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Development and evaluation of a real-time PCR assay for detection of lobster, a crustacean shellfish allergen

Anne C. Eischeid

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Regulatory Science, 5100 Paint Branch Parkway, College Park, MD 20740, USA

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ABSTRACT

Crustacean shellfish are a leading cause of food allergy in American adults, and the Food Allergen Labeling and Consumer Protection Act requires that different types of crustacean shellfish be distinguished from each other. In general ELISA assays are not capable of differentiating crustacean type, but PCR assays are. In this work, a real-time PCR assay for lobster, a crustacean shellfish allergen, was developed and evaluated. Food matrices were spiked with lobster meat at 0.1, 1, 10, 100, 1000, 10^4, and 10^5 parts per million (ppm). In addition to testing of several different food matrices, method performance was determined using conditions which have historically proven challenging for PCR analyses, specifically food matrices with low DNA content and acidic pH levels, as well as foods that were treated with combined high temperature and pressure. Real-time PCR standard curves were generated from spiked, treated foods and analyzed with respect to linear range and reaction efficiency. In most cases, the assay was linear over 6–8 orders of magnitude; lower limits of detection were 0.1–1 ppm and reaction efficiencies were within the preferred range of 100 ± 10%. A notable exception occurred in the case of heat treatment at acidic pH, which resulted in severe delay or complete loss of amplification signals.

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1. Introduction

The Food Allergen Labeling and Consumer Protection Act of 2004, or FALCPA, (21 U.S.C. 343(w)), cites crustacean shellfish as one of eight major allergenic foods in the United States. At least 2% of American adults are estimated to have crustacean shellfish allergy, which typically begins in adulthood and lasts throughout life (Sicherer, Munoz-Furlong, & Sampson, 2004). Allergic reactions to crustacean shellfish can include hives, urticaria, angioedema, trouble breathing, closing of the throat, and even life-threatening anaphylaxis (Sicherer et al., 2004). Detection methods for crustacean shellfish should be capable of the following: 1) detection of very low levels of the target allergenic food in a complex food matrix, 2) differentiation of shrimp, crab, and lobster, and 3) detection of cooked and heat-processed samples. Detection of low levels of the allergenic food are required to protect consumers, because at present the only way allergic individuals can prevent reactions is by completely avoiding foods to which they are allergic. Differentiation of crustacean type is necessary because FALCPA requires that the different types of crustacean shellfish—shrimp, crab, and lobster—be distinguished from each other on package labeling. Detection of cooked or heat-processed samples is also important; crustacean shellfish are nearly always eaten after cooking or heat processing, and therefore allergic individuals get sensitized to these forms.

Research has shown that there are numerous allergenic proteins in crustacean shellfish; their responses to processing are complex but have not been fully elucidated. Studies in shrimp indicate that crustacean shellfish have at least six different types of allergenic proteins, including the major allergen tropomyosin and five minor allergens: arginine kinase, troponin C, sarcoplasmic Ca-binding protein, triosephosphate isomerse, and myosin light chain (Bauermeister et al., 2011). With respect to cooking and food processing: the effects of a given type of processing on different allergenic proteins can vary, and the effect of different types of processing on a given allergenic protein can vary. As tropomyosin is the major crustacean allergen and the first to be discovered, work on specific allergenic proteins has been focused on tropomyosin. Tropomyosin exhibits decreased antibody binding after treatment with combined ultrasound and boiling or combined high temperature and pressure (Yu et al., 2011). Despite apparent breakdown of

E-mail address: Anne.Eischeid@fda.hhs.gov.

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tropomyosin after these combined treatments, however, boiling of whole crustacean shellfish extracts has been shown to 1) increase reactivity and detection using monoclonal antibodies, and 2) increase allergenicity as determined using basophil activation assays, serum IgE binding, and skin prick tests (Abramovitch et al., 2013; Carnes et al., 2007; Kamath, Rahman, Komoda, & Lopata, 2013).

At present, detection of crustacean shellfish is primarily carried out using ELISA assays, which detect proteins directly using antibody binding. ELISA assays are currently the accepted standard in allergen detection and are commercially available for most allergens. Excellent work has been carried out on development and validation of ELISA assays for detection of crustacean shellfish (Sakai, Adachi, Akiyama, & Teshima, 2013; Seiki et al., 2007). However, the ELISA assays do not meet the FALCPA requirement of differentiating shrimp, crab, and lobster. Currently available ELISA assays detect the major allergen tropomyosin, which exhibits approximately 90% similarity between crustacean species, and therefore do not distinguish shrimp, crab, and lobster (Kamath et al., 2013; Poms, Klein, & Anklam, 2004). Detection of processed foods is also a concern with ELISA assays. Structural changes in proteins caused by heat processing can be affected by the nature of the food matrix, and can have variable effects on both antibody binding and protein extraction, thereby influencing ELISA results in sometimes unpredictable ways. Detection of other allergenic proteins besides tropomyosin or development of ELISA assays which recognize denatured (processed) forms of proteins may help alleviate some of these issues. However such work would be complicated by the diverse nature of protein allergens and variations in protein response to food processing. Furthermore, development and production of antibodies for ELISA assays involves significant cell culture and animal work which lead to increased cost and batch-to-batch variation. It is important to develop and evaluate other methods which can be produced cost-effectively, reproduced reliably, and which are robust and consistent in detection of processed samples.

PCR assays, which detect the DNA of allergenic foods, represent another promising but less thoroughly developed detection method. PCR and real-time PCR have several advantages over ELISA assays: DNA is more stable and predictable than proteins in response to heat processing and food matrix characteristics, and PCR methods are capable of distinguishing shrimp, crab, and lobster (Poms et al., 2004). Furthermore, while the performance of ELISA assays is primarily dependent on antibodies which are produced through an inherently costly and variable process, the performance of PCR-based assays is primarily dependent on oligonucleotide primers and probes which are produced through an inexpensive chemical synthesis and do not show batch-to-batch sequence variation. PCR assays do not detect allergenic proteins directly, and they are best used only in cases where the DNA is an accurate indicator of the presence of allergenic proteins, such as crustaceans, fish, tree nuts, and peanuts (Poms et al., 2004; also discussed in Eischeid, Kim, & Kasko, 2013). In those cases for which DNA-based assays are appropriate, they represent a straightforward, cost-effective, and robust detection method for allergenic foods. Few studies have compared ELISA and PCR assays, but PCR methods have performed favorably in such comparisons (Sakai et al., 2013; Taguchi et al., 2011).

DNA-based detection of crustacean shellfish has been carried out using both end-point PCR and real-time PCR (Eischeid et al., 2013: Taguchi et al., 2011). Conventional, end-point PCR is inherently a qualitative method, but real-time PCR can be used for both detection and quantitation of crustacean allergens in complex food matrices (Eischeid et al., 2013). During a real-time PCR reaction, generation of new PCR products is measured at each cycle of the PCR using a fluorescent probe. The C_T value, or cycle number at which a given sample generates signal, is determined during the early exponential phase of the reaction and is related to the amount of target DNA in the sample. A linear standard curve is generated from real-time PCR data by plotting C_T values (on the y axis) as a function of (log) target DNA amount (on the x axis); this provides the basis for quantitation (Adams, 2006; Heid, Stevens, Livak, & Williams, 1996). During an ideal PCR reaction, the amount of target DNA increases 2-fold with each cycle of PCR. Since the ideal, theoretical relationship between x and y axes of the standard curve is known, its slope can be used to assess performance of a real-time PCR assay.

Three food production conditions which present particular challenges to PCR-based allergen detection are 1) heat processing, especially the combined high heat and pressure which occur during canning and autoclaving, 2) food matrices with inherently low levels of DNA, and 3) food matrices with acidic pH. Previous studies indicate that heat processing can adversely affect PCR assays, but that this limitation can be overcome by designing assays to target small regions of the genome. Analysis of meat samples consisting entirely of the target species has demonstrated that qualitative, conventional PCR assays using 270 basepair (bp) PCR products are not affected by extensive boiling or by autoclaving (Arslan, Ilhak, & Calicioglu, 2006). Real-time PCR assays are not affected by boiling but they are affected by autoclaving in a PCR product size-dependent manner, with products up to 116 bp unaffected and products of 350 bp and 565 bp exhibiting either delayed amplification or no amplification (Hird et al., 2006). Previous work from this laboratory has shown that quantitative, real-time PCR assay of shrimp and crab allergens spiked into food matrices was robust to heat treatments, including boiling and autoclaving, when PCR products of approximately 100 bp were used (Eischeid et al., 2013). Food matrices containing inherently low levels of DNA pose another challenge in PCR-based allergen detection. Low sample DNA content can lead to lowered DNA extraction efficiency; but sensitive detection and reliable quantitation require consistently high extraction efficiencies. Addition of carrier nucleic acid has been shown to increase efficiency of silica-based procedures and is commonly used to enhance DNA extraction efficiency (Ausubel et al., 1999; Boom et al., 1990). The effect of carrier nucleic acid is especially apparent in low-DNA content samples and robotic extraction procedures (Kishore, Hardy, Anderson, Sanchez, & Buoncristiani, 2006). A third challenge to PCR-based allergen detection is that of acidic food matrices. DNA is known to undergo acid-catalyzed hydrolysis which may degrade it sufficiently to inhibit PCR-based detection. In foods, accelerated degradation of DNA in acidic conditions, including tomato-based products, has been shown to occur and to affect PCR results (Bauer, Weller, Hammes, & Hertel, 2003; Bergerova, Godalova, & Siekel, 2011).

In this paper, development and evaluation of a method for the detection of lobster allergen is reported. This lobster method has been applied to numerous food matrices using several cooking methods in a manner similar to this laboratory's previous evaluation of shrimp and blue crab allergen assays (Eischeid et al., 2013). In addition, the work described here includes a more thorough investigation of the challenging conditions described above, including boiling and autoclaving in foods with inherently low DNA content and acidic pH levels. Linear range and reaction efficiency are reported for all tested matrices and preparation conditions. The assay was tested over 6–8 orders of magnitude ranging from 0.1 to 10^5 ppm lobster meat. Testing the method across a wide concentration range accounts for trace amounts of allergen which find their way into foods through cross-contact as well as higher amounts of allergen found in foods which have been mislabeled.

2. Materials and methods

2.1. Primer and probe design

Primers and probes for lobster were designed in a manner similar to that previously described for shrimp (Eischeid et al., 2013). Complete mitochondrial genome sequences were obtained from Genbank for *Panulirus japonicus* (accession numbers AB071201, NC_004251), *Panulirus versicolor* (NC_017868), *Panulirus ornatus* (NC_014854), *Panulirus homarus* (NC_016015), *Panulirus stimpsoni* (NC_014339), and *Homarus americanus* (NC_015607). Sequences for 12S genes were manually extracted and aligned; primers and probe were designed from the alignment using Allele ID software (Premier BioSoft, Palo Alto, CA, USA). Primers and probes were designed to specifically target lobster species and avoid other types of crustacean shellfish (e.g., shrimp and crab). Sequences were obtained for two forward primers (5′-CACCTAC-TATGTTACGACTTATCTCATC-3′ and 5′-CCTACTATGTTACGACT-TATTTC-3′) and three reverse primers (5′-TTTTGGTATAAGCT CTAAATTATG-3′, 5′-AAGGTAAAATGATAATAGCTCTAAAGTGTG-3′, and 5′-ATAAGGTAAAATGGTATTAGCTCTAAAGTG-3′), resulting in 80 bp-90 bp PCR products. A single probe (5′-FAM-ACA-TATTGCCCGTCGCTTTCAT-IABkFQ-3′) was obtained for all species. The internal amplification control (IAC) consisted of exogenous, synthetic template DNA with its associated primers and probes; it was developed by Deer, Lampel, and Gonzalez-Escalona (2010) and used as previously described (Eischeid et al., 2013). All primers and probes, as well as template DNA for the IAC, were obtained as custom oligonucleotides from Integrated DNA Technologies (Coralville, IA). Each PCR reaction contained all 5 lobster primers and the single lobster probe, as well as primers and probe for the IAC.

2.2. Sample preparation and DNA extraction for method evaluation in foods

Sample preparation and DNA extraction were carried out as previously described (Eischeid et al., 2013). Food matrices included examples of seafood, deli foods, juices, dry noodles, and canned soups (Table 1 and Table 3). For experiments comparing neutral and acidic conditions, pH was determined prior to spiking, cooking, and homogenization. For all experiments, lobster meat was spiked into foods at levels of 0.1, 1, 10, 100, 1000, 10^4, and 10^5 ppm prior to cooking and homogenization; one sample consisting entirely of lobster meat (10^6 ppm) was included in most experiments as a control. Microwaving was carried out in a 1500 W microwave at full power for 1 ½ minutes. Boiling was carried out on a hot plate, and samples were kept at a low boil for 20 min. Autoclaving was carried out at 121 °C for either 10 min or 20 min, as indicated (Table 3). After spiking and cooking, samples were mixed with 2% SDS buffer (4–5 ml per gram) and thoroughly homogenized using a commercial blender. DNA was extracted from homogenates using a hybrid of the procedure described by Aljanabi and Martinez (1997) and the silica-based DNeasy kit (Qiagen, Valencia, CA) and is described in Eischeid et al. (2013). DNA was eluted in 60 μl Buffer AE and concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Where indicated, 3 μg per sample of *E. coli* carrier DNA (Sigma–Aldrich, cat #D2001) was added prior to DNA extraction (Table 1).

2.3. PCR and data analysis for method evaluation in foods

PCR reactions were set up as previously described (Eischeid et al., 2013) with some modifications. Each PCR reaction contained the following in a 12.5 μl reaction volume: 1 × PCR buffer, 5 mM Mg, 30 nM ROX reference dye, 1.2 mM dNTPs, and 0.625 units Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY, USA). Lobster primers were included at 2000 nM each; the lobster probe was used at 200 nM. Primers and probes for the internal amplification control (IAC) were included at 200 nM, and the internal control template DNA was added to a constant copy number of 5×10^5 in all reactions (Deer at al., 2010; Eischeid et al., 2013). For salmon, macaroni salad, dry noodles, and New England clam chowder, template DNA was included at 50 ng (50,000 pg) per reaction; for clam juice and clam and tomato cocktail; 5 μl of undiluted template DNA was used per reaction. PCR reactions were run on two different platforms: an Mx3005P (Agilent Technologies, Santa Clara, CA, USA) and a 7500 Fast (Applied Biosystems by Life Technologies, San Francisco, CA). Thermal cycling included an initial cycle at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C or 65 °C for 30 s, and extension at 65 °C for 2 min. Annealing temperature did not affect linear range or reaction efficiency at the temperatures used here; fluorescent signal was read at the end of the annealing step. Data obtained from the Mx3005P were analyzed using MxPro software

Table 1
Data from initial validation experiments.

Platform	Food matrix	Processing/extraction	12S	12S	12S	IAC	IAC
			Range	Effic.	R^2	Range	Ct ± SD
Mx3005P	TE buffer[a]	NA[a]	0.05–50,000 pg	92%	0.997	NA*	NA*
Mx3005P	TE buffer[a]	NA[a]	0.005–5000 g	109%	0.994	NA*	NA*
Mx3005P	Salmon (1)[b]	uncooked	0.1–10^6 ppm	102%	0.989	0.1–10^6 ppm	23.83 ± 0.14
7500 Fast	Salmon (1)	uncooked	1 – 10^6 ppm	78%	0.98	0.1–10^6 ppm	21.42 ± 0.20
Mx3005P	macaroni salad (2)	uncooked	0.1–10^5 ppm	82%	0.986	0.1–10^5 ppm	24.68 ± 0.16
7500 Fast	macaroni salad (2)	uncooked	0.1–10^5 ppm	108%	0.984	0.1–10^5 ppm	21.72 ± 0.07
Mx3005P	clam and tomato cocktail (3)	uncooked, with carrier DNA	0.1–10^6 ppm	96%	0.981	0.1–10^6 ppm	23.97 ± 0.29
Mx3005P	clam and tomato cocktail (3)	uncooked, without carrier DNA	0.1–10^6 ppm	103%	0.984	0.1–10^6 ppm	24.97 ± 0.40
7500 Fast	clam and tomato cocktail (3)	uncooked, with carrier DNA	0.1–10^6 ppm	122%	0.978	0.1–10^6 ppm	21.83 ± 0.08
7500 Fast	clam and tomato cocktail (3)	uncooked, without carrier DNA	0.1–10^6 ppm	106%	0.98	0.1–10^6 ppm	22.30 ± 0.21
Mx3005P	dry noodles (4)	uncooked	0.1–10^6 ppm	86%	0.995	0.1–10^6 ppm	22.84 ± 0.21
7500 Fast	dry noodles (4)	uncooked	0.1–10^6 ppm	107%	0.96	0.1–10^6 ppm	20.28 ± 0.33
Mx3005P	New England Clam Chowder (5)	uncooked	0.1–10^6 ppm	115%	0.93	0.1–10^6 ppm	22.74 ± 0.20
7500 Fast	New England Clam Chowder (5)	uncooked	0.1–10^6 ppm	88%	0.97	0.1–10^6 ppm	22.05 ± 0.12
Mx3005P	New England Clam Chowder (6)	microwaved	0.1–10^6 ppm	78%	0.99	0.1–10^6 ppm	20.64 ± 0.26
7500 Fast	New England Clam Chowder (6)	microwaved	1 – 10^6 ppm	73%	0.97	0.1–10^6 ppm	22.84 ± 0.09
Mx3005P	New England Clam Chowder (7)	boiled	0.1–10^6 ppm	89%	0.99	0.1–10^6 ppm	22.87 ± 0.18
7500 Fast	New England Clam Chowder (7)	boiled	0.1–10^6 ppm	99%	0.98	0.1–10^5 ppm	22.65 ± 0.06

[a] NA: not applicable. PCR was carried out directly in TE buffer to test performance of lobster primers and probes.
[b] Numbers in parenthesis indicate which of the 7 independent experiments are represented.

Table 2
Relative standard deviation (RSD) data for initial validation experiments.

Log ppm	Target	RSD(%),Mx3005P	RSD(%), 7500 Fast	Log ppm	Target	RSD (%), Mx3005P	RSD (%), 7500 Fast
−1	12S	7.95	6.50	−1	IAC	5.16	3.50
0	12S	6.08	7.93	0	IAC	5.70	2.60
1	12S	4.49	8.72	1	IAC	5.71	3.52
2	12S	5.48	10.88	2	IAC	6.50	3.33
3	12S	7.41	12.82	3	IAC	5.45	3.62
4	12S	6.51	11.21	4	IAC	5.91	3.44
5	12S	5.96	11.53	5	IAC	6.09	3.80
6	12S	6.97	12.23	6	IAC	6.88	5.32

Table 3
Data for challenging food matrices and processing Conditions.[a]

Food matrix	Matrix description	Processing	12S	12S	12S	IAC	IAC
			Range	Effic.	R^2	Range	Ct + -SD
New England clam chowder	neutral pH/not low DNA	autoclave 10 min	1–10^6 ppm	79%	0.997	0.1–10^6 ppm	23.81 ± 0.16
New England clam chowder	neutral pH/not low DNA	autoclave 20 min	10–10^6 ppm	68%	0.99	0.1–10^6 ppm	23.75 ± 0.08
clam juice	neutral pH/low DNA	autoclave 10 min.	1–10^6 ppm	117%	0.98	0.1–10^6 ppm	23.83 ± 0.14
clam and tomato cocktail[b]	acidic pH/low DNA	autoclave 10 min	amplification only at 10^6 ppm	NA	NA	0.1–10^6 ppm	23.84 ± 0.29
clam and tomato cocktail[b]	acidic pH/low DNA	autoclave 10 min	misc. late amp. except 10^6 ppm	NA	NA	0.1–10^6 ppm	22.86 ± 0.14
clam juice	neutral pH/low DNA	boil 20 min.	0.1–10^5 ppm	101%	0.99	0.1–10^5 ppm	22.85 ± 0.15
clam and tomato cocktail	acidic pH/low DNA	boil 20 min.	misc. late amp. except 10^6 ppm	NA	NA	0.1–10^6 ppm	22.61 ± 0.22

[a] All data in Table 2 were obtained using the Mx3005P.
[b] Two independent experiments.

v4.1.0.0 (Agilent Technologies), and data obtained from the 7500 Fast were analyzed using 7500 Software v2.0.6 (Applied Biosystems/Life Technologies). C_T values were determined using these software programs and graphed versus lobster ppm levels to generate linear standard curves. Statistical R^2 values were calculated for the best-fit line, and reaction efficiency was determined from its slope using the equation $E = 10^{(-1/m)}-1$, where m = slope of the linear standard curve (Adams, 2006; Wilhelm & Pingoud, 2003). For experiments including mock unknowns, ppm levels of unknowns were calculated from their C_T values using the equation from the linear standard curve for that spiking experiment. Since template DNA quantity for the internal amplification control (IAC) was constant for all reactions, it amplified with a constant C_T value regardless of crustacean ppm level; therefore statistical average and standard deviation are reported for the IAC.

2.4. DNA extraction, PCR, and data analysis for cross-reactivity testing

For cross-reactivity testing (Table 4), DNA was extracted from crustacean tissues using the DNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Each PCR reaction contained 250 pg template DNA. Each PCR reaction contained the following in a 12.5 μl reaction volume: 1 × PCR buffer, 5 mM Mg, 30 nM ROX reference dye, 1.2 mM dNTPs, and 0.625 units Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY, USA). Lobster primers were included at 2000 nM each; the lobster probe was used at 200 nM. Primers and probes for the internal amplification control (IAC) were included at 200 nM, and the internal control template DNA was added to a constant copy number of 5×10^5 in all reactions (Deer et al., 2010; Eischeid et al., 2013). Thermal cycling included an initial cycle at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 30 s, and extension at 65 °C for 2 min. All cross-reactivity tests were carried out on the Mx3005P, and data were analyzed using MxPro software v4.1.0.0 (Agilent Technologies). Percent cross-reactivity was calculated from C_T values as described in Rasmussen, Morrissey, and Hanner (2010).

3. Results and discussion

3.1. Analysis of real-time PCR standard curves

All standard curves analyzed for this work were plots of C_T value as a function of ppm lobster meat spiked into the food matrix (e.g., Fig. 1, A–C). C_T values were determined as the PCR cycle number at which amplification plots crossed a threshold level set by the MxPro and 7500 software programs (Fig. 1A and B). The threshold level is set such that amplification for all samples is in the early exponential phase. During early exponential phase, amplification levels are proportional to levels of target DNA in the original sample; that is why PCR product levels can be used to quantify sample DNA content. Standard curves were analyzed with respect to linear range, statistical R^2 value, and reaction efficiency (Adams, 2006; Heid et al., 1996). Ideally, standard curves for real-time PCR assays have linear ranges spanning 6–8 orders of magnitude, and statistical R^2 values of 0.98–1.00. The optimal range for reaction efficiencies is 90%–110%, which corresponds to slopes of −3.59 to −3.10. Slopes are negative because higher amounts of target result in earlier amplification and lower C_T values (Fig. 1A and C; Heid et al., 1996). The ideal value of the slope derives from the fact that during a PCR reaction, the amount of target sequence should theoretically double with each cycle. This means that the relative amount of target is given by 2^n, where n = number of cycles. A 10-fold difference in lobster ppm on the x axis corresponds to a difference of n = 3.32 cycles on the y axis. Therefore a slope of −3.32 is ideal and yields a reaction efficiency of 100%. In the example shown here (Fig. 1C), the lobster target standard curve has a slope of −3.30, yielding a nearly ideal reaction efficiency of 100.9%. Since the IAC was spiked at equal amounts in all samples, amplification was constant regardless of lobster ppm spiking level (Fig. 1B), and the IAC yielded a horizontal line on standard curve plots (Fig. 1C).

3.2. Initial validation experiments

Initial validation experiments indicated that this method performed well in various food matrices, including fish, macaroni

Table 4
Cross-reactivity data.

Common name	Scientific name	C_T value	Delta C_T*	% Cross Reactivity
Target Lobsters				
American Lobster		*24.48 (average)*	*0*	*100.00*
American Lobster	*Homarus americanus*	25.53	1.05	48.30
American Lobster	*Homarus americanus*	23.44	−1.04	205.62
Crabs				
King Crab	*Paralithodes camtschaticus*	No C_T	NA	0.00
Snow Crab	*Chionoecetes opilio*	37.59	13.11	0.01
Snow Crab	*Chionoecetes opilio*	38.75	14.27	0.01
Blue Crab	*Callinectes sapidus*	No C_T	NA	0.00
Blue Crab	*Callinectes sapidus*	No C_T	NA	0.00
Flame box crab	*Calappa flammea*	44.79	20.31	0.00
Yellow box crab	*Calappa sulcata*	38.37	13.89	0.01
Shrimps				
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Whiteleg Shrimp	*Litopenaeus vannamei*	No C_T	NA	0.00
Brown rock shrimp	*Sicyonia brevirostris*	No C_T	NA	0.00
Brown shrimp	*Farfantepenaeus aztecus*	No C_T	NA	0.00
Pink shrimp	*Farfantepenaeus duorarum*	No C_T	NA	0.00
Royal red shrimp	*Pleoticus robustus*	No C_T	NA	0.00
Pink speckled shrimp	*Pennaeopsis serrata*	No C_T	NA	0.00

* for Delta C_T: Delta C_T was calculated as: [C_T value for sample − average C_T value for lobster (24.48)].

salad, dry noodles, canned soup, and clam and tomato cocktail; similar results were obtained for the Mx3005P and 7500 Fast platforms (Table 1). Linear ranges spanned 6–8 orders of magnitude, most R^2 values were 0.98 or higher. Lower limits of detection were 0.1–1 ppm lobster meat in nearly all foods and preparation conditions. In comparison, crustacean ELISA methods have lower limits of 0.3 ppm and 0.9 ppm extracted protein for detection and quantitation, respectively (Seiki et al., 2007). The IAC amplified with C_T values of approximately 20–25 at all ppm levels and in all experiments, consistent with this laboratory's previous work using the same internal control (Table 1; Eischeid et al., 2013). Repeatability was determined for the spiking experiments in Table 1, which represent 7 independent spiking experiments carried out over the course of 6 months by a single analyst. For the lobster 12S target, plots of average C_T value as a function of ppm spiking level yielded reaction efficiencies of 92% and 101%, and R^2 values of 0.99 and 0.98, for the Mx3005P and 7500Fast platforms, respectively (Fig. 2A). For the internal control, plots of average C_T value as a function of ppm level yielded approximately constant C_T values and a horizontal line, as expected (Fig. 2B). Relative standard deviations of the C_T values were approximately 5–12% for the lobster 12S target and 2.5–7% for the internal control (Table 2). For all of the New England Clam Chowder experiments (Table 1), analysis included mock unknowns prepared by spiking in the laboratory. If a method is 100% accurate, then calculated values for mock unknowns are equivalent to expected (spiked)values, and data points fall on a straight line representing this ideal relationship. A plot of calculated ppm versus expected ppm for the mock unknowns (Fig. 3) indicates that: 1) there is no substantial difference between PCR platforms, 2) cooking by boiling and microwaving did not have an adverse effect on quantitation, 3) quantitation was less accurate with decreasing ppm level, and 4) qualitative detection was achieved for all samples, even those spiked at the lowest level (0.1 ppm) and subjected to cooking. Percent accuracy was not calculated for these experiments, because the standard curves themselves were generated via spiking into food matrices.

Several of these initial validation experiments yielded reaction efficiencies outside the ideal range of 90%–110%, in contrast to this laboratory's previous work on shrimp and crab (Table 1; Eischeid et al., 2013). Sample preparation, DNA extraction, and PCR procedures are very similar for the current method and the published shrimp and crab methods. Differences in performance most likely result from primers and probes: in fact, primer concentrations required for optimal performance were higher for the lobster assay (2000 nM) than they were for the previously published shrimp and blue crab assays (200 nM). Though reaction efficiencies in the optimal range of 90%–110% are preferred, accurate determination of unknown ppm levels depends primarily on reaction efficiencies for standards and unknowns being equal to each other—not on their being optimal (Wilhelm & Pingoud, 2003).

3.3. Challenging food matrices and processing conditions

In addition to the initial validation, further experiments were focused on the challenging conditions discussed in the Introduction: combined high heat and pressure, food matrices with inherently low DNA content, and food matrices with acidic pH levels. Since clam and tomato cocktail inherently contains low levels of DNA, the method was tested using carrier DNA to enhance extraction efficiency for this food matrix. Similar results were obtained with and without carrier DNA (Table 1), indicating that carrier DNA is not required for efficient extraction or quantitation using the method described here. Though carrier nucleic acid is known to enhance extraction efficiency in low-DNA content samples, the resulting increase in target DNA quantity is not necessarily relevant for an exponential process like PCR: increases in yield are approximately 20% in manual, silica-based extraction procedures like that used in the work described here (Boom et al.,1990). The difference between data points on lobster standard curves in this work is 10-fold, or 1000%, so a 20% difference in initial template DNA concentration is not expected to have a significant effect on final data. Furthermore, the procedure used in this work included other steps to maximize DNA yield: thorough homogenization of samples in SDS buffer, a salt precipitation step, and increased volumes of guanidine buffer and ethanol are all expected to enhance extraction from processed samples and complex food matrices.

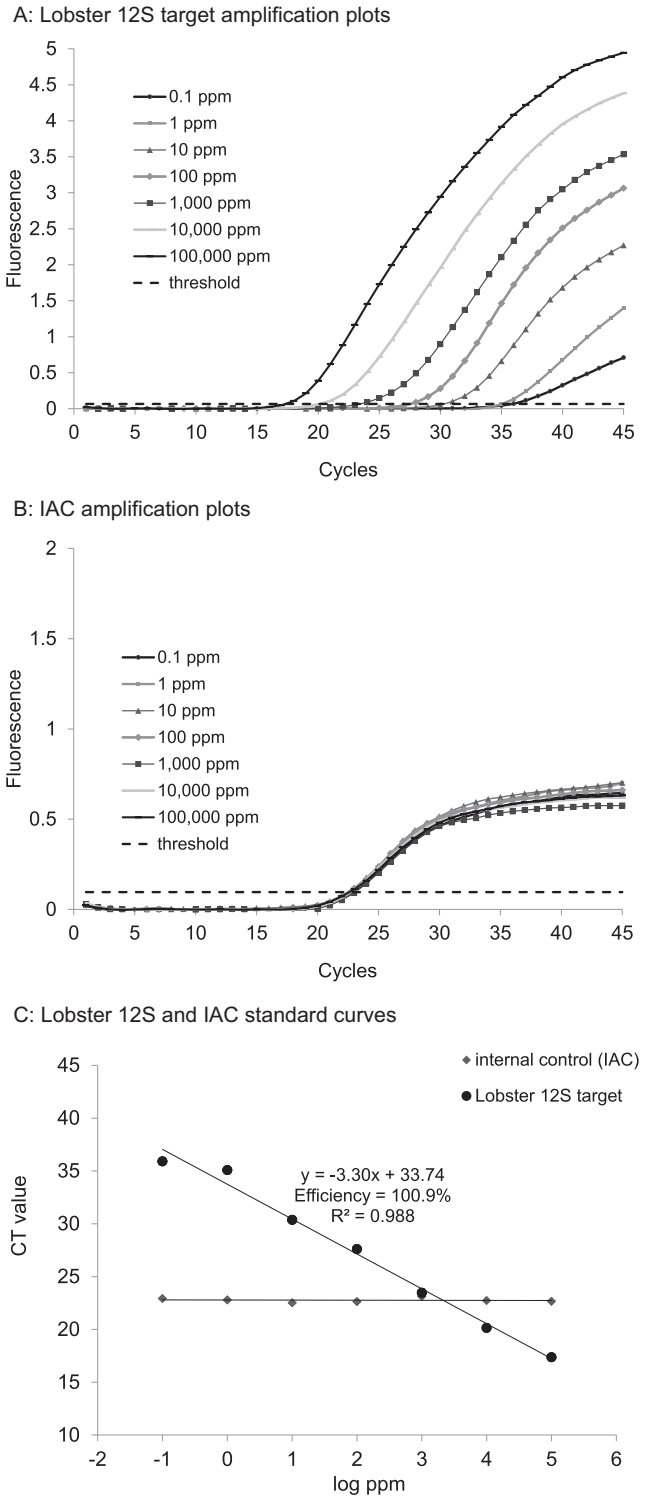


Fig. 1. Example amplification plots and standard curves Data shown are from lobster meat spiked into New England Clam Chowder. A) Amplification plots for the lobster 12S gene target. Plots are evenly spaced and approximately 3 cycles apart, as expected for samples with 10-fold differences in lobster ppm levels. B) Amplification plots for the internal amplification control (IAC). Plots rise above baseline at approximately the same cycle number regardless of lobster ppm level. C) Standard curves generated from the amplification plots in A) and B). C_T values were determined from the point at which amplification plots cross threshold and plotted versus (log) lobster ppm spiking level.

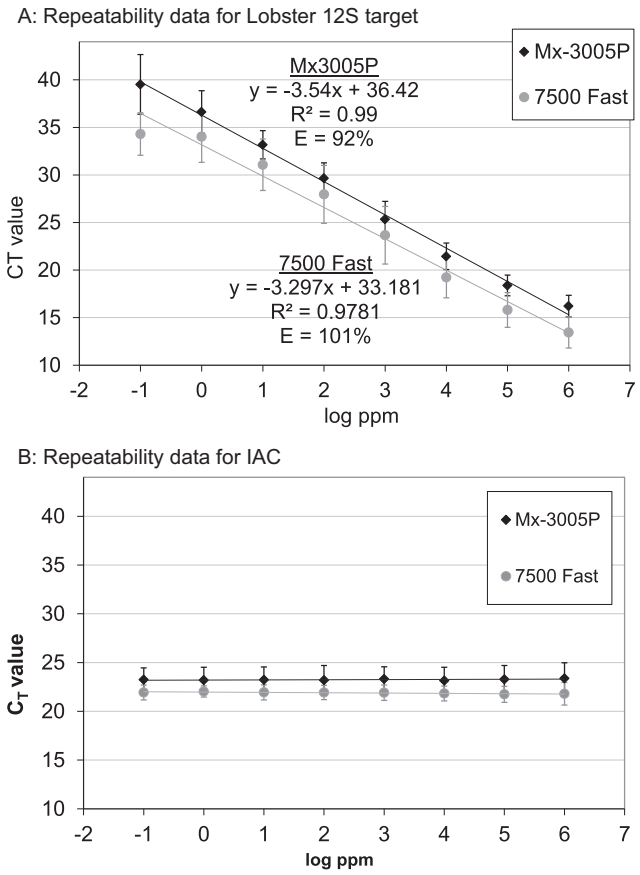


Fig. 2. Repeatability Data for Initial Validation Experiments Combined data from the 7 independent spiking experiments listed in Table 1. Plots show average C_T values as a function of ppm spiking level for the lobster 12S target (Fig. 2A) and the internal control (Fig. 2B); error bars show 1 standard deviation above and 1 standard deviation below the average.

Data at different pH levels indicate that at neutral pH, neither boiling nor autoclaving had an adverse effect on data (Table 3). At acidic pH, however, both boiling and autoclaving resulted in a loss of signal or delayed, non-quantitative amplification from lobster targets (Table 3). In these experiments both the positive control (pure lobster meat, 10^6 ppm) and the IAC amplified consistently and at expected C_T values; therefore losses of signal from the lobster

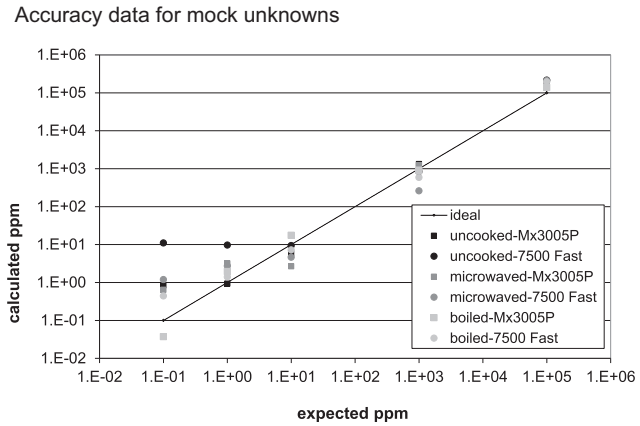


Fig. 3. Accuracy Data for Mock Unknowns Calculated versus expected ppm for mock unknowns. In a method with 100% accuracy, all data points would fall on the ideal line.

target are attributable to DNA degradation rather than issues with the PCR reaction. These data suggest that heat processing by boiling or autoclaving is sufficient to render the 80–90 bp PCR products undetectable in acidic conditions, but not at neutral pH. These results are in agreement with 1) this laboratory's previous work on shrimp and crab PCR methods, which were not adversely affected by heat treatment in non-acidic foods (Eischeid et al. 2013), 2) the work of Hird et al. (2006), who found that real-time PCR in non-acidic conditions was not affected by autoclaving for PCR products up to 116 bp in size, and 3) the work of Bauer et al. (2003), who found that acidic conditions and tomato-based products lead to accelerated DNA degradation during heat treatment.

3.4. Cross-reactivity data

Initial cross-reactivity results indicate that this real-time PCR assay is in fact specific for detection of lobster and does not cross-react with crabs and shrimps (Table 4). These experiments included a single species of lobster, 5 species of crab, and 6 species of shrimp.

Percent cross-reactivity was calculated according to Rasmussen et al. (2010) using the average of C_T values obtained for two different individuals of American Lobster (Table 4). Percent cross-reactivity may appear to have wide variation even within lobster, since it was 48.5% for one lobster and 205% for another. However, it is important to note that these numbers correspond to a difference of only one cycle (delta C_T). As PCR is an exponential process, a small difference in C_T values corresponds to a relatively large percentage. Despite this, delta C_T values for non-target crustaceans were much greater than those for lobster: these ranged from approximately 13–20 and represented negligible (0.01%) cross-reactivity. PCR for cross reactivity tests was carried out using 250 pg template DNA per reaction, which corresponds to 5000 ppm lobster in the standard protocol, where 50,000 pg total DNA is used per reaction (Section 2.3). These tests were carried out using template DNA quantities which far exceed the lower limit of detection (0.1 ppm) for this assay and therefore represent a robust test of cross-reactivity. Further tests of cross reactivity will be carried out as samples from more species become available. Future tests will include more species of lobster, including langostinos and lobsterettes.

4. Conclusions

This paper reports development and validation of a previously unpublished method for detection and quantitation of lobster allergen. To the best of the author's knowledge, it is the most thorough report to date investigating the combined effects of high temperature and pressure, acidic pH and low DNA content on quantitative, real-time PCR based food allergen detection.

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