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Comparison of real-time PCR and ELISA for the detection of crustacean shellfish allergens

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ABSTRACT

Food allergies are a significant public health concern, and crustacean shellfish represent one of the major FDA regulated food allergens. Allergic individuals must avoid foods containing crustaceans, and this necessitates highly sensitive and accurate detection methods. Two of the major methods used are protein-based ELISA and DNA-based real-time PCR. In order to properly compare these very different methodologies, we used identical split samples for a side-by-side comparison and analysed them using four different real-time PCR methods and two different commercial ELISA kits. Three real-time PCR assays targeting the mitochondrial 12S genes of shrimp, crab, and lobster were compared to a commercial ELISA assay for total crustacean protein. A fourth real-time PCR assay targeting the tropomyosin gene of shrimp was compared to an ELISA assay for shrimp tropomyosin. All comparisons were carried out in two different food matrices: Manhattan clam chowder and fish sauce. PCR assays had a more broad dynamic range ($0.1\text{--}10^6$ mg/kg) as compared to ELISA ($200\text{--}4000$ mg/kg) and did not show matrix interference like ELISA. In cases where the ELISA assays did not have matrix interference, there was good qualitative agreement between PCR and ELISA.

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Introduction

Food allergies are a significant public health issue and crustacean shellfish – shrimp, crab, and lobster – are one of the major allergenic foods cited in the Food Allergen Labelling and Consumer Protection Act (FALCPA). Approximately 1–2% of the population is allergic to crustaceans, with some estimates as high as 10% (Fernandes et al. 2015; Sathe et al. 2016; Sicherer and Sampson 2018). Since there is no cure, avoidance remains one of the most effective tools in the management of food allergy (Sicherer and Sampson 2018). This necessitates accurate detection methods. Detection methods developed thus far are primarily based on biomarkers, such as protein and DNA (Prado et al. 2016; Senyuva et al. 2019). Each can be performed in different formats, and each has specific advantages and disadvantages.

Enzyme-linked immunosorbent assay (ELISA), the most commonly used protein-based method, is usually performed in an antibody sandwich format for allergen detection (Prado et al. 2016). ELISAs can

target total protein from the allergenic food of interest or a specific protein, such as the allergen itself (Prado et al. 2016). For crustaceans, commercial ELISA assays are available which determine levels of both total protein and tropomyosin, which is the primary allergenic protein in crustaceans (Fernandes et al. 2015; Sathe et al. 2016). Real-time and endpoint PCR, the most common DNA-based allergen detection methods, are based on amplification and subsequent detection of DNA from the allergenic food. Real-time PCR has the potential to be quantitative and is generally more sensitive and more specific than endpoint PCR. As with ELISA, the exact target analytes can differ: PCR assays can be designed to detect DNA from a gene that codes for the allergen itself or from other regions of either nuclear or organellar genomes. Targeting of high-copy number organellar genomes like mitochondria has been shown to yield more sensitive assays (Eischeid et al. 2013; Prado et al. 2016). For crustaceans, real-time PCR assays targeting mitochondrial genes have been developed in our laboratory for shrimp, crab, and lobster, and a real-time PCR assay targeting the

tropomyosin gene has been developed for shrimp (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018).

PCR and ELISA methods have complementary advantages and disadvantages (reviewed in: Fernandes et al. 2015; Prado et al. 2016). ELISA assesses proteins directly, and most ELISA assays are supplied as ready to use kits which do not require advanced laboratory equipment or high levels of expertise (Senyuva et al. 2019). For these reasons, ELISA has historically been the most commonly used allergen detection method. However, ELISAs have a narrow dynamic range which necessitates sample dilution and can complicate laboratory analyses, and they are often subject to adverse effects from matrix interferences. In addition, rigorous protein extraction protocols may damage the protein targets, rendering them undetectable. The primary disadvantage of PCR is that it does not provide any indication of the presence or quantity of allergenic proteins in food. However, well-designed PCR assays have very high levels of specificity, minimal cross-reactivity, and low limits of detection. Real-time PCR is not affected by matrix interferences and has a broad dynamic range. Moreover, DNA can withstand relatively harsh laboratory extraction methods, which lend a greater chance of success in difficult food matrices. PCR is currently used as a confirmatory method for allergen detection at FDA.

It is beneficial to use both PCR and ELISA-based methods in analysing samples for allergen detection in foods, as this combines the advantages of both techniques and provides greater confidence in the assay outcomes. Furthermore, for crustaceans, FALCPA requires labelling of the specific type of crustacean, shrimp, crab, or lobster; crustacean ELISAs cannot distinguish among them, so PCR is required. Comparing PCR and ELISA results directly can be complicated, as these two methods measure completely different analytes that are extracted by different methods, analysed using different sets of calibration standards, and reported in different units. Comparison, therefore, relies on the generation of empirical data using identical materials. However, available reference materials have primarily been produced using protein extracts and are therefore not suitable for comparison of DNA- and protein-based methods. In order to

compare their performance effectively, it is important to use identical split samples spiked with whole allergenic foods for a side by side comparison. This allows for a direct, straightforward comparison of results without the confounding effects that may result from trying to account for significant differences in measured analytes and reporting units. Studies using split samples have demonstrated that the relative performances of PCR and ELISA depend on the particular ELISA and PCR methods used in the analysis. Most comparisons of quantitative performance, such as dynamic range and limit of detection (LOD), show that real-time PCR has a broader dynamic range than ELISA (Platteau et al. 2011; Fernandes et al. 2018; Piknova et al. 2018), though similar ranges and LODs have been reported in some cases (Costa et al. 2014; Piknova et al. 2018). Relative limits of detection vary and seem to be significantly affected by the specifics of PCR assay design (Stephan and Vieths 2004; Ecker et al. 2013; Costa et al. 2014; Palle-Reisch et al. 2015; Fernandes et al. 2018; Piknova et al. 2018). This variation is not unexpected, as PCR sensitivity can be significantly affected by nearly every aspect of method design: sample preparation, DNA extraction, target abundance, product size, quality of primers and probes, primer concentration, probe concentration, and thermal cycling conditions. Qualitative characteristics such as correlation in data and matrix effects have also been compared. Some reports showed an unexpectedly low level of variation in matrix effects between PCR and ELISA (Ecker et al. 2013; Palle-Reisch et al. 2015; Piknova et al. 2018) though ELISA samples have been noted to require dilution in some cases (Fernandes et al. 2018; Piknova et al. 2018), and most studies have not been focused on matrix variation. Despite the significant differences in the methodology and quantitative performance between PCR and ELISA, there is near universal agreement that their qualitative results are comparable (Stephan and Vieths 2004; Gomez Galan et al. 2010; Ecker et al. 2013; Scharf et al. 2013; Costa et al. 2014; Palle-Reisch et al. 2015; Walker et al. 2018), though some comparisons in market samples have indicated higher levels of variation (Fernandes et al. 2018).

In this study, we report direct comparison of real-time PCR and ELISA for detection of

crustacean shellfish using previously developed real-time PCR methods (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018) and two commercial ELISA kits used by FDA for regulatory purposes. This is one of only two studies (with Fernandes et al. 2018) we know of that compares PCR and ELISA for crustaceans, or any animal-based product, as previous comparison studies were mainly focused on tree nuts and other plant-based products. In this work, we used identical split samples to conduct side-by-side comparison of PCR and ELISA assays that have different targets: real-time PCR assays targeting either the nuclear tropomyosin gene or the mitochondrial 12S ribosomal rRNA (12S rRNA) gene were compared, respectively, with ELISA assays targeting either tropomyosin protein or total crustacean protein. Manhattan clam chowder was used as a model matrix in this study. Further, fish sauce was also used, which is potentially challenging as a low-protein, low-DNA, and high-salt matrix. We compared results of PCR and ELISA methods from the perspective of both the qualitative and quantitative aspects.

Materials and methods

Experimental design

We have carried out two sets of comparisons (Table 1): the first is direct comparison using a Maruha ELISA for total crustacean proteins and three different real-time PCR assays: one each for shrimp (Eischeid et al. 2013), crab (Eischeid and Stadig 2018), and lobster (Eischeid 2016), each targeting

the mitochondrial 12S gene. Separate experiments were conducted for each of the three crustacean types in two different food matrices, Manhattan Clam Chowder and fish sauce. Two completely independent experiments were done for each crustacean type in each food matrix (Table 1). The second comparison used an ELISA Systems assay for tropomyosin protein and real-time PCR targeting the tropomyosin gene (Eischeid et al. 2013) in Manhattan Clam Chowder or fish sauce. This work was carried out using only shrimp, and two completely independent experiments were conducted (Table 1).

Sample preparation

Standards and test samples were spiked and homogenised as described previously (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018), with minor modifications. An initial crustacean homogenate was prepared by mixing 5 g of raw crustacean meat with 15 mL molecular biology grade water, then blended on high speed with the IKA Tube Mill control (IKA, Wilmington, NC, USA) for 1–3 minutes. Additional water was added to reach a total volume of 20 mL and a concentration of 0.25 g (250 mg) of crustacean meat/mL. Subsequently, serial dilutions were prepared with 9 mL of water and 1 mL of the homogenate to produce lower concentrations of crustacean and ensure accurate measurement of even very low amounts of crustacean meat. Standard curves samples for real-time PCR were then spiked by blending 18 mL of water, 5 g of Manhattan Clam Chowder, and 2 mL of crustacean

Table 1. Experimental Design.

Experiment numbers correspond to data in Tables 4–6				
Total Protein ELISA (Maruha kit) and 12S PCR				
Type of crustacean	Food Matrix and Experiment			
	Fish sauce, first expt	Fish sauce, second expt	Manhattan Clam Chowder, first expt	Manhattan Clam Chowder, second expt
Shrimp	Expt 1a	Expt 1b	Expt 2a	Expt 2b
Crab	Expt 1a	Expt 1b	Expt 2a	Expt 2b
Lobster	Expt 1a	Expt 1b	Expt 2a	Expt 2b
Tropomyosin ELISA (ELISA Systems kit) and tropomyosin PCR				
Type of crustacean	Food Matrix and Experiment			
	Fish sauce, first expt	Fish sauce, second expt	Manhattan Clam Chowder, first expt	Manhattan Clam Chowder, second expt
Shrimp	Expt 3a	Expt 3b	Expt 4a	Expt 4b

Table 2. Preparation of crustacean homogenate, spiking levels, and ppm calculations.

homogenate #	mg/mL crustacean meat	mg crustacean meat in 2 mL homogenate	mg food matrix (5 grams)	Crustacean parts per million (ppm)*
1	250	500	5000	100000
2	25	50	5000	10000
3	2.5	5	5000	1000
4	0.25	0.5	5000	100
5	0.025	0.05	5000	10
6	0.0025	0.005	5000	1
7	0.00025	0.0005	5000	0.1
8	0.000025	0.00005	5000	0.01

*parts per million = $10^6 \times (\text{mg crustacean meat} / \text{mg food matrix})$ when 5 g of food matrix is spiked with 2 ml of homogenate.

homogenate to achieve the desired ppm level (Table 2). These standards, consisting of crustacean meat spiked into clam chowder, were used to generate a linear standard curve by plotting C_T value as a function of crustacean level. Crustacean levels in test samples were determined using C_T value for the test sample and interpolation from the standard curve, as is standard practice in real-time PCR. Test samples for PCR-ELISA comparison were prepared in the same manner as standards by spiking Manhattan clam chowder or fish sauce with shrimp, crab, or lobster at 250, 500, 1000, 2000, and 4000 mg crustacean meat per kg food matrix (mg/kg). Homogenised standards and test samples were split into half and stored frozen prior to being used either for PCR or for ELISA.

Real-time PCR

Primers and probes targeting the tropomyosin or 12S rRNA mitochondrial genes (Table 3) were designed as previously described for shrimp (Eischeid et al. 2013), crab (Eischeid and Stadig 2018), and lobster (Eischeid 2016). Full mitochondrial genome sequences were obtained from Genbank (Table 3) and sequences for the 12S rRNA genes were manually extracted from the full mitochondrial genomes. Sequences were aligned, and primers and probe were designed from the alignment using Allele ID software (Premier BioSoft, Palo Alto, CA, USA). Primers and probes were designed specifically to bind target crustacean type sequences and not to bind 12S rRNA gene sequences from other crustaceans.

For real-time PCR, SDS detergent was added to homogenates at a final concentration of 1%; standards and test samples for PCR-ELISA comparison

were subject to DNA extraction, PCR, and initial data analysis as previously described (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018): DNA extraction was carried out using a DNeasy kit (Qiagen, Valencia, CA) and extracted DNA was quantified using a NanoDrop (ThermoFisher Scientific, Wilmington, DE). Each PCR reaction contained 1x PCR buffer, 5 mM Mg, and 1.2 mM dNTPs. Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY) was added to a final concentration of 0.05 units/ μL , or 0.625 units in a 12.5 μL reaction. Template DNA was normalised to 50 ng per reaction. An exogenous internal control developed by Deer, Lampel, and Gonzalez-Escalona (2010) was used as previously described (Table 3; Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018). Primers, probes, and ultramer DNA template for the internal control were purchased from Integrated DNA Technologies (Coralville, IA). Primers and probes for each assay were included at optimal concentrations (Table 3) determined experimentally during development of each assay (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018). Thermal cycling consisted of an initial cycle at 95°C for 3 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 65°C for 2 minutes. Fluorescent signal was read at the end of the annealing step. PCR was carried out on the CFX 96 Real-time PCR system (BioRad, Hercules, CA).

Data were analysed with accompanying CFX Manager software provided by the manufacturer (BioRad, Hercules, CA). For standards, C_T values were plotted versus spiked, known crustacean ppm levels to generate linear standard curves; C_T values

Table 3. Primer and probe sequences, concentrations, and design information.

assay	sequence type	sequence 5' - 3'	final concentration in PCR	species and accession numbers used in design
shrimp	forward primer	TTCTAGGTACACTTCCAGTACACC	200 nM	<i>Penaeus monodon</i> (AF217843), <i>Litopenaeus vannamei</i> (NC_009626, EF584003, DQ534543), <i>Fenneropenaeus chinensis</i> (DQ518969), <i>Farfantepenaeus californiensis</i> (EU497054), <i>Litopenaeus stylirostris</i> (EU517503), <i>Marsupenaeus japonicus</i> (AP006346)
shrimp	reverse primer	TACATCGCCGTCGCCTCTC	200 nM	
shrimp	probe	5'-Cys-5-ACATGTTAGGACTTATCTCGCTT-Iowa Black RQ-3'	200 nM	
crab	forward primer	TCTTCCAGGCTTACTTCCAG	1000 nM	
crab	forward primer	GGCACATCTACTTTGTTACG	1000 nM	
crab	forward primer	TAAACCTACTATGTTACGACTTATCTC	1000 nM	
crab	forward primer	TAAACCTACTATGTTACGACTTATCTC	1000 nM	
crab	forward primer	TAAACCTACTATGTTACGACTTATCTC	1000 nM	
crab	forward primer	CACCTACTTTGTTACGACTTATCTC	1000 nM	
crab	forward primer	CCTACTATGTTACGACTTATCTC	1000 nM	
crab	reverse primer	CTTAGTTAGATAAGCTCTAAATCATG	1000 nM	
crab	reverse primer	GCTAATTAGATAAGCTCTAAAGCATG	1000 nM	
crab	reverse primer	AATTCTGATATAGGCTCTAAATATGTAC	1000 nM	
crab	reverse primer	AATTAAATATGTTAGTAGATATAGC	1000 nM	
crab	reverse primer	AAATTAATATGTTAATTAGATATAAGC	1000 nM	
crab	reverse primer	ATTAAATTTAATCTTGATATAGGC	1000 nM	
crab	reverse primer	AATAGTTAGTAGTATTAGATATAGGC	1000 nM	
crab	probe	5'-FAM-ACATATCGC-ZEN-CCGTGCTTTC-3' - IowaBlack FQ	200 nM	
lobster	forward primer	CACCTACTATGTTACGACTTATCTCATC	2000 nM	<i>Panulirus japonicus</i> (AB071201, NC_004251), <i>Panulirus versicolor</i> (NC_017868), <i>Panulirus ornatus</i> (NC_014854), <i>Panulirus homarus</i> (NC_016015), <i>Panulirus stimpsoni</i> (NC_014339), <i>Homarus americanus</i> (NC_015607).
lobster	forward primer	CCTACTATGTTACGACTTATCTC	2000 nM	
lobster	reverse primer	TTTTGGTAAAGCTCTAAATATG	2000 nM	
lobster	reverse primer	AAGGTAAATGATAATAGCTCTAAAGTGTG	2000 nM	
lobster	reverse primer	ATAAGTAAATGTTAGTATGCTCTAAAGTG	2000 nM	
lobster	probe	5'-FAM-ACATATGCGCGCTGCTTTCAT-IABK-FQ-3'	200 nM	
IAC	forward primer	CTAACCTTCGTGATGAGCAATCG	200 nM	accession numbers not applicable; development of Internal Amplification Control is described in Deer et al. (2010)
IAC	reverse primer	GAT CAG CTA CGT GAG GTC CTA C	200 nM	
IAC	probe	5'-HEX-AGCTAGTGCATGCACTCCAGTCTCTCT 3' -IowaBlackFQ	200 nM	
IAC	template	TACAGCACCTAGCTTGTGATGATCGATCAGTACGTGAGG	5 x 10 ⁵ copies	
		TCCTACGACATCGCAAGCATGCCCTAGCTAAG		
		ATGCATCGATTGCTCATCGATACGTTAGGTGACTAGGAGG		
		ACTGGAGTGCATCGACTAGCTAAGTGGTTCGATTGCTC		
		ATCACGAAGTTAGTGCATACGAAACGAGTGCATGTTAGGTT		
shrimp tropomyosin	forward primer	TGCAGCAACTGAGAACACCTTG	200 nM	<i>Penaeus monodon</i> (AY827100); designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA).
shrimp tropomyosin	reverse primer	TGTCCTCTCCACAAAGCTGGATG	200 nM	
shrimp tropomyosin	probe	5'-FAM-AGGTGCAGGAATCCTTCTGCTGAAGCTA-3'-IowaBlack FQ	200 nM	
shrimp tropomyosin	amplicon	TTTCAACCTTCAGAAAGGATGCAAGCACTTG	varies	
		AGAAAGCCTTGACAGGTGCGAGGAATCTTCTG		
		GAAGGCTAACATCCAGCTTGTGGAGAA		
		GGACAAGGCCCTCTCTAAGCTGAGG		

in test samples were used to interpolate ppm levels from these standard curves. For determination of tropomyosin copy number, a standard curve was generated using known copy numbers of the PCR amplicon (Table 3).

ELISA

The homogenised split taken for ELISA was subject to protein extraction, ELISA, and data analysis per manufacturer's instructions for total crustacean protein (Crustacean Kit Maruha, Maruha-Nichiro, Tokyo, Japan) or tropomyosin protein (# ESCRURD-48, ELISA Systems, Queensland, Australia), both antibody sandwich ELISA assays. With the Maruha ELISA kit for total crustacean protein, samples were prepared by adding 1 g of homogenised sample to 19 ml of extraction solution provided with the kit, shaking at room temperature for 17 hours, and centrifuging at 3000 g for 20 minutes. Supernatant was diluted 20-fold with dilution buffer as instructed by the manufacturer prior to performing ELISA. Calibration standards were prepared via serial dilution of a total crustacean protein standard provided with the kit. Both samples and standards were plated into wells of an ELISA plate precoated with a monoclonal antibody specific to black tiger shrimp tropomyosin and incubated at room temperature for 90 minutes. Unbound substrate was washed away and a secondary, HRP-conjugated detection antibody was added for a 30-min incubation at room temperature. The plate was washed again and TMB substrate was added and allowed 20 minutes at room temperature for reaction with the HRP conjugate. The reaction was stopped by addition of an acidic solution and absorbance of the resulting products was measured at 620 nm.

The ELISA Systems kit to measure tropomyosin protein is also an antibody sandwich design. Samples for this kit were prepared by adding 1 g of sample to 9 mL of extraction solution and shaken at 60°C for 15 minutes. Samples and prepared kit standards containing shrimp tropomyosin were added to wells of an ELISA plate pre-coated with anti-shrimp tropomyosin antibody, incubated at room temperature for 30 minutes, and then washed. Enzyme conjugate solution containing peroxidase-conjugated anti-shrimp tropomyosin antibody was then added to each well, incubated at room temperature for

15 minutes, and washed. Substrate solution containing TMB was then added and allowed 10 minutes at room temperature to react with the enzyme conjugate. The reaction was stopped by addition of an acidic solution and absorbance of the resulting products were measured at 620 nm.

For both kits, ELISA plates were read on an Infinite 200 Plate reader (Tecan, Mannedorf, Switzerland). Standard curves were generated for both ELISA assays by plotting absorbance as a function of protein concentration according to manufacturer's instructions; protein levels in food matrices were determined by interpolation from the standard curves. Protein levels are reported in units provided by kit manufacturers, as ng/mL total crustacean protein for the Maruha ELISA (Tables 4 and Tables 5) or mg/kg crustacean tropomyosin for the ELISA Systems assay (Table 6).

Results and discussion

Total protein ELISA and 12S real-time PCR

Quantitative assessment: dynamic range and LOD

The dynamic range of all three real-time PCR assays used here – shrimp, crab, and lobster – is greater than that of ELISA. Preliminary experiments carried out to establish the reliable quantitative range for ELISA indicated that it was approximately 200–4000 mg/kg crustacean meat in food matrix (data not shown), whereas the linear range for all three PCR assays used here is $0.1\text{--}10^6$ mg/kg crustacean meat in food matrix (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018). Our data show that the dynamic range of the total protein ELISA was more broad for fish sauce (125–4000 mg/kg; Table 4) than for Manhattan clam chowder (250–2000 mg/kg; Table 5): we had to use an additional dilution step in Manhattan clam chowder which may have caused its higher LOD compared to fish sauce.

Previous comparison of crustacean PCR and ELISA has also shown that PCR targeting mitochondrial genes is more sensitive than ELISA and has broad dynamic range (Fernandes et al. 2018) similar to the assays described in this work. Compared to previous PCR-ELISA comparison studies with other food allergens, we have a lower LOD for PCR and a higher LOD for ELISA (Stephan and Vieths 2004; Platteau et al. 2011; Ecker et al. 2013; Palle-Reisch

Table 4. Results for Expt 1, Maruha total protein ELISA and 12S PCR in fish sauce.

Experiment numbers correspond to experimental design in Table 1

Crustacean type and spike level (mg/kg)	Expt 1a ELISA (ng/mL)	Expt 1a ELISA – log	Expt 1b ELISA (ng/mL)	Expt 1b ELISA – log	Expt 1a PCR- CT	Expt 1a PCR- mg/kg	Expt 1b PCR- CT	Expt 1b PCR- mg/kg
Shrimp 4000	18200	4.26	27600	4.44	23.5	1300	23.1	1730
Shrimp 2000	6710	3.83	27000	4.43	24.7	549	24.6	589
Shrimp 1000	4430	3.65	2180	3.34	25.6	277	26	219
Shrimp 500	1870	3.27	1050	3.02	26.6	140	26.8	129
Shrimp 250	1390	3.14	*	NA	27.5	73.7	28	54
Shrimp 125	*	NA	*	NA	28.6	32.3	29.3	22.6
Crab 4000	39400	4.6	68500	4.84	24.1	3590	23	10100
Crab 2000	26100	4.42	32900	4.52	25	1680	24.3	3970
Crab 1000	15200	4.18	9750	3.99	25.9	869	25.2	1990
Crab 500	5120	3.71	5060	3.7	27.2	332	26.7	670
Crab 250	2220	3.35	1630	3.21	28.2	152	27.8	288
Crab 125	804	2.91			29.2	70.2	29.2	104
Lobster 4000	9390	3.97	2620	3.42	26.6	1110	26.5	1130
Lobster 2000	6660	3.82	411	2.61	27.5	586	27.8	437
Lobster 1000	2860	3.46	*	NA	28.2	347	29.3	138
Lobster 500	3390	3.53	*	NA	30.1	84.4	30.1	77.3
Lobster 250	350	2.54	*	NA	29.6	126	30.5	47.5
Lobster 125	142	2.15	*	NA	31.9	23.6	31.4	24.2

*no response in ELISA; NA: not applicable.

Table 5. Results for Expt 2, Maruha total protein ELISA and 12S PCR in Manhattan Clam Chowder.

Experiment numbers correspond to experimental design in Table 1

Crustacean type and spike level (mg/kg)	Expt 2a ELISA (ng/mL)	Expt 2a ELISA – log	Expt 2b ELISA (ng/mL)	Expt 2b ELISA – log	Expt 2a PCR- CT	Expt 2a PCR- mg/kg	Expt 2b PCR- CT	Expt 2b PCR- mg/kg
Shrimp 2000	43700	4.64	127000	5.1	23.8	809	23.2	1410
Shrimp 1000	25900	4.41	85200	4.93	24.9	373	24.2	675
Shrimp 500	13300	4.12	24500	4.39	25.9	174	24.7	442
Shrimp 250	14300	4.16	9050	3.96	27.2	69.2	27.3	72.4
Crab 2000	15800	4.2	50100	4.7	24.6	3730	25.5	1390
Crab 1000	13800	4.14	20900	4.32	25.6	1810	26.9	501
Crab 500	1990	3.3	16100	4.21	26.3	1030	27.8	266
Crab 250	306	2.49	4400	3.64	27.6	423	29.2	99
Lobster 2000	71600	4.85	110000	5.04	25	3270	26.7	1140
Lobster 1000	21400	4.33	66100	4.82	26.2	1430	27.5	602
Lobster 500	17700	4.25	47200	4.67	27.7	446	28.7	244
Lobster 250	6190	3.79	13800	4.14	28.7	202	30.2	75.4

Table 6. Results for Expts 3 and 4, ELISA Systems shrimp tropomyosin ELISA and shrimp tropomyosin PCR.

Experiment numbers correspond to experimental design in Table 1

Fish sauce				
mg/kg shrimp meat	mg/kg tropomyosin	log mg/kg tropomyosin	Copy # tropomyosin	Log copy number
Expt 3a				
4000	*	NA	7650	3.88
2000	*	NA	3580	3.55
1000	*	NA	1370	3.13
500	*	NA	1030	3.01
Expt 3b				
4000	*	NA	15000	4.18
2000	*	NA	6740	3.83
1000	*	NA	4650	3.67
500	*	NA	1860	3.27
Manhattan Clam Chowder				
mg/kg shrimp meat	mg/kg tropomyosin	log mg/kg tropomyosin	Copy # tropomyosin	Log copy number
Expt 4a				
4000	3.56	0.551	14000	4.15
2000	2.87	0.457	7100	3.85
1000	2.22	0.346	3160	3.5
500	0.258	−0.589	1640	3.22
Expt 4b				
4000	3.33	0.522	12900	4.11
2000	3.55	0.55	7100	3.85
1000	2.77	0.442	2790	3.45
500	2.32	0.366	1680	3.22

et al. 2015; Piknova et al. 2018). The lower limit of detection for ELISA was between 125 and 250 mg/kg (Tables 4 and Tables 5) while the lower limit of detection for these real-time PCR assays is between 0.1 and 1 mg/kg (Eischeid et al. 2013; Eischeid 2016; Eischeid and Stadig 2018). This may be a result of the fact that while previous studies focused on plant-based products, our comparison was conducted in animal-based products: other researchers have also found that PCR performs better in animal products (Hsieh et al. 2015). In addition, these PCR assays were designed and optimised for maximal sensitivity: they target regions in the highly abundant mitochondrial genome, which has been shown to yield positive PCR results even in purified proteins (Kim and Lee 2010) and has yielded similar assay performance in other crustacean studies (Fernandes et al. 2018). All aspects of the PCR methods have been thoroughly optimised. The sample preparation includes a thorough grinding step and strong SDS detergent is used in DNA extraction: this results in a rigorous extraction protocol which may yield better extraction and therefore lower detection limits. In contrast, most other PCR assays reported use different sample preparation and extraction methods. Compared to PCR, ELISA must be conducted with relatively mild, and therefore likely less efficient, extraction conditions in order to preserve proteins.

It is also important to note how the practical lower limit of detection for ELISA in a food sample may differ from reported limits of detection for ELISA assays: for this total protein ELISA, the lowest point of the standard curve provided with the kit is 0.78 µg/mL. However, while that may be the lower limit of detection for total crustacean protein on the ELISA plate, the procedure requires multiple dilutions of samples during extraction and plating; even though the values obtained from ELISA are multiplied by a dilution factor to account for this, dilution may still result in some samples falling below the lower limit of detection. The standard procedure for this total protein ELISA kit results in 400-fold dilution of samples, which means that a food product must contain 400 times the detection limit to yield a positive result.

Qualitative assessment: correlation and matrix effects

The ELISA assay appeared to have some matrix interference in fish sauce; however, in general, results showed good qualitative agreement between PCR

and ELISA for all three types of crustaceans in both fish sauce (Figure 1) and Manhattan clam chowder (Figure 2); this is consistent with studies of other food allergens (Stephan and Vieths 2004; Gomez Galan et al. 2010; Ecker et al. 2013; Scharf et al. 2013; Costa et al. 2014; Palle-Reisch et al. 2015; Walker et al. 2018). We also found a strong correlation between PCR and ELISA results, with R^2 values of 0.91 or higher for all cases except for lobster in fish sauce (Figures 1 and Figures 2). In this work, all samples were prepared in the laboratory; reported literature indicates that qualitative agreement may be lower in market samples, especially at higher levels of allergenic contamination (Fernandes et al. 2018).

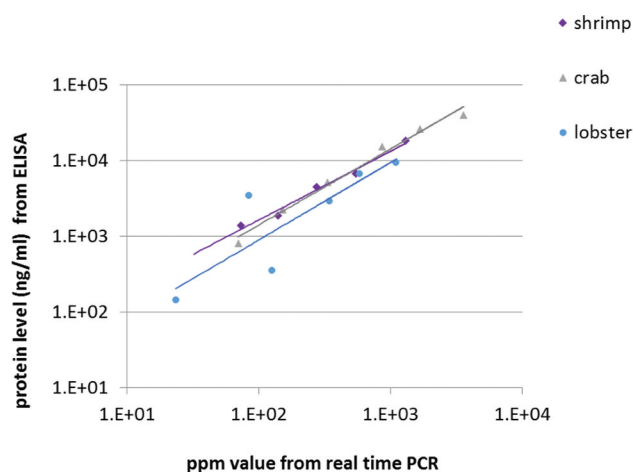


Figure 1. Comparison of 12S PCR and total protein ELISA results in fish sauce showing best fit lines for shrimp ($R^2 = 0.98$), crab ($R^2 = 0.99$) and lobster ($R^2 = 0.73$).

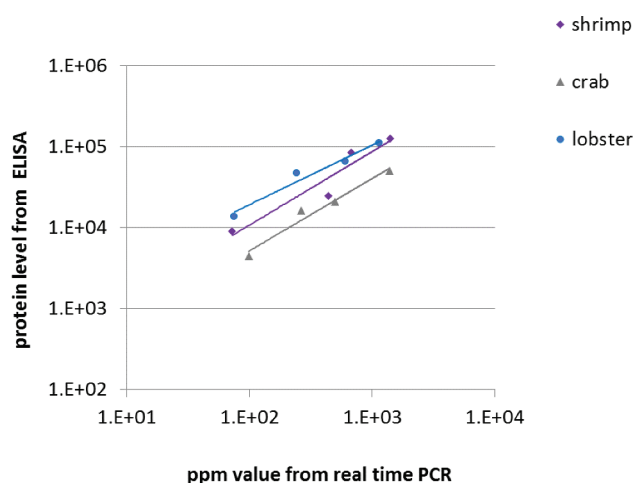


Figure 2. Comparison of 12S PCR and total protein ELISA results in Manhattan Clam Chowder showing best fit lines for shrimp ($R^2 = 0.91$), crab ($R^2 = 0.97$) and lobster ($R^2 = 0.96$).

Compared to shrimp and crab, lobster gave lower protein readings; several samples also were negative for lobster in fish sauce (Table 4). These results suggest some degree of matrix inhibition and highlight differences in response between crustacean type. Matrix inhibition by fish sauce is consistent with that found in the tropomyosin ELISA (see below). Differences in protein readings between shrimp, crab, and lobster are not unexpected; levels of individual proteins, as well as their reactivity with antibodies, can be expected to differ between different types of crustaceans and have been reported for this ELISA (Seiki et al. 2007).

Tropomyosin ELISA and tropomyosin PCR

Quantitative assessment: dynamic range and LOD

The tropomyosin ELISA and tropomyosin PCR results followed a pattern similar to that of the total protein ELISA and 12S PCR. The PCR has a broader dynamic range, from approximately $10 - 10^6$ mg/kg (Eischeid et al. 2013; supplementary material) whereas the dynamic range of this ELISA was determined to be approximately 500–4000 mg/kg (Table 6). Both of these assays have higher limits of detection than the total protein ELISA and 12S PCR, as expected because their respective targets are less abundant.

Qualitative assessment: correlation and matrix effects

In general, we found good qualitative agreement between ELISA and PCR in Manhattan Clam Chowder (Table 6 and Figure 3); this is consistent with our own data discussed above and previous reports (Stephan and Vieths 2004; Gomez Galan et al. 2010; Ecker et al. 2013; Scharf et al. 2013; Costa et al. 2014; Palles-Reisch et al. 2015; Walker et al. 2018). One outlier data point occurred for ELISA results in Manhattan Clam Chowder (Table 6, Expt 4a, 500 mg/kg). We found a dramatic matrix effect with this ELISA assay, as none of the samples produced any signal in fish sauce (Table 6). Fish sauce was intentionally chosen as a potentially problematic, low-protein, low-DNA, high salt matrix. These results highlight the difference in matrix effects between PCR and ELISA as well as between different ELISA assays.

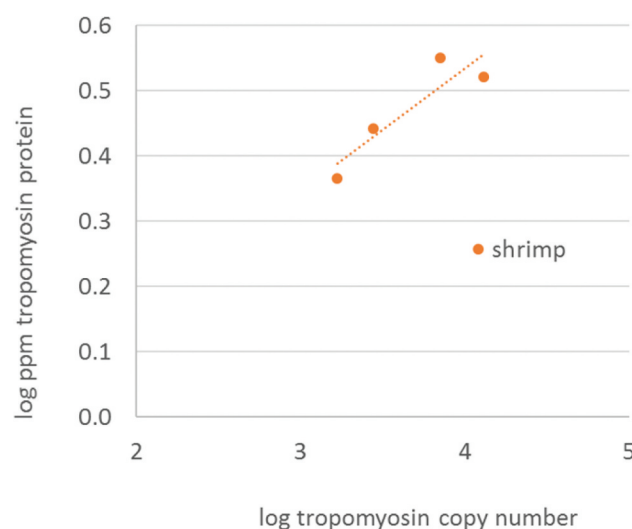


Figure 3. Comparison of tropomyosin PCR and tropomyosin ELISA results for shrimp in Manhattan Clam Chowder ($R^2 = 0.82$).

Conclusions

ELISA- and PCR-based methods for allergen detection can be difficult to compare because they measure different analytes, are calibrated to different types of standards, and are typically reported in different units. Complementary advantages and disadvantages, as well as variation in performance between individual PCR and ELISA methods, highlight the need for direct empirical comparisons and the benefit of using both protein- and DNA-based methods for allergen detection. Approximately 35–40% of FDA recalls result from allergen contamination; yet accurate detection of allergenic foods at trace levels in food products is complex and no single method addresses all of the relevant challenges. The optimal solution from a regulatory standpoint is to have a variety of different, orthogonal analytical methods available. In cases where standard regulatory methods do not produce clear results, samples can be assessed based on the weight of evidence derived from these different approaches to provide the greatest confidence in results.

Author Contributions (CRediT)

AE: conceptualization, methodology, validation, ELISA investigation, ELISA data analysis, comparison data analysis, writing: original draft, review, and editing.

SS: validation, PCR investigation, ELISA investigation, PCR data analysis, writing: review.

PR: ELISA investigation.

Declaration of Interest

This work was funded by the United States Food and Drug Administration. The authors declare no competing interests.

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