

A new real-time PCR assay to specifically detect crustaceans in vegan raw materials and vegan shrimps

Geoffrey Cottenet & Carine Blancpain

To cite this article: Geoffrey Cottenet & Carine Blancpain (2022) A new real-time PCR assay to specifically detect crustaceans in vegan raw materials and vegan shrimps, Food Additives & Contaminants: Part A, 39:4, 645-652, DOI: [10.1080/19440049.2022.2036822](https://doi.org/10.1080/19440049.2022.2036822)

To link to this article: <https://doi.org/10.1080/19440049.2022.2036822>



Published online: 18 Mar 2022.



Submit your article to this journal [↗](#)



Article views: 196



View related articles [↗](#)



View Crossmark data [↗](#)



A new real-time PCR assay to specifically detect crustaceans in vegan raw materials and vegan shrimps

Geoffrey Cottenet  and Carine Blancpain

Institute of Food Safety & Analytical Sciences, Nestlé Research, Lausanne, Switzerland

ABSTRACT

Contamination of vegan products and vegan shrimps with real shrimps and crustaceans must be avoided to comply with vegan claims, even more for preventing strong allergenic reactions. Therefore, the detection of crustaceans must be reliable and sensitive enough for authenticity, traceability, and food safety purposes. A new real-time PCR assay was developed targeting the mitochondrial 16S rRNA gene of crustaceans and was optimised to avoid critical mismatches with primers and probe. By testing several crustacean species and common food ingredients, the method was demonstrated to be specific to crustaceans only. To comply with the limit of non-vegan contamination established at 0.1% (w/w) by the European Vegetarian Union, dedicated cut-off C_T values were determined on vegan raw materials and on vegan shrimps spiked with crustacean materials. The method reached a sensitivity $\leq 0.0005\%$ (w/w), which was further confirmed on reference materials containing a similar amount of crustacean.

ARTICLE HISTORY

Received 2 November 2021
Accepted 20 January 2022

KEYWORDS

Crustacean; detection; DNA;
Vegan; Allergen

Introduction

Crustaceans represent 68000 different species, with the well-known edible representatives being shrimp, lobster, crayfish and crab (Covich et al. 2010). According to FAO report, more than 15 million tons of crustaceans were fished in 2018, with roughly 60% coming from aquaculture (FAO 2020); shrimp and prawn were the most produced crustaceans, either naturally caught or cultivated in aquaculture, with *Litopenaeus vannamei* representing nearly 30% of the total production of crustaceans. Combining this high consumption of shrimps with the growing interest for plant-based alternatives and vegan diets (Vegan Society), vegan shrimps composed of potato starch, pea extract and/or soya extract have been recently commercialized. In these products, producers must ensure the absence of cross-contamination with animal products (Cottenet and Blancpain 2021) but most importantly with real shrimp material which could occur along the supply chain, during the storage or the transportation. Food adulteration and

tampering with shrimp (by-)products must also be considered by food industries and regulators (Cavin et al. 2019). Looking at these risks, the European Vegetarian Union has established that traces of non-vegan or non-vegetarian substances should be present at less than 0.1% (w/w) to conform with vegan labelling (European Vegetarian Union, V-Label). In addition to these authenticity and traceability requirements, crustacean meat is known to be rich in protein and to contain tropomyosin, a well-known allergen; prevalence of shellfish allergy is estimated at 0.5–2.5% of the population, with one of the strongest incidences amongst allergies (Woo and Bahna 2011). Therefore, the detection of undeclared crustacean material in products plays a key role for consumers' safety.

DNA-based methods, especially PCR, are considered as the reference tools for detecting biological adulterants and for species identification (Cavin et al. 2016; Burns et al. 2019). In addition, PCR-based methods have been shown to cover a broader dynamic range and to be more robust

against matrix effects compared to protein-based ELISA approaches when looking for the crustaceans' allergen (Eischeid et al. 2021). Several publications describe the development of (real-time) PCR systems to detect shrimps or crabs (Cao et al. 2011; Eischeid et al. 2013), or to identify specific crustacean species (Fernandes et al. 2017), but only a few describe the development of methods able to detect crustaceans as a whole taxon. These methods usually target the mitochondrial 16S rRNA gene which is considered as the most conserved target within crustaceans to design PCR assays: Brzezinski described a first set of PCR primers (Brzezinski 2005) amplifying a fragment of 205 base pairs (bp) specific to crustaceans, which were then associated with additional probes to increase specificity (Herrero et al. 2012; Santaclara and Espineira 2017; Fernandes et al. 2018). However, one of the primers is based on a region that presents a few critical mismatches on some species that could prevent their efficient amplification. Mäde and Rohmberger designed up to 6 different sets of TaqMan assays to detect a broad range of crustacean species (Mäde and Rohmberger 2017), but this approach complicates the overall usage in routine.

In this study, we developed and optimized a new TaqMan assay based on the work of Brzezinski and Fernandes et al. (2018). The specificity of the new assay was tested against various crustacean species and other materials. Finally, C_T cut-off values were defined at 0.1% (w/w) on vegan raw materials and on vegan shrimps to comply with the vegan V-Label threshold, and the sensitivity was assessed at lower levels for allergen detection.

Materials and methods

Samples

Crustaceans, molluscs, plants, and vegan shrimp samples were obtained from local markets. Meat and fish certified reference DNA samples were purchased at Coring System Diagnostix GmbH (Gernsheim, Germany), and prepared at 10 ng/ μ L with EB buffer (QIAGEN, Hilden, Germany). Crustacean powders from shrimp, langoustine, and lobster, as well as soya and pea protein

concentrate powders commonly used in vegan recipes were obtained from industrial suppliers. Commercial vegan shrimps were collected in supermarkets. Finally, reference materials made of corn flour spiked with 5000 mg/kg of crustacean material (speckled shrimp *Metapenaeus monoceros*, Argentine red shrimp *Pleoticus muel-leri*, and mud shrimp *Solenocera melanthero*) were purchased from Generon SpA (San Prospero, Italy).

For sensitivity trials, mixtures spiked at 1% (w/w) were prepared by adding 10 mg of crustacean material in 990 mg of plant-based matrices, and DNA was extracted from these mixtures.

Animal and plant species used in this work are listed in Table 1.

DNA extraction

DNA from 1 g of sample was mixed with 6 mL of CTAB lysis buffer (Applchem GmbH, Darmstadt, Germany), 660 μ g/mL of proteinase K (Promega, Dübendorf, Switzerland), and 200 μ g/mL of RNase A (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). After incubation for 1 h at 65 °C, the mixture was centrifuged at 3000 g for 5 min, and 500 μ L of supernatant was mixed with an equal volume of chloroform. DNA was then extracted as described by Cottenet et al. (2016). DNA concentration was measured with a Nanodrop (Thermo Fischer Scientific, Waltham, USA), and DNA extracts were diluted at 10 ng/ μ L in EB buffer (QIAGEN). Extracted DNA and reconstituted DNA were stored at -20 °C until further use.

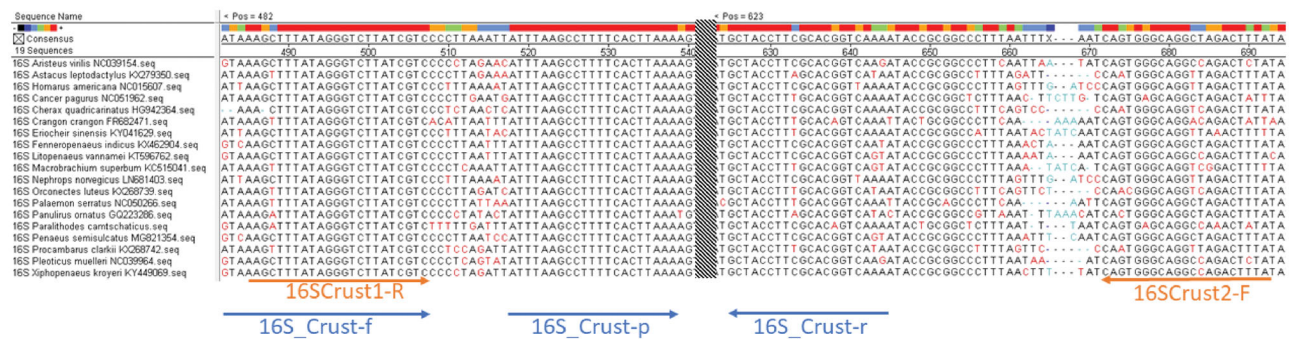
The DNA extracted from the mixtures for the sensitivity trials was further diluted in the DNA from the pure plant-based matrix to reach a level of 0.1% (w/w) down to 0.0005% (w/w). Similarly, the DNA extracted from the Generon reference materials was diluted to lower crustacean concentration in corn DNA. All DNA were prepared at 10 ng/ μ L before dilution.

Design of the crustacean TaqMan real-time PCR assay

Sequences of the mitochondrial 16S rRNA gene of several crustacean species were obtained from

Table 1. List of species used in this study, and average C_T values obtained. Absence of amplifications is indicated with a dash “–”.

Common name	Latin name	C_T	Common name	Latin name	C_T
Northern shrimp	<i>Pandalus borealis</i>	19.5	Scallop	<i>Placopecten magellanicus</i>	–
Whiteleg shrimp	<i>Litopenaeus vannamei</i>	22.1	Chilean mussel	<i>Mytilus chilensis</i>	–
Green tiger prawn	<i>Penaeus semiscultatus</i>	20.5	Blue mussel	<i>Mytilus edulis</i>	–
Giant tiger prawn	<i>Penaeus monodon</i>	20.9	Green mussel	<i>Perna canaliculus</i>	–
Indian prawn	<i>Penaeus indicus</i>	19.7	Oyster	<i>Crassostrea gigas</i>	–
Langoustine	<i>Nephrops norvegicus</i>	19.2	Venus clam	<i>Ruditapes philippinarum</i>	–
Andaman lobster	<i>Metanephrops andamanicus</i>	20.4	Hard Clam	<i>Meretrix lyrata</i>	–
Spiny lobster	<i>Panulirus argus</i>	19.4	Carpet shell	<i>Venerupis decussata</i>	–
American lobster	<i>Homarus americanus</i>	18.1	Common cuttlefish	<i>Sepia officinalis</i>	–
Red king crab	<i>Paralithodes camtschaticus</i>	21.2	Shortfin squid	<i>Illex argentinus</i>	–
Brown crab	<i>Cancer pagurus</i>	18.7	Mitre squid	<i>Uroteuthis edulis</i>	–
Soya	<i>Glycine max</i>	–	Humboldt squid	<i>Dosidicus gigas</i>	–
Maize	<i>Zea mays</i>	–	Big blue octopus	<i>Octopus cyanea</i>	–
Wheat	<i>Triticum aestivum</i>	–	common octopus	<i>Octopus vulgaris</i>	–
Oat	<i>Avena sativa</i>	–	Snail	<i>Helix pomatia</i>	–
Rapeseed	<i>Brassica napus</i>	–	Beef	<i>Bos taurus</i>	–
Oregano	<i>Origanum vulgare</i>	–	Pork	<i>Sus scrofa</i>	–
Basil	<i>Ocimum basilicum</i>	–	Sheep	<i>Ovis aries</i>	–
Parsley	<i>Petroselinum crispum</i>	–	Chicken	<i>Gallus gallus</i>	–
Garlic	<i>Allium sativum</i>	–	Salmon	<i>Salmo salar</i>	–
Tomato	<i>Solanum lycopersicum</i>	–	Sardine	<i>Sardina pilchardus</i>	–
Pea	<i>Pisum sativum</i>	–	Atlantic pollock	<i>Pollachius pollachius</i>	–
		–	Alaska pollock	<i>Theragra chalcogramma</i>	–

**Figure 1.** Alignment of 16S rRNA gene sequences from several crustacean species. The primers described by Fernandes et al. (2018) are 16SCrust2-F and 16SCrust1-R, whereas our new primers are 16S_Crust-f and 16S_Crust-r combined with a TaqMan 16S_Crust-p probe.

the global NCBI nucleotide database (NCBI Nucleotide database), and aligned with MegAlign version 15 (DNASTAR, Wisconsin, United States) (Figure 1). PCR primers and a TaqMan-MGB probe were designed on a region already targeted by Santaclara and Espineira (2017) and Fernandes et al. (2018) who were using reverse complement sequence. Our forward primer 16S_Crust-f was slightly adapted from the reverse primer from Fernandes et al. (2018) to increase its melting temperature from 48 to 56 °C. Although 16S_Crust-f presents a few mismatches on several species, they are exclusively located on its 5'-end and are not expected to have a significant impact on the PCR efficiency (Ghedira et al. 2009). To design our primer reverse, we considered the forward primer from Fernandes et al.

(2018). However, this primer contained several mismatches on its 3'-end that could prevent an efficient detection of popular crustaceans, such as the brown crab (*Cancer pagurus*), the red king crab (*Paralithodes camtschaticus*) and the Louisiana crawfish (*Procambarus clarkii*) which is the 2nd most produced crustacean in aquaculture (FAO 2020). A similar primer described by Santaclara and Espineira (2017) also presented critical mismatches on crab species. Therefore, a new reverse primer 16S_Crust-r was designed without 3'-end mismatches, allowing the amplification of a fragment of approximately 157–158 bp. Compared to the 203-bp fragment amplified by Fernandes et al. (2018), this shorter fragment is even more likely to be amplified successfully in highly processed samples (Hird et al.

2006). Finally, a TaqMan MGB probe 16S_Crust-p was newly designed on a well-conserved region of the crustaceans' 16S rRNA gene, presenting no wobbles.

Real-time PCR runs

The real-time PCR consisted of a 20- μ L amplification mix containing 10 μ L of 2x TaqMan[®] FAST Advanced Master Mix (Thermo Fisher Scientific), 900 nM each of 16S_Crust-f (5'-GTCAAGCTTTATAGGGTCTTATCGTC-3') and 16S_Crust-r (5'-TTTGACCGTGCDAAAGGTAGC-3') primers (Thermo Fisher Scientific), 250 nM of the crustacean 16S_Crust-p probe (5'-FAM-ATTTAAGCCTTTTCACTTAAA-MGB-3') (Thermo Fisher Scientific), 2 μ L of 10x Exogenous IPC-VIC mix (Thermo Fisher Scientific), 0.4 μ L of 50x Exogenous IPC DNA (Thermo Fisher Scientific), and 5 μ L of sample DNA at 10 ng/ μ L. Each extracted DNA was amplified in duplicate, and the average C_T value was considered. Real-time PCR runs were performed on a QuantStudio 7 instrument (Thermo Fisher Scientific) using a FAST mode. Following a first step at 50 °C for 2 min to activate the UNG-glycosylase and a second step at 95 °C for 20 s to activate the hot-start DNA Taq polymerase, the cycling program consisted in a denaturation step for 1 s at 95 °C and an annealing/elongation step for 20 s at 60 °C. For vegan compliance, 35 cycles were sufficient, whereas 40 cycles were applied for allergen detection. The QuantStudio Real-Time PCR software version 1.3 (Thermo Fisher Scientific) was used to analyse FAM and VIC fluorescent signals with an automatic baseline and a manual threshold of 0.1 and 0.04, respectively.

Assessment of the method sensitivity

To comply with the European Vegetarian Union threshold of 0.1% (w/w), C_T cut-off values were established for raw materials frequently used in vegan recipes and for finished products, by analyzing a minimum of 5 samples spiked at 0.1% (w/w). For raw materials, shrimp powder, langoustine powder and lobster powder were spiked into a soya and a pea protein concentrate

powders, leading to 6 individual samples. The C_T threshold was calculated as the 95% upper confidence interval of the average C_T value obtained. This cut-off value was then confirmed by analyzing a minimum of 20 individual spiked samples at 0.1% (w/w), applying this C_T cut-off to consider a sample as positive or negative. Similarly, for finished products, commercial vegan shrimp products were spiked at 0.1% (w/w) with 5 different species of shrimp (*Penaeus monodon*, *P. indicus*, *P. semiscultatus*, *Pandalus borealis* and *L. vannamei*), and a cut-off value was determined and then verified.

The limit of detection of the method (LOD) is the amount of analyte at which its presence is detected at least 95% of the time (LOD_{95%}) (CODEX 2010); therefore a minimum 20 replicates must be analysed at the lowest concentration. For allergen testing, the DNA extracted from the previous spiked mixtures prepared for vegan claim were further diluted down to 0.0005% (w/w) in the matrix DNA.

Results and discussion

PCR efficiency and specificity of the crustacean real-time PCR assay

To determine the PCR efficiency of the new crustacean assay, the DNA from the most frequent shrimp, *Litopenaeus vannamei* (FAO 2020), was serially diluted and amplified in triplicates. The coefficient of regression showed a satisfactory linearity higher than 0.99, and the slope of -3.449 indicated a reliable amplification efficiency equivalent to 94.9% (Figure 2).

To evaluate the specificity of the new crustacean design several species of crustacean, molluscs, fish, meat and plant were tested. All the crustacean species led to early amplifications between 18 and 22 C_T values (Table 1). This wide range of C_T values can be mainly explained by two factors: (i) the crustacean samples leading to the latest C_T values of 20 to 22 were actually purchased as frozen products, but already cooked, which has certainly damaged the DNA as compared to raw products (Hird et al. 2006); (ii) the difference in number of mitochondrial DNA

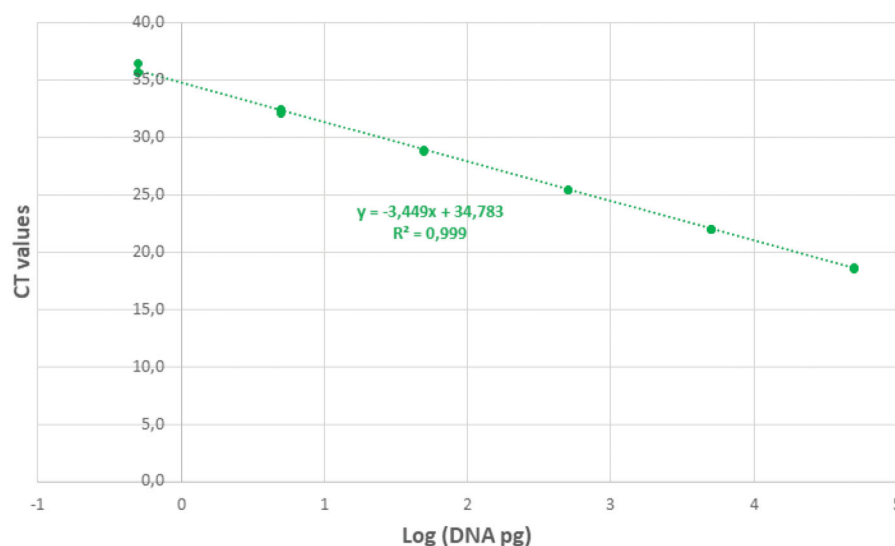


Figure 2. Linear regression between the amount of *Litopenaeus vannamei* DNA in the PCR (Log pg) (X-Axis) and the obtained C_T values (Y-axis) for validation of assay efficiency. All data points were analysed in triplicate.

copies per species and per types of tissue as compared to nuclear DNA (Ballin et al. 2009).

When replacing our primer reverse 16_Crust-r by the primer forward 16SCrust2-F from Fernandes et al. (2018) (Figure 1), C_T values obtained on the red king crab, the brown crab and the spiny lobster switched from 21.2, 18.7, and 19.4 to 34.4, 24.7 and 33.8, respectively (data not shown). This clearly illustrated the negative impact on the amplification of the 3'-end mismatches observed on the theoretical alignment and explained in the design of the assay. On the contrary, our assay enabled a consistent amplification of the tested crustacean species with an average C_T value of 20.0.

When testing all the other non-crustacean species, no amplifications were observed (Table 1) which demonstrated the specificity of our design on crustacean species only. No PCR inhibitions were seen on the internal positive control IPC-VIC on any of the tested samples (data not shown).

Vegan C_T cut-off and sensitivity of the method

For vegan labelling claim, a C_T cut-off was defined at a level of 0.1% (w/w) corresponding to the V-label tolerance. Crustacean powders from shrimp, langoustine and lobster were individually spiked into a soya powder and a pea powder frequently used as raw materials in vegan recipes.

These 6 samples led to an average C_T value of 30.9 and an upper confidence interval at 95% of 32.6, which was defined as the vegan C_T threshold (Table 2). To validate this cut-off value, 21 individual samples were prepared and analysed by spiking the 3 crustacean powders in 4 different soya and 3 different pea powders. Amongst the 21 results obtained, 20 samples led to C_T values lower or equal to the cut-off value of 32.6, and only 1 led a C_T value higher (32.7) (Table 2). These results led to a detection rate of 95.2%, confirming the value of 32.6 as a reliable threshold to consider a raw material as vegan or not.

A similar approach was applied to determine a C_T cut-off for finished products by spiking vegan shrimps with 5 different species of shrimp at 0.1% (w/w). The C_T cut-off value was first established with these 5 samples, leading to an average C_T value of 28.0 and an upper confidence interval at 95% of 28.8 (Table 2). To verify this vegan cut-off value, 4 replicates of the similar shrimp mixtures were prepared and analysed leading to 20 values. Only 1 value exceeded this 28.8 threshold, which led to a 95% detection rate and confirmed this vegan cut-off value for finished products.

In addition, to assess the $LOD_{95\%}$ of the method, DNA from the previous powder mixtures and those from the spiked vegan shrimps were further diluted down to 0.0005% (w/w) and analysed. Out of these 41 data points, 2 samples

Table 2. C_T values obtained on spiked samples at 0.1 % (w/w) to determine and verify the vegan C_T cut-off values in raw materials and in finished products. For raw materials, soya and pea isolate proteins were spiked with shrimp, langoustine and lobster powders, whereas 5 raw shrimps were spiked into vegan shrimps for finished products. C_T values exceeding the C_T cut-off values are indicated in bold.

Determination of the C _T cut-off		C _T values	Verification of the C _T cut-off		C _T values
Raw materials					
Shrimp	in soya	29.7	Shrimp	in soya 1	31.2
Langoustine	in soya	30.6	Langoustine	in soya 1	32.1
Lobster	in soya	30.6	Lobster	in soya 1	32.5
Shrimp	in pea	30.9	Shrimp	in soya 2	30.4
Langoustine	in pea	31.9	Langoustine	in soya 2	31.0
Lobster	in pea	31.9	Lobster	in soya 2	31.1
Average		30.9	Shrimp	in soya 3	29.8
Standard deviation		0.86	Langoustine	in soya 3	30.8
Vegan C _T cut-off value		32.6	Lobster	in soya 3	30.7
(= 95% upper confidence interval)			Shrimp	in soya 4	30.3
			Langoustine	in soya 4	31.4
			Lobster	in soya 4	32.2
			Shrimp	in pea 1	31.1
			Langoustine	in pea 1	32.1
			Lobster	in pea 1	32.2
			Shrimp	in pea 2	31.3
			Langoustine	in pea 2	32.7
			Lobster	in pea 2	32.3
			Shrimp	in pea 3	31.7
			Langoustine	in pea 3	32.5
			Lobster	in pea 3	32.6
Finished Products					
<i>P. monodon</i>	in vegan shrimp	28.6	<i>P. monodon</i>	in vegan shrimp 1	28.6
<i>P. indicus</i>	in vegan shrimp	28.0	<i>P. indicus</i>	in vegan shrimp 1	28.2
<i>P. semisculatus</i>	in vegan shrimp	27.7	<i>P. semisculatus</i>	in vegan shrimp 1	27.7
<i>P. borealis</i>	in vegan shrimp	28.1	<i>P. borealis</i>	in vegan shrimp 1	28.6
<i>L. vannamei</i>	in vegan shrimp	27.4	<i>L. vannamei</i>	in vegan shrimp 1	27.6
Average		28.0	<i>P. monodon</i>	in vegan shrimp 2	28.5
Standard deviation		0.42	<i>P. indicus</i>	in vegan shrimp 2	28.3
Vegan C _T cut-off value		28.8	<i>P. semisculatus</i>	in vegan shrimp 2	27.6
(= 95% upper confidence interval)			<i>P. borealis</i>	in vegan shrimp 2	28.5
			<i>L. vannamei</i>	in vegan shrimp 2	27.7
			<i>P. monodon</i>	in vegan shrimp 3	28.6
			<i>P. indicus</i>	in vegan shrimp 3	28.2
			<i>P. semisculatus</i>	in vegan shrimp 3	27.7
			<i>P. borealis</i>	in vegan shrimp 3	28.6
			<i>L. vannamei</i>	in vegan shrimp 3	27.6
			<i>P. monodon</i>	in vegan shrimp 4	28.9
			<i>P. indicus</i>	in vegan shrimp 4	28.4
			<i>P. semisculatus</i>	in vegan shrimp 4	28.0
			<i>P. borealis</i>	in vegan shrimp 4	28.7
			<i>L. vannamei</i>	in vegan shrimp 4	27.6

amplified very late giving C_T values of 39.9, whereas the other replicates successfully amplified (Figure 3(a)) leading to an average C_T value of 37.1 ± 2.6 . This confirms a $LOD_{95\%} \leq 0.0005\%$ (w/w), equivalent to ≤ 5 mg/kg. This sensitivity matches completely with what is usually achieved for allergen detection (Monaci and Visconti 2010).

Analysis of reference materials

To confirm the reliability and the applicability of the method, 3 reference materials were analysed mimicking real-life samples. These reference materials contained 5 mg/kg of *Metapenaeus*

monoceros, *Pleoticus muelleri*, and *Solenocera mel-antho* spiked in a corn-based matrix. Ten replicates of each sample were tested and led to successful amplifications (Figure 3(b)). A few replicates led to C_T values between 39 and 39.5 which confirmed the sensitivity of the method previously determined at 0.0005% (w/w) or 5 mg/kg, as well as the applicability of the method for routine testing.

Conclusions

A new real-time assay was designed on the crustacean 16S rRNA gene by optimizing the location of the primers and the TaqMan probe to reduce

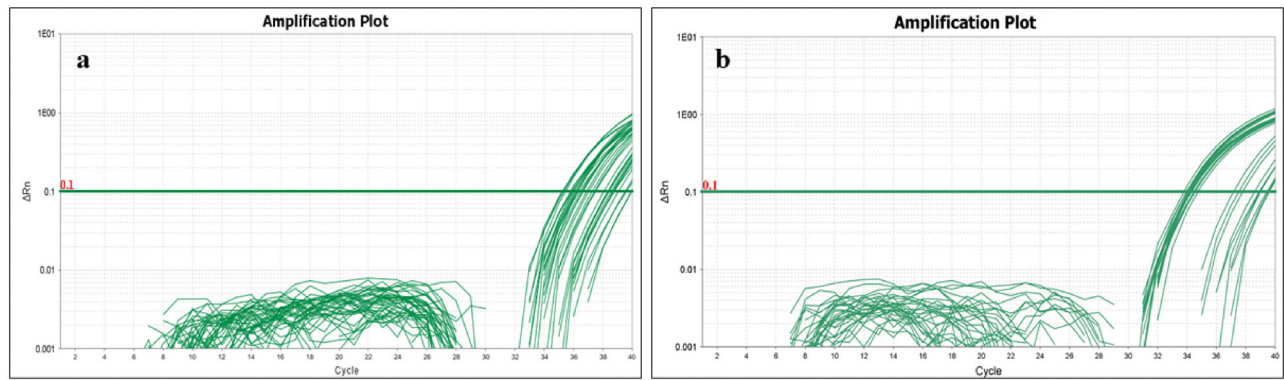


Figure 3 Real-time PCR amplification curves obtained on 41 samples of soya powders, pea powders and vegan shrimps spiked with crustacean at 0.0005% (w/w) (3a), and amplification curves obtained on reference materials containing 0.0005% (w/w) of *M. monoceros*, *P. muelleri*, and *S. melanthero* analysed in 10 replicates each (3b). The number of cycles and the fluorescence are in X- and Y-axes, respectively.

critical mismatches of primers previously published. The new assay successfully amplified crustaceans only, including crabs and spiny lobster that could individually be detected by PCR primers previously published. Our new TaqMan PCR method was adapted to vegan claims by defining dedicated C_T cut-off values on vegan raw materials frequently used in vegan recipes, and on vegan shrimps. These C_T cut-off values will enable the detection of non-vegan material above the V-Label threshold of 0.1% (w/w) established by the European Vegetarian Union. When further diluted, crustacean material was detectable at $\leq 0.0005\%$ (w/w) which is well adapted to allergen detection. This limit of detection was confirmed on reference materials containing similar amount of crustacean material. Further experiments would need to be carried out to broaden the scope of the method to other vegan finished products such as vegan fish fillets and vegan burgers.

Acknowledgement

Authors would like to thank Generon SpA for preparing reference materials with low amount of shrimp tissues.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Geoffrey Cottenet  <http://orcid.org/0000-0002-4293-8158>

References

- Ballin NZ, Vogensen FK, Karlsson AH. 2009. Species determination – can we detect and quantify meat adulteration? *Meat Sci.* 83(2):165–174.
- Brzezinski JL. 2005. Detection of crustacean DNA and species identification using a PCR-restriction fragment length polymorphism method. *J Food Prot.* 68(9): 1866–1873.
- Burns M, Foster L, Walker M. 2019. DNA techniques to verify food authenticity – applications in food fraud. United Kingdom: The Royal Society of Chemistry.
- Cao J, Yu B, Ma L, Zheng Q, Zhao X, Xu J. 2011. Detection of shrimp-derived components in food by real-time fluorescent PCR. *J Food Prot.* 74(10):1776–1781.
- Cavin C, Cottenet G, Blancpain C, Bessaire T, Frank N, Zbinden P. 2016. Food adulteration: from vulnerability assessment to new analytical solutions. *Chimia.* 70(5): 329–333.
- Cavin C, Cottenet G, Fuerer C, Tran L-A, Zbinden P. 2019. Food fraud vulnerabilities in the supply Chain: an industry perspective. In: *Reference Module in Food Science*. Elsevier B. V.
- CODEX CAC/GL 74-2010. 2010. Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods [accessed 2021 June 07]. http://www.fao.org/fileadmin/user_upload/gmfp/resources/CXG_074e.pdf.
- Cottenet G, Blancpain C. 2021. A real-time PCR method to assess the presence of vertebrate material in plant-based products. *Food Control.* 125:108001.
- Cottenet G, Sonnard V, Blancpain C, Ho HZ, Leong HL, Chuah PF. 2016. A DNA macro-array to simultaneously

- identify 32 meat species in food samples. *Food Control*. 67:135–143.
- Covich AP, Thorp JH, Rogers DC. 2010. Introduction to the subphylum Crustacea. In: Thorp JH and Covich AP, editor. *Ecology and classification of North American Freshwater Invertebrates*, 3rd ed. London: Academic Press; p. 695–723.
- Eischeid AC, Kim B-H, Kasko SM. 2013. Two quantitative real-time PCR assays for the detection of Penaeid shrimp and blue crab, crustacean shellfish allergens. *J Agric Food Chem*. 61(24):5669–5674.
- Eischeid AC, Stadig SR, Rallabhandi P. 2021. Comparison of real-time PCR and ELISA for the detection of crustacean shellfish allergens. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 38(4):563–572.
- European Vegetarian Union. 2021. Criteria of the V-Label: regulations [accessed 2021 June 16]. https://www.v-label.eu/regulations_text.
- FAO, Food and Agriculture Organization of the United Nations. 2020. *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome, Italy: FAO.
- Fernandes TJR, Costa J, Oliveira MBPP, Mafra I. 2018. A new real-time PCR quantitative approach for the detection of shrimp crustaceans as potential allergens. *J Food Compost Anal*. 72:7–14.
- Fernandes TJR, Silva CR, Costa J, Oliveira MBPP, Mafra I. 2017. High resolution melting analysis of a COI mini-barcode as a new approach for Penaeidae shrimp species discrimination. *Food Control*. 82:8–17.
- Ghedira R, Papazova N, Vuylsteke M, Ruttink T, Taverniers I, De Loose M. 2009. Assessment of primer/template mismatch effects on real-time PCR amplification of target taxa for GMO quantification. *J Agric Food Chem*. 57(20): 9370–9377.
- Herrero B, Vieites JM, Espineira M. 2012. Fast real-time PCR for the detection of Crustacean allergen in foods. *J Agric Food Chem*. 60(8):1893–1897.
- Hird H, Chisholm J, Sanchez A, Hernandez M, Goodier R, Schneede K, Boltz C, Popping B. 2006. Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. *Food Addit Contam*. 23(7):645–650.
- Mäde D, Rohmberger D. 2017. Development of sensitive and specific real-time PCR systems for the detection of crustaceans in food. *Eur Food Res Technol*. 243(12): 2105–2113.
- Monaci L, Visconti A. 2010. Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends Food Sci Technol*. 21(6): 272–283.
- NCBI Nucleotide database. 2013. National library of medicine (US). National Center for Biotechnology Information [accessed 2021 Feb 8]. <https://www.ncbi.nlm.nih.gov/nuccore?itool=toolbar>.
- Santaclara FJ, Espineira M. 2017. Fast real-time PCR for the detection of crustacean allergens in foods. *Methods Mol Biol*. 1620:163–171.
- Vegan Society. 2021. Statistics [accessed 2021 June 16]. <https://www.vegansociety.com/news/media/statistics>.
- Woo CK, Bahna SL. 2011. Not all shellfish “allergy” is allergy! *Clin Transl Allergy*. 1(1):3.