DEVELOPMENT OF HIGH SENSITIVE REAL-TIME PCR TO DETECT MUSTARD AND OTHER ALLERGENS OF THE FAMILY *BRASSICACEAE* IN FOOD SAMPLES

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Abstract
Mustard is a commonly used condiment including in production of other food products. As mustard is an allergen, it is necessary to control its presence. The development of PCR test-systems for its detection is complicated by the fact that this condiment can be made from seeds of various plant species (Brassica juncea, Brassica nigra, Sinapis alba) of the family Brassicaceae that are not closely related. This family includes other plant species such as white cabbage (Brassica oleracea) and rapeseed (Brassica napus), which can cause the allergic reaction, although seldom. In this connection, many authors use primers specific to many species of this family, including to allergens, to detect mustard. In this work, we used the similar strategy. To increase sensitivity, primers for the mitochondrial COX gene were selected. To increase PCR stability in analysis of deeply processed products, primers were selected for a region with a length of 61 base pair. In the work, the specificity and sensitivity of the developed PCR method was confirmed. Analyses of different products, including those that underwent deep technological processing, were carried out with these primers. Also, primers were selected to detect white mustard (S. alba). When analyzing products on the presence of white mustard, characteristic regional preferences were demonstrated: this species is used in manufacturing products mainly in the UK and USA.

Introduction

Mustard is one of the most commonly used condiments. It is used in many food types, such as spice mixtures, condiments, sauces, marinades, including for meat baking, finished meat products and gastronomy products. Mustard can be prepared from plant seeds of different species: brown mustard (*Brassica juncea*), black mustard (*Brassica nigra*) and white mustard (*Sinapis alba*). Irrespective of a species, which seeds were used for production, mustard is an allergen. Information about its content should be indicated on a label according to TR CU022/2011 [1] and Regulation (EU) No 1169/2011 of the European Parliament and of the Council [2]. More than half of patients with an allergy to mustard also have an increased sensitivity to several other plant-derived foods and pollen [3]. It is necessary to note that the family *Brassicaceae* also includes such allergens as rapeseed, cabbage and broccoli.

The main mustard allergens are 2S albumins of white and brown mustard seeds (Sin a 1 and Bra j 1, respectively) [4,5]. These two albumins have the similar structure and immunological properties. Therefore, when studying mustard as an allergen in a product composition, the species origin of raw materials is of no importance. For *S. alba*, several other allergens were revealed: 11S globulin of seeds (Sin a 2) [6], non-specific lipid transfer protein (Sin a 3) and profilin (Sin a 4) [7]. It is not likely that new allergens will be established for *B. juncea*. An allergy to pollen of white cabbage (*Brassica oleracea*) is rare [8]. With that, about half of patients showed cross-sensitivity to mustard. In addition, the search for potential allergens of *B. nigra* has been performed [9].

At present, to control the mustard presence in foods, several methods based on enzyme-linked immunosorbent assay (ELISA) [10,11] and polymerase chain reaction (PCR) are used. With that, ELISA methods showed false positive reactions when analyzing rapeseed [10] or egg protein and soy [11]. When developing PCR methods, the authors propose primer pairs for detection of individual species *B. juncea* and *S. alba*, as well as multiplex test-systems to detect these species together with other allergens. For example, Palle-Reisch *et al.* selected a primer pair for detection of *B. juncea* and *B. nigra* [12]. However, the reaction efficiency was different (100.6% and 85.3%, respectively) when studying DNA of each of these species. In this connection, the limit of detection for *B. nigra* was lower (0.005% DNA in a sample). In another work, Palle-Reisch *et al.* proposed a primer-probe system for the duplex real-time PCR assay for detection of all three mustard species [13]. Sensitivity of this system was 0.0005%. The use of multiplex test-systems is in high-demand for detection of allergens. For example, Köppel et al. proposed hexaplex real-time PCR to detect allergens including mustard [14]. In this work, primers proposed by Mustorp *et al.* were used, which were specific not only to possible mustard species, but also to rapeseed (*Brassica napus*), cabbage (*B. oleracea*), radish (*Raphanus sativus*). The detection limit of this reaction was 0.0032%.

The models of sausage meat heated at 75–78 °C for 15 min. were used in [12,13]. Nevertheless, mustard is used in the composition of deeply processed products including canned foods and marinades. This impact leads to a significant DNA degradation. One of the approaches that increase stability

of a PCR method in analysis of degraded DNA is reducing a length of the obtained amplicon [15].

Taking into account the presence of several allergens in the family *Brassicaceae*, impossibility to select a DNA region specific only to species *B. juncea*, *B. nigra* and *S. alba*, and the experience of several researchers, we proposed a primer pair specific to several species of this family. To increase PCR reliability in analysis of non-degraded DNA, primers were selected for a region with a length of 61 base pair.

It is considered that mainly species of the genus *Brassica* are used in Europe and Asia for mustard production, while *Sinapis alba* is used in the USA and Canada. In this work, we also assessed the share of mustard produced using seeds of *Sinapis alba* on the Russian market.

Materials and methods
Objects of investigation
Seeds of white mustard, brown mustard and white cabbage were taken as positive controls. Soy, corn and wheat were used as the negative control.

Samples of mustard available on the Russian market at retail and produced in Russia (3), the Netherlands (1), Germany (1), Poland (1), France (1), USA (3) and UK (1) were studied. The following samples of products containing mustard seeds were taken: marinated vegetables (3), sauce (3), baking chicken marinade (1) and mustard oil (1). Mustard seeds and brine were taken from samples of marinated vegetables.

Sampling and DNA extraction
Seeds were ground in a homogenizer LB20E (Waring Commercial, Torrington, Connecticut, USA). When necessary, food products were minced in a homogenizer GRINDOMIX GM 200 (Retsch, Haan, Germany). Mustard oil (5 ml) was settled for one hour in a centrifuge SIGMA 3–18K (Sigma Zentrifugen, Osterode am Harz, Germany) with acceleration of 29700g at a temperature of 10 °C. After that, the supernatant was removed and sediment was mixed in the 20% Tween 80 solution. For DNA extraction, 100 mg of food products and control samples were taken. Liquid samples were taken with a volume of 200 μl. DNA extraction was carried out using Sorb-GMO-B kit (Syntol, Moscow, Russia) according to the instruction.

Primer design
The primer pairs Brass_fam and Sin_alb were complimentary to the mitochondrial COX gene and retrotrans-

poson Sal-T1 (Table 1), respectively. The choice of the mitochondrial DNA region for identification of the family Brassicaceae was conditioned by its multi-copy nature, which enhanced sensitivity of the method. The DNA regions were available in the GenBank database [16]. For system design, the programs Primer-BLAST [17] and Oligo-Analyzer v. 3. were used [18].

Conditions for real-time PCR
Real-time PCR was carried out using an amplifier ANK-32 (Syntol, Moscow, Russia). The reaction mixture (30 μl) contained primers with a concentration of 300 nM, 2.5 mM MgCl2, dNTPs with a concentration of 0.25 mM each, SynTaq polymerase with a concentration of 2.5 activity units and 5 μL of extracted DNA. The components of the reaction mixture were produced by Syntol. The parameter of PCR was as follows: initial denaturation at 95 °C for 7 min and 45 cycles of amplification (60 °C, 40 s and 95 °C, 15 s). All samples were investigated in triplicate. The obtained data were analyzed using the software ANK-32 (Syntol).

Based on the results of PCR with primers Brass_fam, semi-quantitative analysis of the mustard content in the test samples was carried out by the equation:

$$x = E^{(Cq_K - Cq_T)} \qquad (1),$$

where
E is the reaction efficiency;
Cq_K is the threshold cycle of the amplification curve of the positive control;
Cq_T is the threshold cycle of the amplification curve of the sample.

Data analysis was carried out using Microsoft Excel 2016 [19].

Results and discussion
Detection of efficiency, specificity and cut-off cycles of positive results
The short length of the PCR amplicon was used for increasing method stability when analyzing samples with degraded DNA.

To detect the reaction parameters and limit of detection, PCR with the selected primer pairs was carried out.

For analysis of primers for the Sin_alb gene, DNA of white mustard seeds and its decimal dilutions of up to 0.0001% were used. For analysis of primers Brass_fam, we used a dilution of DNA of white mustard seeds with a concentration of 100% to 0.0001%. The detection limit of PCR

Table 1. Sequences and positions of the primers used in the study

Primer	Primer sequence (5'–3')	Amplification region	Amplification size, bp
Brass_fam-F	GCCGAGATCAAGGTTCAAACAAA	COX	61
Brass_fam-R	CTTAAAATGTCCTTCTTCCCCGC		
Sin_alb-F	GTACGTCTCTAATCGGCATGGAT	Retrotransposn Sal-T1	107
Sin_alb-R	CTGCTGTTCTCTGTTTCGTGAAG		

bp = base pairs

with primers for Brass_fam was 0.001% of the target template in a sample. This value was not lower than in similar test-systems [12,13,14]. The calculated coefficient of correlation of PCR was $R^2 = 0.99$; PCR efficiency was $E = 2.01$. The equation of linear regression is the following:

$$y = -3,317\,x + 44,97 \qquad (2)$$

The calculated coefficient of correlation of PCR with primers Sin_alb was $R^2 = 0.99$; PCR efficiency was $E = 2.03$. The equation of linear regression is the following:

$$y = -3,2\,x + 51,145 \qquad (3)$$

When using Brass_fam and Sin_alb, no non-specific annealing with samples of soy, corn, wheat was found. Non-specific annealing was absent in the reaction between primers Sin_alb and DNA of white cabbage.

Analysis of mustard condiments

The PCR result for the sample of the US organic mustard powder was expectedly close to the Cq values of the control. Relative quantitative analysis of condiments showed that in the samples of domestic mustard, its content was in a range of 0.5 to 4.5% in reference to ground seeds. This value was 0.22%, 1.8% and 13.9% in mustard produced by Germany, the Netherlands and Poland, respectively. The highest content of the mustard powder was in the condiment produced by France (29.6%) and the UK (71%). In one US sample, a mustard proportion was 5.9%, in another 0.004%. Such a low value can be explained by replacement of the mustard powder with some other aromatic raw materials. It is necessary to note that product pH influences the ultimate value of the PCR result [20]. As vinegar is used, as a rule, in production of this condiment, the obtained values can be insignificantly lower than real.

Analysis of other food products

The authors of previous studies tested their methods on different products: cooked sausage models, sauces, spice mixes, meat spreads, fried noodles, nuggets and so on [13,14]. However, there were no deeply processed products among these objects. In our work, we took samples of brine from canned marinated vegetables and mustard oil besides sauces and marinades.

The PCR method allowed detecting mustard in the sauce compositions: 0.6–0.4% of the content relative to the positive control. The mustard content was 10% in the sample of marinade for baking.

The result of the quantitative analysis of the mustard oil composition was 0.005% relative to the positive control,

which is explained by the technology of vegetable oil purification. This result shows that it is impossible to detect mustard oil in the composition of food products using this method. Nevertheless, this method can be used for establishing vegetable oil falsification.

Mustard DNA was revealed in the samples of marinade of canned vegetables at a level of 0.001–0.0015%. With that, it is necessary to note that whole mustard seeds were added. Therefore, it was shown that sensitivity of the developed method allows identifying mustard in the composition of deeply processed products, in particular, canned foods.

Analysis of the use of S. alba seeds in production of ready condiments and other food products

Samples with the mustard content of not lower than 0.1% were used for analysis. It was linked with lower sensitivity of PCR with primers Sin_alb. Whole mustard seeds were taken from the brine of canned products. S. alba was qualitatively detected in the products from Poland and the Netherlands as well as USA and UK. In the US and UK mustard samples, mainly white mustard was detected. With that, it should be noted that the condiment sample from the USA was denoted as Braun Mustard. In the European products *S. alba* was detected in lower quantities. It is connected with the fact that these samples belonged to the recipe of "French" mustard, which composition envisages the use of both white mustard and brown or black mustard.

Conclusion

In this work, we proposed a primer pairs to detect mustard in food samples by the PCR method. High sensitivity of the method was shown. This, in particular, allowed detecting mustard traces in deeply processed samples such as canned foods. Also, successful PCR with the sample of DNA extracted from mustard oil was carried out. Therefore, this method can be used for detection of vegetable oil falsification.

Ready condiments and products were compared regarding the use of *S. alba* seeds. Despite availability of raw materials, European manufacturers traditionally use brown and black mustard, while in the UK and USA, white mustard has been detected in condiments declared as brown mustard. Nevertheless, there are condiment types, in which seeds of the genera *Brassica* and *Sinapis* are used. In this connection, it is impossible to rely on the geographical origin of a product and use one primer pair specific only to the genus *Brassica* or *Sinapis*, when analyzing food on the mustard presence.

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