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OPTIMIZATION OF STANDARD SAMPLES DILUTION FOR DETERMINATION OF CRUSTACEANS BY THE PCR METHOD

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ABSTRACT

A current trend indicates the importance of protecting consumers by accurate product allergen labelling. Despite allergen labelling legislation consumers look for non-allergenic foods or products without trace allergen declaration rather than products including allergen. Therefore, food companies make an effort to minimize presence of allergens in products by effective cleaning in the production process so that the traces of allergens do not need to be declared on product labelling.A detection limit specified by optimalization of Real Time PCR. Reached value represents detection limit 10 ppm with quantitation range of reaction from 1 000 000 ppm to 10 ppm. The effectiveness of the method is 99.1% with a standard deviation of 2.27%. Reliability of the method determines the coefficient of determination 97.73% that is calculated based on the standard deviation of 0.0227. This method is suitable for precise designation of varieties of Family *Crustaceae*.

Keywords: *Crustacea*, optimalization, Real Time PCR, allergen, labelling

INTRODUCTION

Polymerase chain reaction (PCR) is a method enabling highly efficient multiplication of specific DNA segments bordered by two primers in vitro conditions. DNA polymerase is able to synthesize the complementary strand by a single-stranded template. Amplification takes place in 3 steps - denaturation, annealing of primers to the template DNA and polymerization (**Židek, 2012**). The main precondition for PCR analysis is DNA of good quality and quantity. While PCR is not able directly to detect the allergic component in the product, it can be used to detect the species from which allergenic ingredients originate **(Bošiak *et al.*, 2009).**
The most typical crustaceans include shrimps, lobsters, crabs etc. They are a rich source of proteins and low in fat. Their cholesterol content is very low and, therefore, they are food suitable for people with higher blood level of cholesterol **(Murdock, 2002)**. Of course, this group may not suffer allergy to crustaceans or molluscs. In Asia, the prevalence of allergic diseases has increased from 0.8 % to 29.1 % for asthma, 40 % of allergic rhinitis **(Hajeb - Selamat, 2011)**. Extensive panel on representative of Crustacea shrimp (*Crangoncrangon*), that is also referred to as garnet and is very commercially marketed, observed **Bauermeister *et al.* (2011)**. Identified were 6 recombinant proteins of crustaceans TM, AK, SCP, MLC, Troponin C and TIM. Tropomyosin is extremely stable in its allergenic potential after heat treatment, but *in vitro* conditions cleavage by protease is effective at reducing the binding of allergen and IgE binding **(Guang - Ming *et al.*, 2011).**
Crustaceans can cause food allergic reactions. They can cause problems in gastrointestinal tract (GIT), but also a systemic allergic reaction. **(Steensma, 2003).**
Over the last few years a lot of allergens have been identified in seafood, including crustaceans. Three different research teams analyzed major allergen in shrimp as muscle protein tropomyosin**(Daul*et al.*, 1994; Shanti *et al.*, 1993; Leung *et al.*, 1994).** Subsequent studies have confirmed that this protein is also found in crabs, lobsters and cockroaches or shellfish. **Lehrer *et al.* (2003)** followed the work of **Daul *et al.* (1994)**, who confirmed the presence of 36 kDa of protein. This protein was present not only in meat of shrimp, but also in broth, where the shrimps were cooked. Immunoblot analysis of **Motoyama*et al.* (2007)** confirmed the presence of tropomyosin in 6 species of crustaceans - *Penaeusmonodon, Penaeusjaponicus, Farfantepenaeusnotialis, Paralithodescamtschaticus, Chionoecetesopilio, Erimacrusisenbeckii.*

MATERIAL AND METHODS

Samples preparation

As the samples were used three raw materials that are commonly used for preparing of dehydrated soups containing allergen of crustaceans. Powder of lobster in dehydrated state - *Pandalus borealis*
Crab flavor in dehydrated state - *Cancer pagurus*.
Powder of marine lobster in the dehydrated state - *Nephropsnorvegicus*
We performed 10 dilutions, which surveyed a sample representing mix 3 ingredients containing crustaceans are diluted with distilled water (Figure 1) for the resulting concentrations in ppm (mg.kg^{-1}) (Table 1).

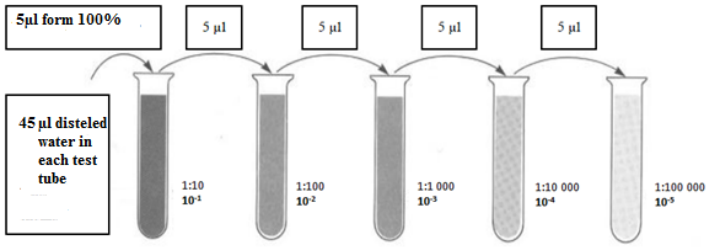

Figure 1 The standard procedure of decimal dilution with water

Table 1 Concentration of standard samples (mg.kg^{-1})

Dehydrated sample	Content of the sample	Conversion in ppm
1	100	1 000 000
2	10	100 000
3	1	10 000
4	0.1	1000
5	0.01	100
6	0.001	10
7	0.0001	1
8	0.00001	0.1
9	0.000001	0.01
10	0.0000001	0.001

Qualitative detection of allergens was performed using Real-Time PCR method in laboratories of the Department of Food Hygiene and Safety at the Slovak University of Agriculture.

Preparation of the reaction mixture

Cao *et al.* (2011) published primers which were used in our work. These are primers that hybridize to the mtDNA (mitochondrial DNA). Since in the work had been tested several primers, we selected Shrimp2-R and Shrimp2-F from Biotech company and probe Shrimp2Probe. These primers are bordering a segment long 119 bp of the mitochondrial DNA. To mastermix composition were added TaqMan probe and distilled water.

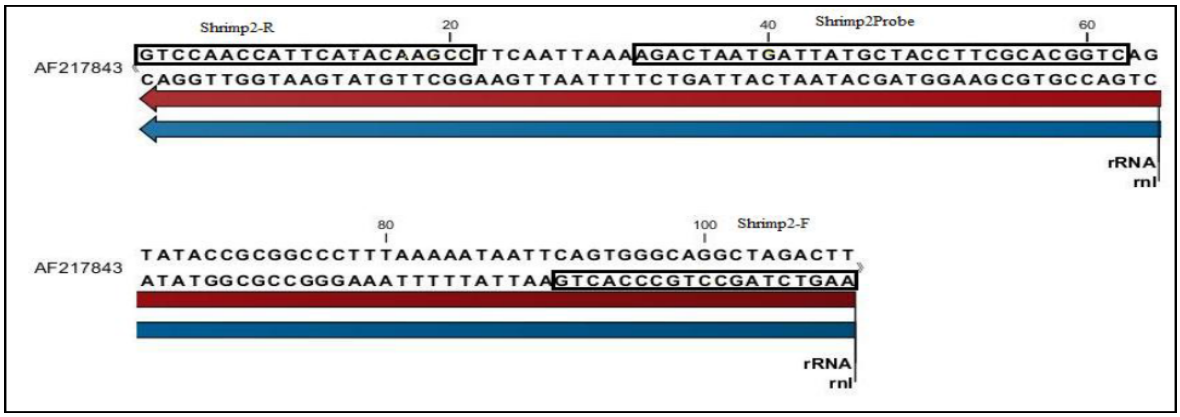

Figure 2 Primers identifying segment amplified by the Real - Time PCR with probes

For analysis kit LightCycler ® TaqMan ® Master (Roche) was used.The cycle of PCR started by initial denaturation at 95 °C for 10 minutes. Subsequently 50 cycles were repeated with alternating two steps (Fig. 3) and measured by the fluorescence signal.

1.step	**t=95°C**	**time=5s**
2.step	**t=60°C**	**time=34s**

Figure 3 Elongation at 50 cycles with the parameters in each step

The final cooling was for 30 s. at 40° C. PCR reaction was performed in capillary cycler LightCycler ® 1.5 (Roche) using LightCycler software version 5.4 (Roche).

RESULTS AND DISCUSSION

The result of our optimization of PCR method and verifying of the designed primers Shrimp2-R, Shrimp2- F and specific probes Shrimp2 Probe showed an increase in the intensity of fluorescence Light Cycler Software 4.5 dilution up to 10 ppm (sample No. 6). Reached value represents detection limit 10 ppm with quantitation range of reaction from 1 000 000 ppm to 10 ppm. Samples no. 7-10 did not exceed nonspecific background, fluorescence increase was not detectable (Fig. 4). By extrapolation of the samples to the crossing point we can conclude that the samples with number 1 and 2, at concentrations 1 000 000 and 100 000 ppm exceeded unspecific background in the range of cycles 19 – 21.The authors Espiñeira*et al*. (2010) and Cao *et al*. (2011) were designed specific primer sets so that they can detect DNA from the lowest possible detection limit. Mentioned authors in their work moving detection limit of 10 ppm. In our work, we have reached the limit of detection 10 ppm. We used the same probe from Roche TaqMan of identical nucleic acid sequence as Cao et al. (2011). TaqMan probe oligonucleotides containing a fluorescent dye, is usually longer than the primers used. Compared with other works, we achieved an error rate of less than 4%, whereas the addition of two specific primers Shrimp2 Shrimp2-F and-R were used as components in PCR and specific probe Shrimp2Probe.

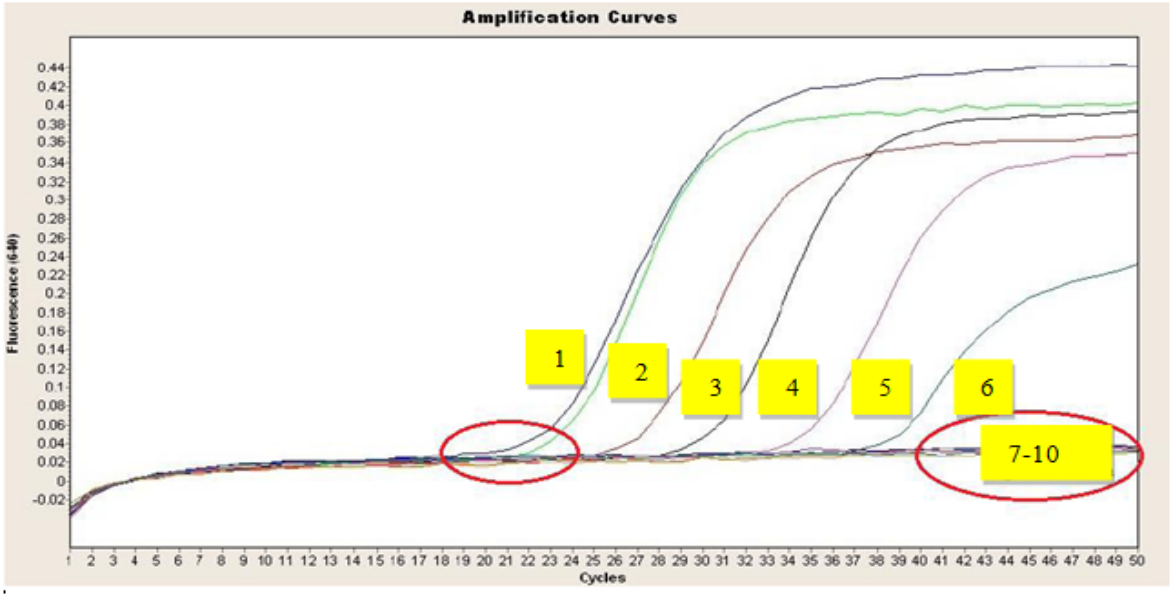

Figure 4 Optimization of PCR methods for sample dilution with water

The effectiveness of the method is 99.1% with a standard deviation of 2.27% as can be seen in Figure 5. Reliability of the method determines the coefficient of determination 97.73%, that is calculated based on the standard deviation of 0.0227 (Fig. 5).

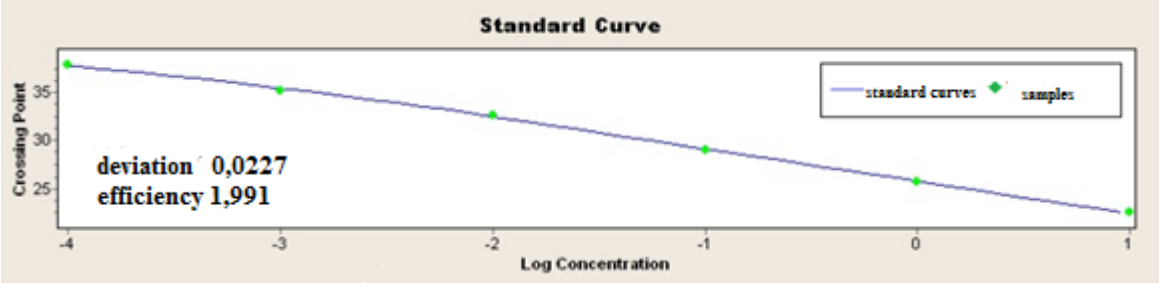

Figure 5 Standard curve representing the PCR method

CONCLUSION

Optimization of PCR method and verification of primers Shrimp2-R, Shrimp2-F and specific Shrimp2Probe and by monitoring fluorescence intensity by LightCycler Software 4.5 we obtained a detection limit of 10 ppm with a quantification range of reactions from 1 000 000 ppm to 10 ppm. At samples no. 7-10 was not exceeded nonspecific background, fluorescence increase was not detectable. The effectiveness of the method is 99.1% with a standard deviation of 2.27%.This method allows to detect the presence of allergens of crustaceanin foods and thereby reduce the risk of adverse allergic reactions by contamination in the production process

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