https://doi.org/10.17221/28/2017-CJFS

Development of a Triplex Real-Time PCR Assay for Simultaneous Detection of Allergenic Ingredients in Processed Food

*Wenju ZHANG, Yulei ZHAO, Qingjin XU and Qin CHEN**

Shanghai Key Laboratory of Bio-Energy Crops, School of Life Sciences, Shanghai University, Shanghai, P.R. China

**Corresponding author: chenqincc@staff.shu.edu.cn*

Abstract

Zhang W., Zhao Y., Xu Q., Chen Q. (2018): Development of a triplex real-time PCR for simultaneous detection of allergenic ingredients in processed food. Czech J. Food Sci., 36: 22–27.

SYBR Green real-time or quantitative PCR (Q-PCR) is a suitable system in which to establish a multiplex method to detect allergenic ingredients in food. In this study, a triplex Q-PCR method was developed to detect trace amounts of peanut, soybean and sesame in processed food. Specific PCR primer sets were designed and the concentration of the primers used in the triplex PCR was optimised. The triplex method showed high specificity and sensitivity which were similar to those of the simplex method, and it was applied for the detection of allergenic ingredients in commercially available processed food. The results demonstrate that the developed triplex Q-PCR is a quick, reliable and efficient method for the detection of allergenic ingredients in processed food.

Keywords: peanut; sesame; soybean; SYBR Green; triplex Q-PCR

Food allergies can lead to a series of symptoms such as acute vomiting, hives, shock or even death in sensitive individuals (Sicherer & Sampson 2014). Until now, there is no universal effective therapy for food allergies. The treatment strategy for most allergic diseases is based on pharmacological therapy, and on rare occasions, allergen-specific immunotherapy (Bousquet *et al*. 1998). Thus, the most effective option for sensitive individuals is to avoid the consumption of allergen-containing food (Zhang *et al*. 2015). Several factors in the food production process can lead to the contamination of the food product by allergenic ingredients, such as the contamination of raw food or the sharing of the production line with the allergenic food (Redmond & Griffith 2003; Sicherer & Sampson 2007). Therefore, it is important to develop reliable, quick, and inexpensive multiplex methods to detect the various kinds of allergenic ingredients in food.

Conventional food analysis techniques include protein-based immunoassays and enzyme-linked immunosorbent assays (ELISAs), which are prone to cross-reactivity with non-target proteins (Poms *et al*. 2004). In addition, food processing procedures often employ high temperature or pressure, which could denature the target protein and influence the detection process (Davis *et al*. 2001; Rastogi *et al.* 2007). Compared to protein, DNA content in the food matrix appears to be more stable during the food processing procedure (Hernandez *et al.* 2005). Thus, DNA-based detection methods, such as real-time or quantitative PCR (Q-PCR), has attracted the attention of researchers in this field and commercial kits are now available for the detection of several food

Supported by the National Natural Science Foundation of China, Grant No. 31201306, the project of the Food Science Discipline Construction of Shanghai University, and National Key Technology Support Program of China, Grant No. 2013BAD12B06.

https://doi.org/10.17221/28/2017-CJFS

allergens (Herrero *et al.* 2014). Generally, Q-PCR methods include the Taqman and SYBR-Green assays. The Taqman assay provides higher sensitivity than that provided by the SYBR Green assay, but it is more expensive and not suitable for the establishment of multiplex detection methods because of the possible extensive overlap of the emission spectrum of the dye commonly used in Taqman assays (Pafundo *et al.* 2010; Navarro *et al.* 2015). However, SYBR Green Q-PCR is a suitable system for the development of a multiplex PCR method as distinct melting temperatures (T_m) can be established for each amplicon of the chosen target genes. Several SYBR Green multiplex PCR methods have been established for detecting various ingredients in foods (Pafundo *et al.* 2010; Agrimonti *et al.* 2015; Palle-Reisch *et al.* 2015; Shin *et al.* 2016; Xu *et al.* 2017).

Here, we described the development of a triplex SYBR Green Q-PCR method to detect trace amounts of peanut, soybean and sesame ingredients in food. The chosen targets were those genes that encode allergenic proteins. Following optimisation, specificity and sensitivity analysis, this method was applied for the detection of allergenic ingredients in commercial food.

MATERIAL AND METHODS

Sample preparation and pre-treatment. All samples used in this study were purchased from a local supermarket. The samples, which included peanut (*Arachis hypogaea*), soybean (*Glycine max*), sesame (*Sesamum indicum*), sorghum (*Sorghum bicolor*), corn (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), buckwheat (*Fagopyrum esculentum*), oat (*Avena sativa*), rice (*Oryza sativa*), millet (*Setaria italica*), pea (*Pisum sativum*), celery (*Apium graveolens*), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), and pear (*Pyrus bretschneideri*) plants as well as two kinds of biscuits were ground into powder form for DNA extraction.

DNA extraction. Samples of plant DNA (including mixed samples used for sensitivity analysis, except sesame), were extracted by the modified cetyltrimethylammonium bromide (CTAB) method (Zhang *et al.* 2015). Then, 0.2 g of samples were ground and incubated in 800 µl TES (with 100 mM Tris, pH 8.0, 10 mM EDTA, and 2% SDS), including 50 µg Proteinase K at 60°C for 1 hour. The salt concentration was adjusted to 1.4 M with 5 M NaCl; 1/10 volume 10% CTAB was added followed by incubation for 30 min at 65°C and centrifugation for 10 minutes. Supernatant was transferred to a new tube and an equal volume of phenol-chloroform-isoamyl alcohol (phenol : chloroform : isoamyl alcohol, 25 : 24 : 1, v/v) was added followed by centrifugation. The supernatant was transferred to a fresh tube, and an equal volume of SEVAG (chloroform : isoamyl alcohol, 24 : 1, v/v) was added followed by centrifugation. The supernatant was again transferred to a fresh tube, 3 µl RNase (10 mg/ml) was added, and the sample was incubated at 37°C for 30 minutes. Then, 0.7 volume isopropanol was added to precipitate DNA, and the sample was incubated at –20°C for 1 h followed by centrifugation. The supernatant was removed and pellets were washed twice with cold 70% ethanol, dried at ambient temperature and dissolved in 50 µl of double distilled water. The Plant Genomic DNA Kit (China) was used to extract DNA from sesame, according to the manufacturer's instructions. The GMO food DNA Extraction Kit (Tiangen, China) was used to extract DNA from various commercial food products according to the manufacturer's instructions. The DNA extractions were repeated three times and the DNA was pooled for the following experiments. DNA concentrations were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA).

Primers. Primer sets for soybean, sesame and peanut were designed using Primer Premier 5.0 (Premier, USA) based on the *soy28k* (AB046874), *ses i 1* (AF240005) and *Ara h 1* (AF432231) gene sequences. The sequences of the specific 18S rRNA-F/R primers based on the nuclear 18S rRNA gene were derived from previous research (Zhang *et al.* 2015). All primers were synthesised by Sangon Biotech (China). Primer sequences are the following: *Soy28k*-F, CTAGAAACATTGGAAACACC; *Soy28k*-R, ATCACATACCCTCAAGACAT; *Ses i 1*-F, TGAGGAACGTGGACGAGAG; *Ses i 1*-R, CCCTAGCCCTCTGGTAAACC; *Ara h 1*-F, CCATCATTTCACCATCCACAC; *Ara h 1*-R, CTCTCATTGCTCCTGCTACTA; 18S rRNA-F, TCTGCCCTATCAACTTTCGATGGTA; 18S rRNA-R, AATTTGCGCGCCTGCTGCCTTCCTT.

Real time-PCR. The Q-PCR reaction was performed in a final volume of 20 µl, containing 10 µl of 2 × SYBR Green Real-time PCR Master Mix (Toyobo, Japan), 1 µl of each primer (according to the description in the text), 50 ng of DNA template and double distilled water to make up the final volume to 20 µl.

https://doi.org/10.17221/28/2017-CJFS

The reaction was carried out in a CFX96 Real-time PCR System (Bio-Rad, USA), using the following PCR programme: initial denaturation at 95°C for 30 s; 40 cycles consisting of denaturation at 95°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for 15 s; data acquisition of the signal took place every 0.5°C, continuously for 5 s during the annealing and elongation steps of each cycle from 55–95°C.

RESULTS AND DISCUSSION

DNA extraction and PCR targeting 18S rRNA. The purity of the DNA is the basis of PCR technology. The processing of commercial food under high temperature or pressure leads to the clumping or aggregation of the ingredients in food. This procedure usually renders the extraction or purification of the DNA difficult (Hird *et al.* 2003). Thus, in this study, proper DNA extraction methods were applied for the different kinds of samples. On the other hand, in order to avoid the appearance of the 'false negative results' resulting from DNA impurities that could inhibit PCR amplification, 18S rRNA primer sets were used to amplify an amplicon of about 140 bp, which could be obtained from all the eukaryotic samples (Wei *et al.* 2014; Zhang *et al.* 2015). The results showed that all the DNA samples that were extracted from plant materials (Table 1) or the commercial foods (Table 3) yielded amplicons of similar size (about 140 bp), indicating that all the samples harboured DNA that was PCR amplification-competent.

Simplex PCR. Specific primer pairs were designed based on the genomic sequences of *soy28k* (soybean), *2S albumin* (sesame) and *Ara h 1* (peanut) genes, and amplicon lengths were 147, 126, and 82 bp, respectively. The specificity of the simplex PCR assay was verified by using the DNA from 16 common plant species. Only peaks were observed for the targets genes, demonstrating that the assay possessed good specificity for detecting soybean, sesame and peanut ingredients (Table 1). For sensitivity limit detection, two kinds of dilutions were employed. Firstly, the target DNA solution was serially diluted with the DNA solution from wheat, and the other two plant species and distilled water was used as a control solution. The results showed that the detection limit reached 0.1 ng for peanut DNA and 0.01 ng for soybean and sesame DNA, and the dilution solution had no obvious impact on the detection limit of the target DNA. Secondly, the target plant was ground to powder form and diluted with different kinds of plant powders; this mixture was used for DNA extraction and Q-PCR analysis. The results showed that the detection

Table 1. Specificity

Plant specie	Simplex PCR			Triplex PCR			18S rRNA
	peanut	soybean	sesame	peanut	soybean	sesame	
Peanut	+	–	–	+	–	–	+
Soybean	–	+	–	–	+	–	+
Sesame	–	–	+	–	–	+	+
Sorghum	–	–	–	–	–	–	+
Corn	–	–	–	–	–	–	+
Barley	–	–	–	–	–	–	+
Wheat	–	–	–	–	–	–	+
Buckwheat	–	–	–	–	–	–	+
Oat	–	–	–	–	–	–	+
Rice	–	–	–	–	–	–	+
Millet	–	–	–	–	–	–	+
Peas	–	–	–	–	–	–	+
Celery	–	–	–	–	–	–	+
Tomato	–	–	–	–	–	–	+
Apple	–	–	–	–	–	–	+
Pear	–	–	–	–	–	–	+

(+) melting peak at the corresponding position; (–) indicates no melting peak at the respective position

https://doi.org/10.17221/28/2017-CJFS

Table 2. Sensitivity

The dilution ways of the samples		Simplex PCR			Triplex PCR		
		peanut	soybean	sesame	peanut	soybean	sesame
DNA samples[1] (ng)	distilled water	0.1	0.01	0.01	0.1	0.01	0.1
	wheat	0.1	0.01	0.01	0.1	0.01	0.1
	peanut	–	0.01	0.01	–	0.01	0.1
	soybean	0.1	–	0.01	0.1	–	0.1**
	sesame	0.1	0.01	–	0.1	0.01	–
Plant material powders[2] (%)	wheat	0.05	0.005	0.005	0.05	0.005	0.005
	peanut	–	0.005	0.005	–	0.005	0.05**
	soybean	0.05	–	0.005	0.05	–	0.005
	sesame	0.05	0.005	–	0.05	0.005	–

[1]target DNA samples were serially diluted with water and reference DNA (in amounts of 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng target DNA in a total of 50 ng DNA, respectively); [2]target plant tissue powders were serially diluted with reference plant powers, at percentages of 5, 0.5, 0.05, 0.005, and 0.0005, respectively; total amount of DNA for each PCR was also 50 ng; **when applied in triplex PCR, the LOD of sesame was increased compared to the simplex PCR in some circumstances, to 0.1 ng and 0.05%

limit was 0.05% for peanut and 0.005% for soybean and sesame. There was no obvious influence of the mixing of plant powders on the detection limit of peanut, soybean and sesame.

Triplex PCR. The SYBR Green Q-PCR method is suitable for the construction of a multiplex method, because each amplicon generates a single peak in the melting curve and each specific peak indicates a distinct PCR amplicon (Pafundo *et al.* 2010; Wei *et al.* 2014). However, in order to make the peaks more distinguishable, the gaps in T_m values between the different amplicons should be greater than 3°C. In this study, the Q-PCR results showed that the T_m values were 73.5, 79.3 and 82.7°C for each amplicon, which was suitable for the establishment of a triplex PCR method.

Several parameters could influence the amplification efficiency in a simplex PCR reaction, including the size of the amplicon, primer concentration and GC content of the primer (Reed *et al.* 2007). However,

Table 3. Application

Commercial food product	Labeling			Simplex/triplex PCR		
	soybean	sesame	peanut	soybean	sesame	peanut
Biscuit – 1	–	–	*	– / –	– / –	+ / +
Biscuit – 2	+	–	+	+ / +	– / –	+ / +
Chocolate – 1	+	–	–	+ / +	– / –	– / –
Chocolate – 2	–	*	*	– / –	– / –	+ / +
Chocolate – 3	–	–	*	– / –	– / –	+ / +
Chocolate – 4	–	–	–	– / –	– / –	+ / +
Chocolate – 5	–	–	–	– / –	– / –	– / –
Chocolate – 6	–	*	*	– / –	– / –	+ / +
Sauce – 1	+	+	+	+ / +	+ / +	+ / +
Sauce – 2	+	–	+	+ / +	– / –	+ / +
Sauce – 3	–	–	–	– / –	– / –	– / –
Sauce – 4	+	+	–	+ / +	+ / +	– / –
Sauce – 5	–	–	–	– / –	– / –	– / –

In the 'precautionary' labelling: (+) contains this ingredient; (–) not mentioned on the label; *may contain this ingredient or related food products have been processed on the same production line; in other cases: (+), (–) positive or negative result in the test

https://doi.org/10.17221/28/2017-CJFS

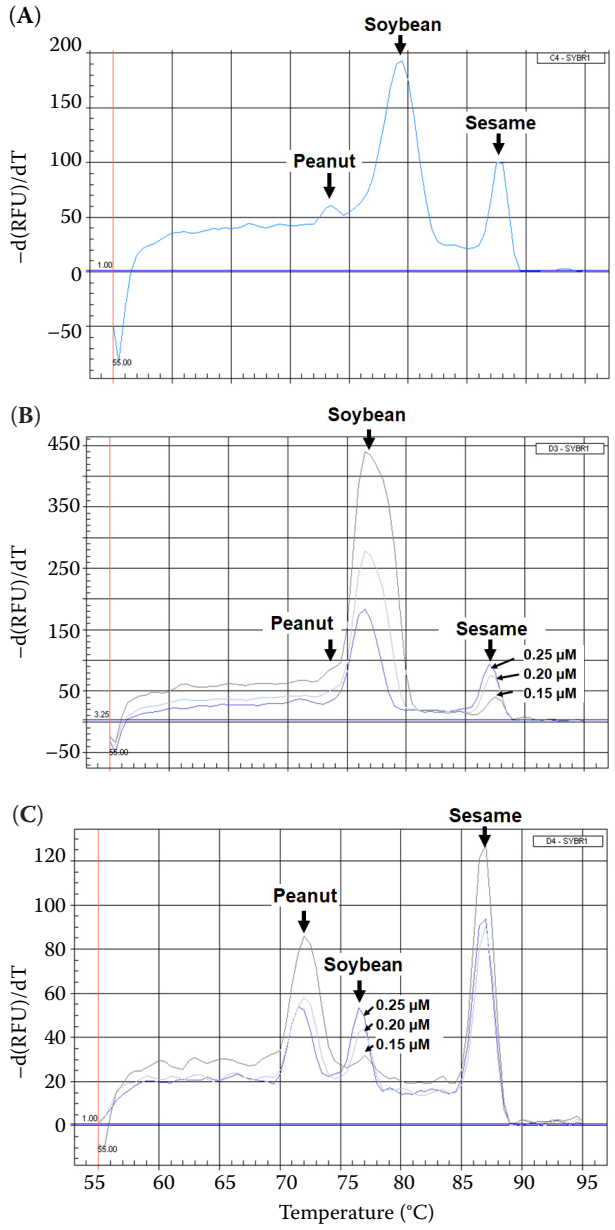

Figure 1. Optimisation of the concentration of primer sets for triplex Q-PCR: (**A**) all primer pairs were used at concentration of 0.3 µM, (**B**) and (**C**) the concentrations of the primer pairs for sesame and soybean were reduced to 0.25, 0.2, and 0.15 µM, respectively

when a multiplex PCR system is constructed, except for the parameters mentioned above, the dominant factor which would influence the detection efficiency would be the preference for the amplification of a specific amplicon during the PCR process, due to competition between individual PCR amplifications (Pafundo *et al.* 2010; Agrimonti *et al.* 2015). In order to reduce the negative influence of competition on the detection limit, the key parameters of the triplex Q-PCR should be optimised. In this study, the concentration of each primer pair was found to have a significant impact on the detection limit of the target. Firstly, an equal amount of each primer pair of 0.3 µM was used, and the results showed that the peak for peanut was significantly inhibited (Figure 1A). Then, we attempted to reduce the primer pair concentration for sesame and soybean. When the concentration of the sesame primer pair was reduced, the peak for peanut was still inhibited (Figure 1B). However, when the primer pair concentration of soybean was reduced to 0.2 µM, there was a significant enhancement in the peanut signal (Figure 1C). In conclusion, the triplex Q-PCR method was able to detect peanut, soybean and sesame in foods efficiently when the concentration of the primer pairs for peanut, soybean and sesame were 0.3, 0.2, and 0.25 µM, respectively. Further, the specificity and the sensitivity of the triplex method were analysed. The results are shown in Table 2. The triplex PCR method showed good specificity, similar to the simplex method. As for the sensitivity, the detection limit of the triplex method was identical to that of the simplex method in most cases with the following exceptions: when the dilution solution was soybean DNA, the detection limit of sesame was reduced to 0.1 ng DNA, and when sesame was diluted with peanut powders, the detection limit of sesame was raised to 0.05%.

Application. The established simplex and triplex Q-PCR methods were applied to detect the peanut, soybean and sesame ingredients in processed food. The results are listed in Table 3. All results from the triplex method were consistent with the simplex method, indicating that the established triplex method was reliable and accurate. Most of the results were consistent with the precautionary labels. Among all the samples, all three kinds of allergenic ingredients could be detected in sauce 1. However, the peanut ingredient was detected in the chocolate 4 sample, even though there was no clear declaration that this food contained peanut. This suggests that stringent investigation of allergen information remains an important priority.

CONCLUSIONS

In this study, A SYBR Green-based triplex Q-PCR method was constructed to detect trace amounts of peanut, soybean and sesame ingredients in processed

https://doi.org/10.17221/28/2017-CJFS

food. The method showed high specificity and sensitivity. Applying the method to the analysis of commercial foodstuffs demonstrated that the established method was a reliable, accurate and effective way to detect allergenic ingredients in processed food.

References

Agrimonti C., Pirondini A., Marmiroli M., Marmiroli N. (2015): A quadruplex PCR (qxPCR) assay for adulteration in dairy products. Food Chemistry, 187: 58–64.

Bousquet J., Lockey R., Malling H.J. (1998): Allergen immunotherapy: Therapeutic vaccines for allergic diseases. A WHO position paper. Journal of Allergy and Clinical Immunology, 102: 558–562.

Davis P.J., Smales C.M., James D.C. (2001): How can thermal processing modify the antigenicity of proteins? Allergy, 56: 56–60.

Hernandez M., Esteve T., Pla M. (2005): Real-time polymerase chain reaction based assays for quantitative detection of barley, rice, sunflower, and wheat. Journal of Agricultural and Food Chemistry, 53: 7003–7009.

Herrero B., Vieites J.M., Espineira M. (2014): Development of an in-house fast real-time PCR method for detection of fish allergen in foods and comparison with a commercial kit. Food Chemistry, 151: 415–420.

Hird H., Lloyd J., Goodier R., Brown J., Reece P. (2003): Detection of peanut using real-time polymerase chain reaction. European Food Research and Technology, 217: 265–268.

Navarro E., Serrano-Heras G., Castano M.J., Solera J. (2015): Real-time PCR detection chemistry. Clinica Chimica Acta, 439: 231–250.

Pafundo S., Gulli M., Marmiroli N. (2010): Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. Analytical and Bioanalytical Chemistry, 396: 1831–1839.

Palle-Reisch M., Hochegger R., Cichna-Markl M. (2015): Development and validation of a triplex real-time PCR assay for the simultaneous detection of three mustard species and three celery varieties in food. Food Chemistry, 184: 46–56.

Poms R.E., Klein C.L., Anklam E. (2004): Methods for allergen analysis in food: a review. Food Additives & Contaminants, 21: 1–31.

Rastogi N.K., Raghavarao K., Balasubramaniam V.M., Niranjan K., Knorr D. (2007): Opportunities and challenges in high pressure processing of foods. Critical Reviews in Food Science and Nutrition, 47: 69–112.

Redmond E.C., Griffith C.J. (2003): Consumer food handling in the home: A review of food safety studies. Journal of Food Protection, 66: 130–161.

Reed G.H., Kent J.O., Wittwer C.T. (2007): High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics, 8: 597–608.

Shin S.P., Ishitani H., Shirakashi S. (2016): Development of a multiplex PCR to detect *Kudoa* spp. and to distinguish *Kudoa septempunctata* in olive flounder *Paralichthys olivaceus*. Aquaculture, 464: 37–41.

Sicherer S.H., Sampson H.A. (2007): Peanut allergy: Emerging concepts and approaches for an apparent epidemic. Journal of Allergy and Clinical Immunology, 120: 491–503.

Sicherer S.H., Sampson H.A. (2014): Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. Journal of Allergy and Clinical Immunology, 133: 291–307.

Wei S., Zhao H., Xian Y.Y., Hussain M.A., Wu X.Y. (2014): Multiplex PCR assays for the detection of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* with an internal amplification control. Diagnostic Microbiology and Infectious Disease, 79: 115–118.

Xu Y.G., Sun L.M., Wang Y.S., Chen P.P., Liu Z.M., Li Y.J., Tang L.J. (2017): Simultaneous detection of *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafood using dual priming oligonucleotide (DPO) system-based multiplex PCR assay. Food Control, 71: 64–70.

Zhang W.J., Cai Q., Guan X., Chen Q. (2015): Detection of peanut (*Arachis hypogaea*) allergen by Real-time PCR method with internal amplification control. Food Chemistry, 174: 547–552.

Received: 2017–01–20
Accepted after corrections: 2017–11–23
Published online: 2018–02–07