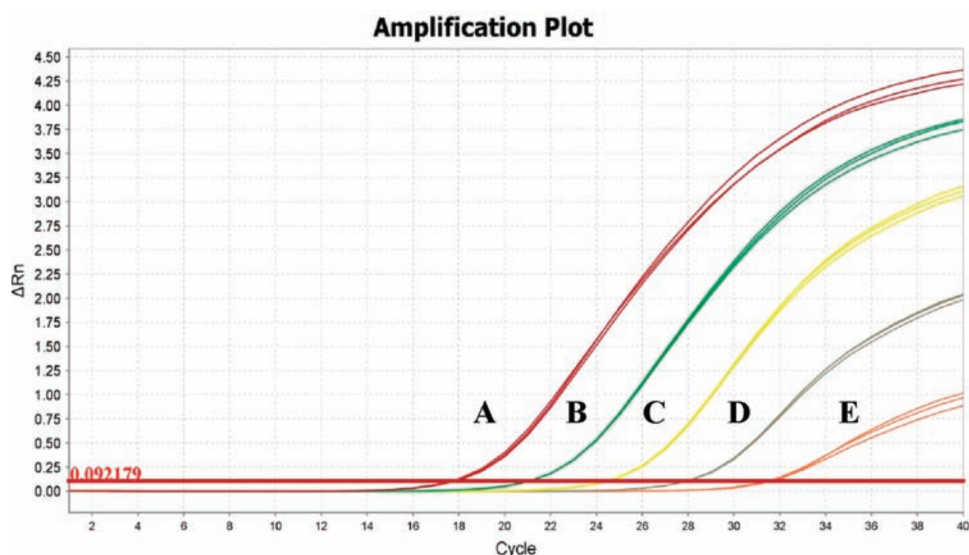

Figure 2. Efficiency of the real-time PCR assay: (A) 125 ng; (B) 12.5 ng; (C) 1.25 ng; (D) 125 pg; (E) 12.5 pg of total DNA from crustacean.

efficiency of the amplification. To ensure the amplification, some authors establish a maximum fragment size of about 200 bp. Moreover, the presence of certain sauces, acids, fats, and other additives can also affect the reaction as inhibitors of PCR or produce higher DNA degradation.[27] In the present work the size of the fragment used ensures the correct amplification; however, all of these factors can affect incrementing the value of C_t in these types of processes. In all samples that contain crustacean, the C_t values obtained were 18 ± 1, when 125 ng of fresh and frozen template was used; in processed products such as canned or pickled products spiked with crustacean cooking water, this C_t was major, between 28 and 34, but always <35. This is due to the thermal treatments and low pH these products are subjected to. In the cross-reactivity analysis no false-positive results were observed, even under the most severe conditions used in the test, as documented by C_t values of >35.

These results demonstrate that low quantities of crustaceans present in different processed products are easily detected with the present methodology, which can be applied to frozen, canned, fried, cooked, battered, and elaborated products such as cakes.

The fast real-time PCR herein developed provides advantages relative to accuracy, sensitivity, specificity, dynamic range, high-throughput capacity, and lower risk of contamination and reduces the time of assay to 40 min. It also presents an advantage over ELISA techniques in that DNA is much more stable, which allows the detection of crustacean and trace amounts of crustacean in all kinds of products including those that have undergone aggressive treatments such as high temperatures or pressures, which can cause structural changes in the proteins and problems in the reaction with the antibodies. Also, in the ELISA are possible cross-reactions between closely related proteins. The present method can be useful in questions regarding food quality and security control and in the verification of the implementation of labeling requirements to protect consumer rights.

■ ASSOCIATED CONTENT

Supporting Information

Figure 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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