



Original Research Article

A new real-time PCR quantitative approach for the detection of shrimp crustaceans as potential allergens



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ABSTRACT

Allergy to crustaceans is an increasingly important food safety issue. To protect people from experiencing adverse allergic reactions, reliable methodologies are necessary to verify the labelling of processed seafood. In the present work, two new DNA-based approaches targeting the 16S rRNA mitochondrial gene are proposed to detect crustaceans in foods using a qualitative PCR assay specific for crustaceans (shrimps, lobsters and crabs) and a quantitative real-time PCR assay specific for shrimp crustaceans. The real-time PCR system allowed the detection and quantification down to 0.1 pg and 0.0001% (w/w) of shrimp DNA and shrimp in model mixtures, respectively. The method exhibited high performance for quantitative analysis in the range of 0.0001% to 50% as inferred by the calibration curve parameters being effectively validated with blind mixtures. The qualitative PCR assay can provide a simple, fast and high throughput tool for screening the presence of crustaceans in processed foods, while the proposed real-time PCR method proved to be a useful tool for the accurate detection and quantification of shrimp in foods at trace levels.

1. Introduction

The international trade of seafood species and products has been growing over the years, mainly due to the high nutritive value and popularity of these foods across many countries. However, the increased consumption of seafood has been followed by more frequent reports of allergic reactions among consumers (Lopata et al., 2010). Recent data suggest that shellfish allergy can affect up to 2.5% of the general population (especially among adults) (Woo and Bahna, 2011). Crustaceans, such as shrimps, crabs or lobsters, represent a major food resource with high commercial value, being also responsible for eliciting the majority of the allergic reactions related to shellfish (Pedrosa et al., 2015; Thalayasingam and Lee, 2015; Khora, 2016). About 60% of all crustaceans (13.9 million tonnes), based on global capture and production, belong to shrimps/prawns, from which *Litopenaeus vannamei* is the main crustacean species, with a production of 3.9 million tonnes in 2015 (FAO, 2017).

Crustacean-allergic individuals can experience moderate to strong adverse immunological reactions, including anaphylaxis. Accordingly, for the protection of at least 95% of this population group, a reference dose of 10 mg of shrimp protein (corresponding to 44 mg of shrimp) has been recently proposed using, in the case of shrimp, threshold data of $n < 80$ individuals (Allen et al., 2014). The establishment of reference doses for allergenic food residues, including shrimps, is part of the

VITAL (Voluntary Incidental Trace Allergen Labelling) program of The Allergen Bureau of Australia & New Zealand (ABA), in order to guide advisory labelling decisions to use on food labels (Allen et al., 2014; Taylor et al., 2014). Therefore, allergic individuals should rely on the labelling information to avoid the consumption of any offending food. Besides, cross-contamination occurrences during production, storage or transport may lead to the inadvertent presence of allergens in foods (Herrero et al., 2012; Fernandes et al., 2017). Hence, in order to protect sensitised persons, the labelling of fourteen allergenic ingredients (including crustaceans) is demanded by the European Union legislation (Directive 2007/68/EC, Regulation (EU) No 1169/2011). To comply with it, several producers use frequently the precautionary labelling “may contain traces of...”, which often causes some indecision or rejection by food allergic consumers. Therefore, sensitive analytical methods aimed at detecting trace levels of the allergenic ingredients in complex matrices are needed to verify the labelling of crustaceans.

According to the Regulation (EU) No 1169/2011, the detection of the allergenic food is demanded, but the target analyte does not necessarily have to be the allergenic protein itself. In this sense, both protein- and DNA-based methods have been widely used for allergen detection (Zhenxing et al., 2010; Eischeid et al., 2013; Prado et al., 2016). For protein analysis, the enzyme-linked immunosorbent assay (ELISA) is by far the most widely used immunochemical technique to identify and quantify allergens in food (Lopata et al., 2010; Zhenxing

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et al., 2010; Wang et al., 2011; Gomaa and Boye, 2015). In spite of the major advantages of this methodology associated with simplicity, speed, high specificity and sensitivity inherent to the antigen/antibody interaction, it also presents some drawbacks. Cross-reactivity phenomena can lead to the occurrence of false positive results, while conformational changes of proteins caused by food processing can lead to false negative results (Costa et al., 2014; Prado et al., 2016). On the other hand, DNA-based methods meet some advantages in comparison with immunochemical assays. DNA molecules are almost ubiquitous in any organic matter and more resistant to processing than proteins, which allows their analysis from difficult matrices, such as processed and complex foods (Herrero et al., 2012; Eischeid et al., 2013). For allergen analysis, real-time PCR-based methods have been widely applied (Mafra et al., 2008; Prado et al., 2016; Costa et al., 2017). Their specificity has relied mainly on primers and probes targeting sequences of genes encoding for allergenic proteins, but mitochondrial genes have also been demonstrated to be useful targets (Prado et al., 2016; Costa et al., 2017). In the case of crustacean detection and differentiation, the mitochondrial genes, such as 16S rRNA (Cao et al., 2011; Pascoal et al., 2011; Herrero et al., 2012; Eischeid et al., 2013; Mäde and Rohmberger, 2017; Zagon et al., 2017; Wilwet et al., 2018), 12S rRNA (Eischeid et al., 2013; Eischeid, 2016; Eischeid and Stadig, 2018) and cytochrome oxidase subunit I (COI) (Eischeid et al., 2013; Fernandes et al., 2017) have been used as specific markers, taking advantage of their high copy number that usually results in more sensitive assays. From the available reports on real-time PCR, several of them allow high sensitivity levels of detecting particular groups of crustacean species (Cao et al., 2011; Herrero et al., 2012; Eischeid et al., 2013; Eischeid, 2016; Mäde and Rohmberger, 2017; Zagon et al., 2017; Eischeid and Stadig, 2018). However, only a few reports are based on quantitative real-time PCR methods using model mixtures simulating low levels of spiked crustaceans (Eischeid et al., 2013; Eischeid, 2016; Eischeid and Stadig, 2018). Eischeid et al. (2013) describes two real-time PCR systems able to quantify DNA from penaeid shrimps and crabs. Eischeid (2016) reports a real-time PCR quantitative system specific for lobsters based on model mixtures as calibrants, followed by a similar approach for crabs (Eischeid and Stadig, 2018), both methods being effective for allergen quantification.

Considering the scarce methods for crustacean allergen quantification by DNA-based methods, the development of a quantitative real-time PCR system specific for a wide range of shrimp species is proposed, validated and applied to verify labelling compliance of foods. Additionally, the performance of quantitative analysis of the new method is compared with ELISA.

2. Materials and methods

2.1. Sample collection and preparation

Several crustacean specimens from different species ($n = 18$) (whiteleg shrimp – *Litopenaeus vannamei*; speckled prawn – *Metapenaeus monoceros*; Indian white prawn – *Fenneropenaeus indicus*; giant tiger prawn – *Penaeus monodon*; Jinga shrimp – *Metapenaeus affinis*; green tiger prawn – *Penaeus semisulcatus*; Argentine red shrimp – *Pleoticus muelleri*; razor mud shrimp – *Solenocera melanthero*; Udang merah – *Solenocera crassicornis*; knife shrimp – *Haliporoides triarthrus*; common prawn – *Palaemon serratus*; scarlet shrimp – *Aristaeopsis edwardsiana*; Norway lobster – *Nephrops norvegicus*; European lobster – *Homarus gammarus*; Caribbean spiny lobster – *Panulirus argus*; crab – *Portunus validus*; velvet swimming crab – *Necora puber*; edible crab – *Cancer pagurus*) (Table 1) were purchased from local markets or provided by Marfresco (Loures, Portugal) and Brasmar Seafood Companies (Trofa, Portugal), who identified most of the species through morphological characteristics. The identity of *L. vannamei*, *M. Monoceros*, *F. indicus*, *P. monodon*, *M. affinis* was previously confirmed by sequencing (Fernandes et al., 2017). The selection of species was made regarding the relevance

in terms of consumption and availability in different commercial areas of the Portuguese market.

A total of 62 non-crustacean species was also used (pre-existing extracts or tissues) to evaluate the assay specificity: seafood species ($n = 28$) (Atlantic cod, Pacific cod, Alaska pollock, saithe, ling, Atlantic salmon, gilt-head bream, common sole, European pilchard, yellowfin tuna, European hake, North Pacific hake, Argentine hake, whiting, deepwater hake, ray, European seabass, whiting-pout, Atlantic horse mackerel, tadpole codling, rose fish, rock ling, Pacific mackerel, black scabbardfish, Nile perch, squid, mussel, undulated surf clam), meat species ($n = 15$) (wild boar, duck, partridge, hare, quail, pheasant, red deer, rabbit, chicken, turkey, lamb, ostrich, cow, horse, pig) and plant species ($n = 19$) (onion, garlic, parsley, pepper, bay leaf, sweet chili, tomato, maize, soybean, potato, manioc, lupin, chestnut, walnut, broad bean, rye, wheat, rice, pumpkin).

Several seafood products ($n = 18$), including shrimp patties, seafood broth/soups, surimi and pre-cooked dishes, were also acquired at local markets for assay applicability.

The species tissues and food samples were ground and homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) and stored at -20°C until further analysis. All containers and grinding mill knives used during this procedure were previously treated with DNA decontamination solution (DNA-ExitusPlus™; AppliChem, Darmstadt, Germany).

2.2. Preparation of model mixtures

The preparation of binary model mixtures was intended to simulate a processed shrimp stuffing/filling (e.g., shrimp patty fill). Shrimp meat (*L. vannamei*) was boiled in water for 5 min, drained and minced in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). A mixture containing 50% (w/w) of ground-cooked shrimp kernel in béchamel sauce (milk, milk cream, wheat flour, maize starch and salt) was prepared by the addition of 200 g of shrimp to the same amount of sauce. Afterwards, successive additions of béchamel sauce to the 50% homogenised mixture enabled the preparation of the following binary proportions containing 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% (w/w) of shrimp in béchamel. Identically, for method validation, blind mixtures were prepared to contain 8.0, 4.0, 2.5 and 0.25% (w/w) of shrimp in béchamel. The binary mixtures were stored at -20°C until further analysis.

2.3. Primer and probe design

Sequences of the 16S rRNA gene were selected from NCBI database from a set of 17 different crustacean species (Fig. 1). However, it was not possible to find available sequences for the *P. validus* species. Sequence alignment was performed with BioEdit v.7.2.5 software (Ibis Biosciences, Canada) using ClustalW (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and examined for suitable regions for primer and probe design. The primers were designed to amplify as many species as possible within crustacean families; therefore regions of the 16S rRNA gene with very low intra- and interspecific variability, and also low intra- and intergenus variability were chosen (Eischeid et al., 2013; Mäde and Rohmberger, 2017). The primers (16SCrust2-F: TAA AGT CTG GCC TGC CCA CTG; 16SCrust1-R: AGC TTT ATA GGG TCT TAT CGT C) and probe (16SCrust1-P: FAM-TTA ATT GAA GGC TTG TAT GAA TGG TTG GAC-BHQ1) (fragment of 203 bp) were then submitted to a basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), in order to identify regions of local similarity among homologue sequences of different species and to calculate the statistical significance of the matches. Primer properties, self-hybridisation and the absence of hairpins were evaluated using the software OligoCalc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). The oligonucleotides were synthesised by STABVIDA (Lisbon, Portugal).

Table 1

Qualitative PCR results for eukaryotic and crustacean-specific amplifications and real-time PCR cycle threshold values for several crustacean species.

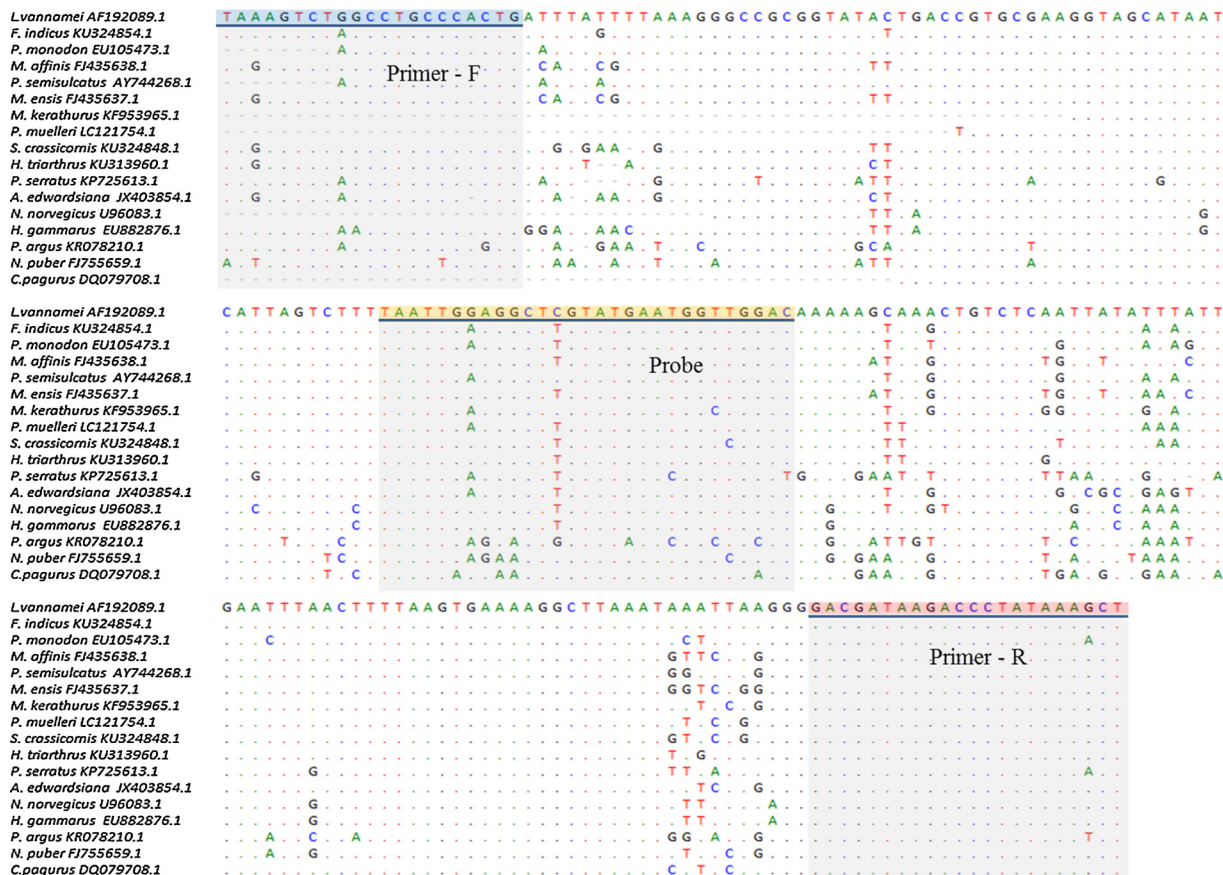
order	suborder	infraorder	superfamily	family	species	scientific name	qualitative PCR ^a		real-time PCR
							eukaryotic (18S rRNA)	crustacean (16S rRNA)	
Decapoda	Dendrobranchiata		Penaeoidea	Penaeidae	whiteleg shrimp	<i>Litopenaeus vannamei</i>	+	+	20.20 ± 0.30
					speckled prawn	<i>Metapenaeus monoceros</i>	+	+	23.53 ± 0.36
					Indian white prawn	<i>Fenneropenaeus indicus</i>	+	+	23.40 ± 0.21
					giant tiger prawn	<i>Penaeus monodon</i>	+	+	22.17 ± 0.09
					Jinga shrimp	<i>Metapenaeus affinis</i>	+	+	20.22 ± 0.09
					green tiger prawn	<i>Penaeus semisulcatus</i>	+	+	23.88 ± 0.16
				Solenoceridae	Argentine red shrimp	<i>Pleoticus muelleri</i>	+	+	21.79 ± 0.21
					razor mud shrimp	<i>Solenocera melanthero</i>	+	+	22.01 ± 0.07
					coastal mud shrimp	<i>Solenocera crassicornis</i>	+	+	20.60 ± 0.38
					knife shrimp	<i>Haliporoides triarthrus</i>	+	+	20.53 ± 0.28
					scarlet shrimp	<i>Aristaeopsis edwardsiana</i>	+	+	20.47 ± 0.11
	Pleocyemata	Caridea	Palaemonoidea	Palaemonidae	common prawn	<i>Palaemon serratus</i>	+	+	–
					Norway lobster	<i>Nephrops norvegicus</i>	+	+	20.28 ± 0.06
		Astacidea	Nephropoidea	Nephropidae	European lobster	<i>Homarus gammarus</i>	+	+	21.14 ± 0.12
					Caribbean spiny lobster	<i>Panulirus argus</i>	+	+	–
		Brachyura	Portunoidea	Portunidae	smooth swimming crab	<i>Portunus validus</i>	+	+	–
					velvet swimming crab	<i>Necora puber</i>	+	+	–
			Cancroidea	Cancridae	edible crab	<i>Cancer pagurus</i>	+	+	–

^a (+) positive qualitative PCR amplification.^b Mean values of cycle threshold (Ct) ± standard deviation (SD) of replicate assays (n = 4); (–) non-detected amplification.

2.4. DNA extraction

The DNA from all crustacean species and binary mixtures (100 ng) was extracted using the SureFood® Prep advanced kit (CONGEN Biotechnologie GmbH, Berlin, Germany), according to the manufacturer's instruction of Protocol 1. All DNA extractions were done in duplicates and the extracts were kept at –20 °C until further analysis.

The concentration and purity of the extracts were assessed by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc.).

**Fig. 1.** Sequence alignment of NCBI 16S rRNA gene entries showing primer (16SCrust2-F/16SCrust1-R) and probe (16SCrust1-P) design regions.

The quality of extracted DNA was also analysed by electrophoresis in a 1.0% agarose gel containing GelRed 1× (Biotium, Hayward, CA) for staining and carried out in STGB 1× (GRISP, Porto, Portugal) for 25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories).

2.5. Qualitative PCR amplification

The following PCR protocol was used for DNA amplification: 25 µL of total reaction volume containing 2 µL of DNA extract (100 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.01% of Tween 20 (Genaxxon Bioscience, Ulm, Germany), 200 µM of each dNTP (Bioron, Ludwigshafen, Germany), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl₂ (Genaxxon Bioscience, Ulm, Germany) and 200 nM of each primer (16SCrust2-F/16SCrust1-R). The amplifications were performed in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA) using the following cycling program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

To guarantee the absence of any false negative results, the amplification capacity of all the DNA extracts was verified by PCR with universal eukaryotic primers targeting the nuclear 18S rRNA gene (18SRG-F/18SRG-R), as described by Costa et al. (2013). The PCR products were then visualised by electrophoresis in a 1.5% agarose gel, as described in the previous section.

2.6. Real-time PCR amplification

For the real-time PCR amplifications, 20 µL of total reaction mixture included 1× of SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA), 300 nM of each primer (16SCrust2-F/16SCrust1-R), 200 nM of probe (16SCrust1-P) and 2 µL of DNA extract. The following temperature conditions performed on a fluorometric thermal cycler CFX96 real-time PCR detection system (Bio-Rad Laboratories) were used: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 60 °C for 45 s, with collection of fluorescence signal at the end of each cycle. Fluorescence data were analysed using the software Bio-Rad CFX Manager 3.1. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at automatic threshold settings.

For the determination of absolute limit of detection (LOD) and limit of quantification (LOQ) a series of 10-fold serially diluted shrimp DNA samples were prepared (100 ng to 0.01 pg), while the binary mixtures (0.0001–50%) allowed the determination of the relative LOD and LOQ of shrimp meat in food matrix. The LOD was considered as the lowest amplified level for 95% of the replicates and the LOQ was established as the lowest amplified level within the linear or dynamic range of the calibration curve (ENGL, 2015).

2.7. ELISA

Two different ELISA kits for the detection of crustaceans in foods were used for comparative purposes with the proposed real-time PCR assay. The MonoTrace™ Crustacea ELISA Kit (BioFront Technologies, Tallahassee, FL) is a monoclonal antibody (Mab)-based assay used for the quantification of crustacean (1–40 mg/kg) presence/contamination in several food matrices, while RidaScreen® Fast Crustacean (R-Biopharm AG, Darmstadt, Germany) claims the detection of crustacean proteins (mainly tropomyosin) in a range of 20–160 mg/kg.

The extraction of proteins from model mixtures and food samples used in both ELISA kits was carried out according to the manufacturers' description. Briefly, a pre-heated extraction buffer (9 mL or 20 mL for MonoTrace or RidaScreen kits, respectively) was added to 1 g (double extraction for each sample) of previously ground sample and incubated

for 10 min (42 °C or 60 °C for MonoTrace or RidaScreen kits, respectively) with continuous mixing. The sample was centrifuged (2000g or 2500 g for MonoTrace or RidaScreen kits, respectively) and the aqueous phase was transferred to new tubes for subsequent analysis.

Afterwards, similar protocols were performed using both ELISA kits according to the manufacturers' instructions. The absorbance values were plotted against the decimal logarithmic concentration of the crustacean protein standard solutions. A non-linear regression function was carried using a sigmoid four parametric logistic function:

$$B \equiv Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^b} + D$$

Y being the optical density (absorbance), A the maximum absorbance, b the slope of the calibration curve in linear range, C the 50% inhibition-concentration – IC₅₀ (µg/L), D the minimum absorbance, and X the analyte concentration (µg/L) (Fig. S1, Supplementary material). Each sample was analysed in triplicate.

3. Results and discussion

3.1. Specificity of primers targeting the 16S rRNA gene

A region of the mitochondrial 16S rRNA gene was used as a group specific target for the crustacean detection. This evidence was firstly demonstrated by *in silico* analysis, which enables the identification of homologue regions of crustaceans among the available sequences at NCBI. The specificity of primers (16SCrust2-F/16SCrust1-R) were assessed with primer-Blast tool, targeting the nucleotide collection (nt) of organisms from Penaeidae, Solenoceridae, Palaemonidae, Nephropidae, Palinuridae, Portunidae, Aristeidae and Cancridae families. Fig. 1 presents the sequence alignment of NCBI 16S rRNA gene entries, using only one for each species for simplicity, though there are several accesses at NCBI. For instance, in the case of *L. vannamei*, there are about 20 sequences for the same query coverage 99–100%, with the exact identity (99–100%). Results from this analysis (over 13,300 Blast hits) predicted a fragment with an expected size of 203 bp for the species presented in Fig. 1, although the length of the product may vary between 199 and 206 bp for other crustacean species. The results from Nucleotide Blast analysis (using megablast algorithm) over the query sequence of 203 bp verified a query coverage of > 97% with 95–100% of identity and E-value < 3 × 10^{−85} for the GenBank accessions of the targeted nucleotide collection of organisms, thus confirming the *in silico* specificity of the primers for crustaceans. Experimentally, the PCR amplification with the proposed primers showed a fragment with an expected size of approximately 200 bp for all the tested crustacean species (Table 1, Fig. S2, Supplementary material). The specificity of primers was further assessed using the DNA of 62 food-related species (seafood, other animal species and plants), previously described (Section 2.1). Since no cross-reactivity was observed with any of the non-target species, the specificity of the assay was confirmed for crustacean species (Table S1, Supplementary material). As the extracts were verified as containing DNA suitable for PCR amplification with universal eukaryotic primers, false negative results can be excluded.

3.2. Real-time PCR method development

A real-time PCR probe system targeting the 16S rRNA gene was proposed for the detection and quantification of crustacean species, focusing on the group of shrimps. Considering the results of qualitative PCR (Section 3.1), the specificity of the real-time PCR system was verified for all tested crustacean species, the cycle threshold (Ct) values being presented in Table 1. As can be observed, Ct values ranged from 20.20 (*Litopenaeus vannamei*) to 23.88 (*Penaeus semisulcatus*) and five crustacean species were not amplified by real-time PCR, namely *Palaemon serratus*, *Panulirus argus*, *Portunus validus*, *Necora puber* and

Cancer pagurus (Table 1). The negative amplifications are supported by the 16S rRNA gene sequence alignment (Fig. 1), particularly when the probe region is considered. In fact, *C. pagurus* and *P. serratus* presented 4 nucleotide differences comparing with the reference sequence (*L. vannamei*), while *N. puber* and *P. argus* showed 5 and 8 nucleotide mismatches, respectively. Generally, the real-time PCR system was effective in detecting all the species from the Penaeoidea superfamily, in which are included the most commonly consumed shrimp species. Only one shrimp species (*P. serratus*) was not amplified with the probe system because it belongs to a more distantly related taxonomic group at the level of suborder (Pleocyemata). On the other hand, in the same suborder, the species of Nephropidae family were amplified, while crab species from Palinuridae, Portunidae and Cancridae families were not. The cross-reactivity of species of Nephropidae family also occurred in the method of Eischeid and Stadig (2018) specific for crabs, probably because they share common features to both crabs and shrimps. Therefore, the proposed real-time PCR method can be considered as a specific approach for the detection and quantification of the most common shrimp species, particularly those included in the superfamily of Penaeoidea (Penaeidae, Solenoceridae and Aristeidae).

For the development of the real-time PCR quantification approach, the requirements set by the European Network of GMO Laboratories (ENGL) document “Definition of minimum performance requirements for analytical methods of GMO testing” (ENGL, 2015) and the guidelines of “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (Bustin et al., 2009) were taken into consideration. The real-time PCR system was applied to a 10-fold serially diluted shrimp DNA extract (100 ng to 0.01 pg) that allowed the establishment of a dynamic range of 7 orders of magnitude and an absolute LOD of 0.1 pg (Table 2, Fig. 2). The LOD could also be considered as the LOQ since it was within the linear range of the calibration curve (Fig. 2). The calibration curve parameters, namely PCR efficiency (97.4%), r^2 (0.997) and slope (-3.371) were all within the acceptance criteria, suggesting a high performance of the assay (Table 2) (Bustin et al., 2009; ENGL, 2015). These results are in good agreement with a

reported method that enabled the detection of penaeid shrimp DNA down to 0.1 pg (Eischeid et al., 2013).

To propose a system that enables estimating the amount of shrimp crustaceans in foods, the use of binary mixtures as reference standards with known amounts of spiked shrimp is crucial. Their use allowed a new calibration curve with a dynamic range of 6 orders of magnitude, covering the tested concentration range of 0.0001–50% (w/w) to be established (Table 2, Fig. 2). Accordingly, both LOD and LOQ were identical, being down to 0.0001% (1 mg/kg). The calibration curve parameters (PCR efficiency of 97.7%, slope of -3.380 and r^2 of 0.987) suggested again a high performance of the assay (Table 2) (Bustin et al., 2009; ENGL, 2015). These findings are in good agreement with the levels of quantification obtained by other authors for crabs (Eischeid and Stadig, 2018) and lobsters (Eischeid, 2016), which ranged between 0.00001% and 10% (w/w). Regarding the relative quantification of shrimp meat using model mixtures as calibrants, no data were found in the literature.

For assay validation, the developed real-time PCR system was evaluated for its ability to estimate the shrimp content using blind mixtures with 8%, 4%, 2.5% and 0.25% (w/w) of shrimp. The coefficient of variation expressing the relative standard deviation of the estimated values ranged between 13.0% and 19.9%, which indicates that the method is precise, according to ENGL (2015) performance criteria ($\leq 25\%$) (Table 3). Comparing the actual values with the estimated ones, there is evidently a high proximity between them. The measured trueness, expressed as bias (0.83–12.6%), is within $\pm 25\%$ of the actual value over the tested dynamic range, demonstrating the high accuracy of the method (ENGL, 2015). Therefore, in the present work, a real-time PCR method able to quantify shrimp crustacean meat in food is proposed and validated for the first time.

3.3. Analysis of processed foods by real-time PCR and ELISA

To verify the applicability of the proposed PCR methods, 18 food samples containing crustaceans or labelled as susceptible to containing them were analysed (Table 4). Concerning the qualitative PCR results, as expected, most samples were positive producing the 203 bp fragment of the 16S rRNA gene. The exceptions were the sample #17 (Brás-style codfish) with a faint band, and samples #12 (noodles) and #18 (codfish “patanisca”) that were negative. This complies with the labelled information, since none of the samples declares shrimp as an ingredient (samples #17 and #18 may contain crustaceans and sample #12 only contains shrimp flavour). The real-time PCR results confirmed these findings, with Ct values ranging from 21 to 36, allowing the shrimp content in all samples to be estimated (Table 4). The foods containing the highest amounts of shrimp were #15 (shrimp “feijoada”) and #16 (shrimp “açorda”), which is in good agreement with the composition of these traditional dishes. The different samples of tested patties (#1–#5) reveal variable shrimp contents, which might be related to the addition of shrimp extracts/flavours instead of shrimp meat. The same finding can be attributed to the samples of shellfish soups (#6 and #7) and surimi (#10 and #11), while samples #9, #13 and #14 show a very low proportion of shrimp among other seafood species.

For comparative purposes, two ELISA kits for crustacean detection, namely MonoTrace and RidaScreen, were also applied, to verify their feasibility to estimate crustaceans. The calibration curve of MonoTrace ELISA Kit was determined according to manufacturer instructions with the provided ready-to-use crustacean standards (0, 1, 4, 10, 20 and 40 mg/kg) with an r^2 of 0.999 (Fig. S1A, Supplementary material). Similarly, the calibration curve of RidaScreen ELISA kit was built using crustacean standards (0, 20, 40, 80 and 160 mg/kg) with an r^2 of 1.00, which is presented in Fig. S1B (Supplementary material). From the tested samples, 9 were selected to be analysed by both kits in $n = 3$ replicates, based on the low estimated contents obtained by real-time PCR, since the kits are optimised for trace amounts (Table 4). All of the analysed samples were positive for the presence of crustaceans. When attempting

Table 2

Real-time PCR results targeting the 16S rRNA gene of crustaceans for the absolute detection of shrimp DNA using 10-fold serially diluted extracts and for the relative determination of shrimp meat using binary model mixtures.

absolute amount (pg shrimp)	Ct $\pm$ SD ^a	relative amount (%) w/w of shrimp in béchamel)	Ct $\pm$ SD ^a
100 000	17.74 $\pm$ 0.33 (16/16)	50	18.24 $\pm$ 0.28 (12/12)
10 000	21.21 $\pm$ 0.21 (16/16)	10	20.22 $\pm$ 0.48 (12/12)
1 000	24.79 $\pm$ 0.26 (16/16)	5	22.05 $\pm$ 0.29 (12/12)
100	28.21 $\pm$ 0.24 (16/16)	1	22.96 $\pm$ 0.45 (12/12)
10	31.56 $\pm$ 0.22 (16/16)	0.5	25.53 $\pm$ 0.28 (12/12)
1	34.74 $\pm$ 0.50 (16/16)	0.1	27.44 $\pm$ 0.45 (12/12)
0.1	38.06 $\pm$ 0.57 (16/16)	0.05	29.27 $\pm$ 0.38 (12/12)
0.01	nd ^b	0.01	30.93 $\pm$ 0.20 (12/12)
		0.005	31.68 $\pm$ 0.22 (12/12)
		0.001	34.49 $\pm$ 0.37 (12/12)
		0.0005	36.47 $\pm$ 0.99 (12/12)
		0.0001	37.28 $\pm$ 0.49 (12/12)
		0 (béchamel sauce)	nd
PCR efficiency (%)	97.4 $\pm$ 0.9	PCR efficiency (%)	97.7 $\pm$ 2.3
correlation coefficient (r^2)	0.997 $\pm$ 0.000	correlation coefficient (r^2)	0.987 $\pm$ 0.003
slope	-3.371 ± 0.023	slope	-3.380 ± 0.056

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) of replicate assays, in brackets (positive amplifications/total replicates).

^b nd, not detected.

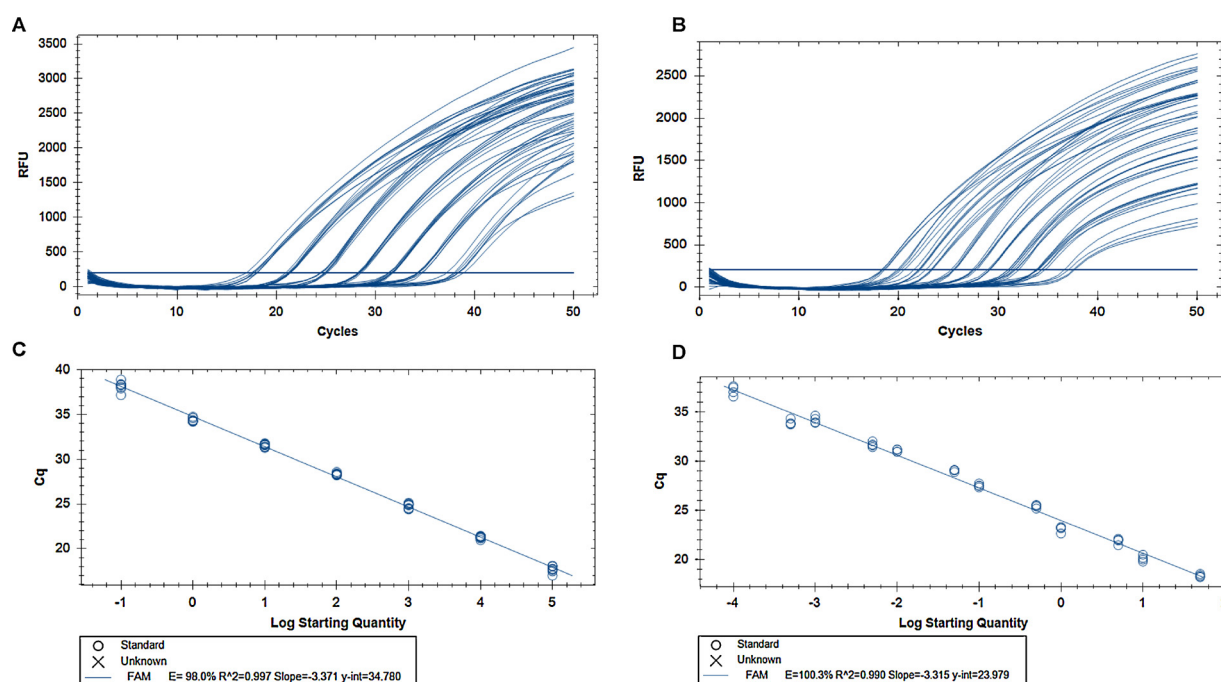


Fig. 2. Real-time PCR amplification (A, B) and calibration (C, D) curves with a TaqMan probe targeting the 16S rRNA gene using a serially diluted shrimp-DNA extract (100,000–0.1 pg) (A, C) and DNA extracts from binary model mixtures of cooked shrimp in béchamel sauce (0.0001–50% (w/w)) (B, D).

Table 3

Results for the validation of the quantitative real-time PCR assays using blind samples.

blind samples	crustacean content% (w/w)		CV (%) ^b	bias (%) ^c
	actual value	estimated value (mean ± SD) ^a		
A	8.0	7.13 ± 0.93	13.0	−10.9
B	4.0	4.51 ± 0.59	13.0	12.6
C	2.5	2.52 ± 0.42	16.5	0.83
D	0.25	0.28 ± 0.06	19.9	11.3

^a Mean values ± standard deviation (SD) of replicate assays ($n = 12$) from three independent real-time PCR runs.

^b CV, coefficient of variation.

^c Bias = ((mean estimated value – true value)/true value) × 100.

to estimate the crustacean content, most samples needed to be diluted (from 10- to 50-fold) and even so, some were out of the quantitative range using the MonoTrace kit (#5, #7, #9 and #14). The non-quantified or high estimated contents for samples #2, #5, #7, #9 and #14 are not really well correlated with the real-time PCR determinations. The non-correlation of ELISA and real-time PCR results has already been reported in the determination of other food allergens, such as almond (Röder et al., 2011) and hazelnut (Platteau et al., 2011). It is important to mention that these assays are optimised to quantify trace levels of crustaceans, rather than crustaceans as ingredients in foods. Moreover, these kits are optimised to target tropomyosins from crustaceans (instruction manuals of MonoTrace™ Crustacea ELISA Kit and RidaScreen® Fast Crustacean), based on their strong resistance to food processing and high abundance. However, tropomyosins are also present in molluscs, which are ingredients in samples #9 and #14, suggesting potential cross-reactivity in these cases, as reported in the kits (instruction manuals of MonoTrace™ Crustacea ELISA Kit and RidaScreen® Fast Crustacean) and in other reports (Werner et al., 2007). The presence of spices, such as curcumin, and other condiments are also prone to develop cross-reactivity with ELISA kits (instruction manual of RidaScreen® Fast Crustacean), justifying the high estimates of samples

#2, #5, #7 and #14. The low estimated amounts for surimi samples (#10 and #11) with both kits are according to the real-time PCR results, highlighting again the absence of crustacean meat that is replaced by flavours. The results for the samples with precautionary labelling for crustaceans (#17 and #18) were also at trace or non-quantifiable levels with both kits, agreeing with the PCR results.

4. Conclusion

In the present work, two new DNA-based approaches targeting the 16S rRNA mitochondrial gene are proposed to detect crustaceans in foods: a qualitative PCR assay specific for the most often consumed crustaceans (shrimps, lobsters and crabs) and a quantitative real-time PCR assay specific for shrimp. The proposed real-time PCR system enabled the detection and quantification down to 0.1 pg and 0.0001% (w/w) of shrimp DNA and shrimp in model mixtures, respectively. The validation results with blind mixtures showed that the system was accurate and precise in the tested range.

Both qualitative PCR and real-time PCR methods were successfully applied to analyse commercial food samples and verify labelling compliance. The comparison of shrimp estimates with two ELISA kits highlights that, at trace amounts of crustaceans, real-time PCR and ELISA are well correlated. However, for the higher contents (crustaceans or shrimps as ingredients), both ELISA kits display much higher estimates than those of real-time PCR, suggesting potential cross-reactivity of immunoassays with other food components, such as molluscs and spices.

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Table 4
Results for the processed food samples analysed by qualitative PCR, real-time PCR and ELISA for the detection and quantification of crustaceans.

sample	type of food	relevant label information	qualitative PCR		real-time PCR ^a		ELISA	
			eukaryotic (18S rRNA)	crustacean (16S rRNA)	Ct ± SD	estimated amount ± SD (mg/kg)	MonoTrace Estimated amount ± SD (mg/kg) ^b	RidaScreen Estimated amount ± SD (mg/kg) ^b
1	shrimp patties	shrimp, shrimp powder	+	+	27.04 ± 0.34	892 ± 22	nt ^c	nt
2	shrimp patties	shrimp, shrimp powder	+	+	27.37 ± 0.08	710 ± 37	1392 ± 44 [*]	3071 ± 55 [*]
3	shrimp patties	shrimp, shrimp powder	+	+	25.34 ± 0.49	2570 ± 360	nt	nt
4	shrimp patties	shrimp, shrimp powder	+	+	25.09 ± 0.32	3110 ± 680	nt	nt
5	shrimp and hake patties	Shrimp, fish	+	+	28.76 ± 0.39	168 ± 17	> LQ ^d	3618 ± 841 [*]
6	shellfish soup	shrimp, lobster extract	+	+	26.66 ± 0.34	770 ± 116	nt	nt
7	shellfish soup	shrimp, lobster extract	+	+	27.58 ± 0.87	307 ± 54	> LQ	5061 ± 881 [*]
8	shellfish broth	shrimp, shrimp and lobster extract	+	+	24.63 ± 0.62	4510 ± 560	nt	nt
9	shellfish rice preparation	crustaceans	+	+	30.37 ± 0.40	65.7 ± 17.9	> LQ	828.3 ± 31.1 [*]
10	surimi	crab (extract and flavour)	+	+	36.03 ± 0.88	1.16 ± 0.17	1.24 ± 0.07	79.8 ± 3.8
11	surimi	crab (extract and flavour)	+	+	33.99 ± 0.75	4.71 ± 0.54	3.60 ± 0.08	< LQ (4.94 ± 0.44)
12	shrimp noodles	crustaceans, shrimp flavour	+	–	–	–	nt	nt
13	seafood risotto	shrimp, squid, mussels, fish, crustacean extract	+	+	27.03 ± 0.63	483 ± 72	nt	nt
14	paella	shrimp, squid, mussels, chicken	+	+	29.65 ± 0.63	147 ± 43	> LQ	4245 ± 365 [*]
15	shrimp “feijoada”	shrimp, lobster extract	+	+	22.54 ± 0.74	11400 ± 900	nt	nt
16	shrimp “açorda”	shrimp	+	+	21.05 ± 0.20	43200 ± 5500	nt	nt
17	Brás-style codfish	may contain crustaceans	+	± ^c	–	–	< LQ	< LQ
18	codfish “patanisca”	may contain crustaceans	+	–	–	–	28.4 ± 7.0	82.6 ± 8.4

^a Mean values ± standard deviation (SD) of replicate assays (n = 8).

^b Mean values ± standard deviation (SD) of replicate assays (n = 3).

^c ±, faint band.

^d LQ, limit of quantification.

^e nt—not tested.

* Estimated amounts were obtained from previously diluted protein extracts.

by FSE and MCTES). The authors acknowledge the kind supply of shrimp species by Marfresco (Loures, Portugal) and Brasmar Seafood Companies (Trofa, Portugal).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jfca.2018.05.012>.

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Supplementary material

Table S1. Results of cross-reactivity testing of primers targeting the crustacean 16S rRNA gene with non-crustacean species

Species	Scientific name	Qualitative PCR ^a	
		Eukaryotic (18S rRNA gene)	Crustacean (16S rRNA gene)
Seafood species			-
Atlantic cod	<i>Gadus morhua</i>	+	-
Pacific cod	<i>Gadus macrocephalus</i>	+	-
Alaska pollock	<i>Theragra chalcogramma</i>	+	-
Saithe	<i>Pollachius virens</i>	+	-
Ling	<i>Molva molva</i>	+	-
European hake	<i>Merluccius merluccius</i>	+	-
Argentine hake	<i>Merluccius hubbsi</i>	+	-
North Pacific hake	<i>Merluccius productus</i>	+	-
Deepwater hake	<i>Merluccius paradoxus</i>	+	-
Whiting	<i>Merluccius capensis</i>	+	-
Atlantic salmon	<i>Salmo Salar</i>	+	-
European pilchard	<i>Sardina pilchardus</i>	+	-
Yellowfin tuna	<i>Thunnus albacares</i>	+	-
Gilt-head bream	<i>Sparus aurata</i>	+	-
European seabass	<i>Dicentrarchus labrax</i>	+	-
Whiting-pout	<i>Trisopterus luscus</i>	+	-
Pacific mackerel	<i>Scomber japonicus</i>	+	-
Atlantic horse mackerel	<i>Trachurus trachurus</i>	+	-
Rose fish	<i>Sebastes marinus</i>	+	-
Black scabbardfish	<i>Aphanopus carbo</i>	+	-
Nile perch	<i>Lates niloticus</i>	+	-
Common sole	<i>Solea solea</i>	+	-
Ray	<i>Raja</i> spp.	+	-
Tadpole codling	<i>Salilota australis</i>	+	-
Rock ling	<i>Genypterus blacodes</i>	+	-
Squid	<i>Loligo vulgaris</i>	+	-
Mussel	<i>Mytilus edulis</i>	+	-
Undulated surf clam	<i>Paphia undulata</i>	+	-
Meat species			
Wild boar	<i>Sus scrofa scrofa</i>	+	-
Duck	<i>Anas platyrhynchos</i>	+	-
Partridge	<i>Alectoris rufa</i>	+	-
Hare	<i>Lepus granatensis</i>	+	-
Quail	<i>Coturnix coturnix</i>	+	-
Pheasant	<i>Phasianus colchinus</i>	+	-
Red deer	<i>Cervus elaphus</i>	+	-
Rabbit	<i>Oryctolagus cuniculus</i>	+	-
Chicken	<i>Gallus gallus</i>	+	-

Turkey	<i>Meleagris gallopavo</i>	+	-
Lamb	<i>Ovis aries</i>	+	-
Ostrich	<i>Struthio camelus</i>	+	-
Cow	<i>Bos taurus</i>	+	-
Horse	<i>Equus caballus</i>	+	-
Pig	<i>Sus scrofa</i>	+	-
Plant species			
Onion	<i>Allium cepa</i>	+	-
Garlic	<i>Allium sativum</i>	+	-
Parsley	<i>Petroselinum crispum</i>	+	-
Pepper	<i>Piper nigrum</i>	+	-
Bay leaf	<i>Laurus nobilis</i>	+	-
Sweet chili	<i>Capsicum annuum</i>	+	-
Tomato	<i>Solanum lycopersicum</i>	+	-
Maize	<i>Zea mays</i>	+	-
Soybean	<i>Glycine max</i>	+	-
Potato	<i>Solanum tuberosum</i>	+	-
Manioc	<i>Manihot esculenta</i>	+	-
Lupine	<i>Lupinus albus</i>	+	-
Chestnut	<i>Castanea sativa</i>	+	-
Walnut	<i>Juglans regia</i>	+	-
Broad bean	<i>Vicia faba</i>	+	-
Rye	<i>Secale cereale</i>	+	-
Wheat	<i>Triticum aestivum</i>	+	-
Rice	<i>Oryza sativa</i>	+	-
Pumpkin	<i>Cucurbita pepo</i>	+	-

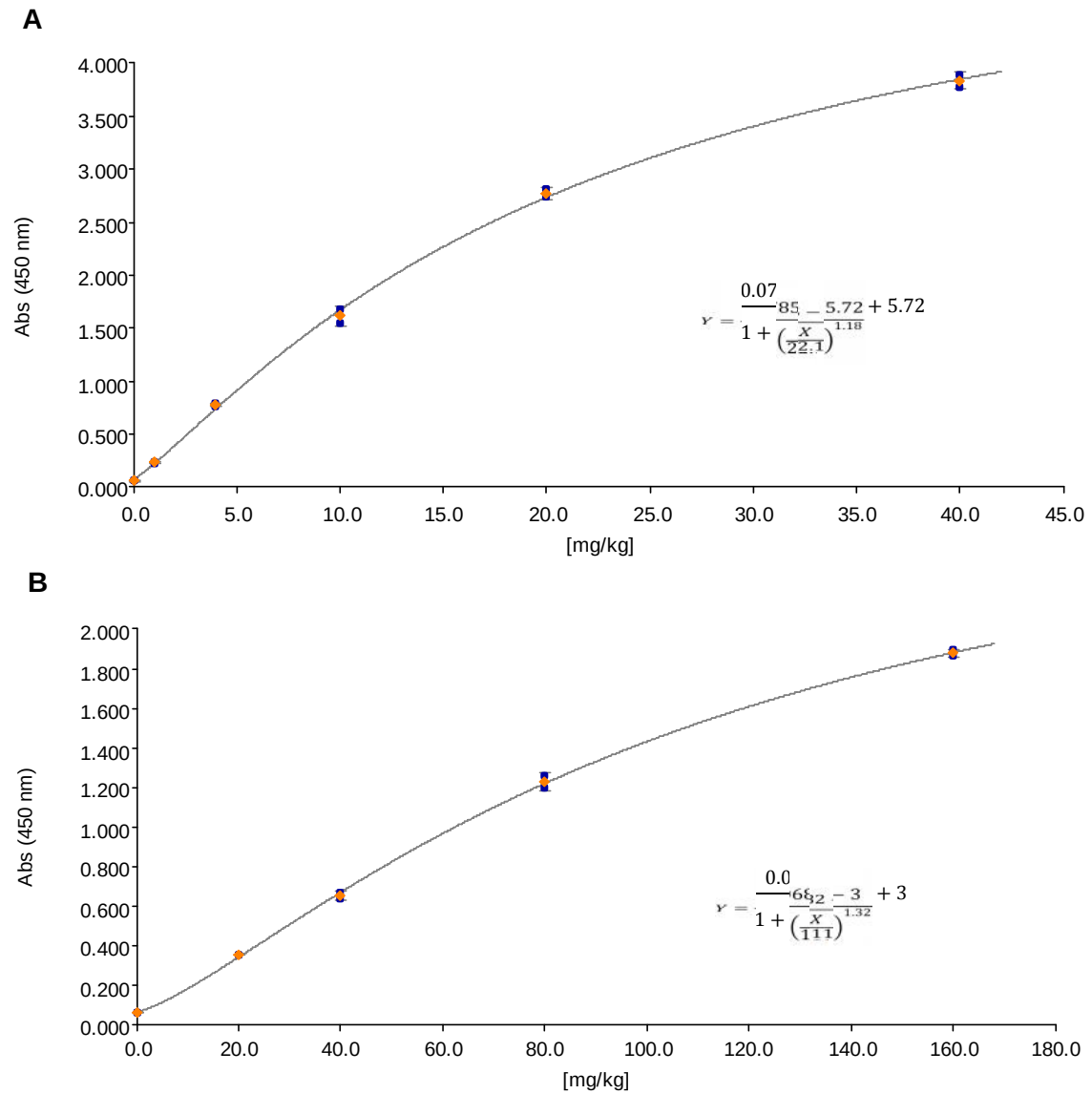


Figure S1. Calibration curves and respective sigmoid four parametric logistic functions for both ELISA kits. (A) MonoTrace and (B) RidaScreen. Mean Abs (450 nm) values (♦) and standard deviation (I).

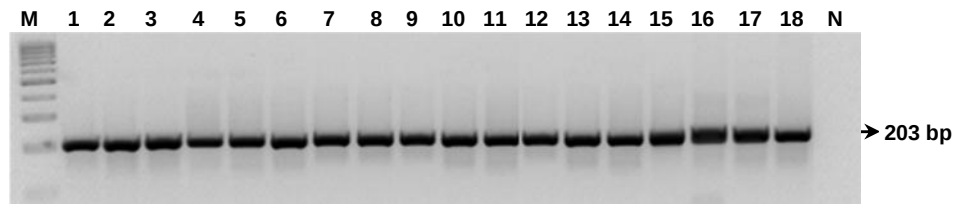


Figure S2. Agarose gel electrophoresis of PCR amplification products targeting the 16S rRNA gene of crustaceans for specificity testing. Legend: M, 100 bp DNA ladder (Bioron, Ludwigshafen, Germany); 1, *L. vannamei*; 2, *M. monoceros*; 3, *F. indicus*; 4, *P. monodon*; 5, *M. affinis*; 6, *P. semisulcatus*; 7, *P. muelleri*; 8, *S. melanthero*; 9, *S. crassicornis*; 10, *H. triarthrus*; 11, *P. serratus*; 12, *A. edwardsiana*; 13, *N. norvegicus*; 14, *H. gammarus*; 15, *P. argus*; 16, *P. validus*; 17, *N. puber*; 18, *C. pagurus*; N, negative control.