



Simultaneous quantification of the food allergens soy bean, celery, white mustard and brown mustard via combination of tetraplex real-time PCR and standard addition

Florian Luber ^a, Anja Demmel ^b, Katrin Pankofer ^a, Ulrich Busch ^b, Karl-Heinz Engel ^{a,*}

^a Technische Universität München, Lehrstuhl für Allgemeine Lebensmitteltechnologie, Maximus-von-Imhof-Forum 2, D-85350 Freising-Weihenstephan, Germany

^b Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit LGL, Veterinärstraße 2, D-85764 Oberschleißheim, Germany

ARTICLE INFO

Article history:

Received 20 December 2013

Received in revised form

29 June 2014

Accepted 30 June 2014

Available online 11 July 2014

Keywords:

Quantification

Food allergen

Tetraplex real-time PCR

Mustard

Celery

Soy bean

ABSTRACT

Food allergies are a significant public health concern throughout the world with various symptoms that range from mild to severe or even life-threatening. As the only option for the allergic consumer is the strict avoidance of the allergenic food, in the European Union the declaration of the most frequent food allergens on the labels of products is required. However, traces of food allergens that may be present in foods due to cross-contact are not included in this regulatory provision. Therefore, thresholds above which labeling is mandatory are being discussed. The surveillance of such thresholds requires specific, sensitive and quantitative methods. For this purpose, a tetraplex real-time PCR method was established to quantify simultaneously trace amounts of the four allergens soy bean, celery, white and brown mustard. The quantification is achieved by using the standard addition method. The approach was validated with DNA extracted from lysate mixtures of boiled sausage and the standard materials at concentration levels of 1, 5, 10, 20, 40, 100 and 400 mg/kg. The parameters recovery, repeatability and robustness were evaluated and the limits of quantification of soy bean (8.5 mg/kg), brown mustard (2.6 mg/kg), celery (3.7 mg/kg) and white mustard (36.8 mg/kg) were determined. The method was applied to commercial food products labeled with one or more of the analytes or with a “may contain traces of ...” statement.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Food allergy is a significant public health concern throughout the world affecting about 3–6% of the population (Sicherer, 2011). Various symptoms can be elicited by a food allergy; they may range from mild to severe or even be life-threatening (Boye, Danquah, Thang, & Zhao, 2012). A cure of food allergies is not yet possible, so the strict avoidance of the allergenic food is the only option for patients (Burks et al., 2012). In order to protect the consumers from an unintentional consumption, in the European Union (EU) the most common allergenic ingredients need to be labeled according to the Directive 1169/2011 (European Parliament and Council of the European Union, 2011). In this Directive, 14 foods and food groups

are listed for which labeling is mandatory; the list includes celery, mustard and soy bean. However, this regulatory provision does not consider traces of allergens which entered the product unintentionally through cross-contact. Due to their duty of care, manufacturers often label their products with the term “may contain”. The excessive usage of such precautionary labeling leads to uncertainty amongst consumers. Consequently, thresholds concerning allergens arising from cross-contacts are being discussed (Richter, Rubin, & Lampen, 2012), as the implementation of thresholds above which labeling is mandatory might help to reduce the uncertainty of consumers and to facilitate the assessment of the product safety by manufacturers. However, for the surveillance of potential future thresholds, specific and quantitative methods are necessary.

In routine analysis of allergens in foods, enzyme-linked immune sorbent assays (ELISA) and methods based on polymerase chain reactions (PCR) play the most important roles (Fuchs, Cichna-

* Corresponding author. Tel.: +49 (0)8161 71 4250; fax: +49 (0)8161 71 4259.
E-mail address: k.h.engel@wzw.tum.de (K.-H. Engel).

Markl, & Hohegger, 2010). Several PCR methods have already been published for the detection of celery (Fuchs, Cichna-Markl, & Hohegger, 2012; Hupfer, Waiblinger, & Busch, 2007; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008), soy bean (Gryson, Messens, & Dewettinck, 2008; Köppel et al., 2010), white mustard (Fuchs et al., 2010) and brown mustard (Palle-Reisch, Wolny, Cichna-Markl, & Hohegger, 2013). Kits allowing for the quantification of soy bean and celery (CONGEN, 2013a; CONGEN, 2013b) and the detection of mustard (CONGEN, 2013c) are commercially available. For the detection and quantification of soy bean and white mustard, respectively, singleplex real-time PCR systems based on an external calibration have been published (Federal Office of Consumer Protection and Food Safety (BVL), 2013). The disadvantage arising from the fact that this approach is matrix-dependent has been compensated for by using the same sample and calibration materials. In routine analysis, however, this might be difficult to achieve, owing to the high diversity of potential food matrices.

Köppel et al. (2010) established a tetraplex real-time PCR system to simultaneously detect and quantify DNA from celery and soy bean besides hazelnut and peanut. The same authors extended this tetraplex real-time PCR system to a hexaplex system for the detection and quantification of DNA from soy bean, celery, white mustard, cashew, peanut and hazelnut. However, the included mustard system shows some cross-reactivity with other members of the *Brassicaceae* family. In addition, the method does not allow to quantify the allergen concentration in food, because the content of the allergenic food cannot be extrapolated from the DNA amount (Köppel, Velsen-Zimmerli, & Bucher, 2012).

Besides the quantification with an external calibration, two further DNA-based quantification approaches have been developed in order to calculate quantitative data from the cycle threshold values and to overcome the dependency on appropriate standard materials corresponding to the respective food matrices. One of the quantification methods is based on the addition of a unique internal standard material (Hirao, Hiramoto, Imai, & Kato, 2006), the other one on the principle of standard addition (Eugster, 2010).

The aim of this study was the establishment of a tetraplex real-time PCR method allowing the simultaneous quantification of trace amounts of celery, soy bean, white and brown mustard. The method should be based on a modified standard addition procedure in order to be independent from an external calibration. Method development was performed using boiled sausages as matrix; the suitability of the method was demonstrated by investigation of commercially available foods containing celery, mustard or soy bean as ingredients or being labeled with the term “may contain traces of celery, mustard and soy bean”.

2. Material and methods

2.1. Material

2.1.1. Sample material

Boiled pork sausages (boiling temperature: 100 °C; boiling time 1.5 h) of the type Lyoner (French: cervelas) were provided by the CVUA Freiburg (Chemisches- und Veterinäruntersuchungsamt, Freiburg, Germany). White mustard seeds, brown mustard seeds, celery seeds and soy flour were obtained from local supermarkets. Commercially available samples named “mixed spices for fried noodles Thai style”, “fried noodles with chicken”, “mashed potatoes with meatballs” and “noodles Asian style” were purchased in a local supermarket. They were either labeled to contain one or more of the analytes as ingredients or with a “may contain traces of ...” statement (“may contain traces of celery, soy bean and mustard”/“may contain traces of mustard”). The sample materials were ground with a hand blender (M160, ESGE, Metten, Switzerland) and stored at –20 °C until use.

2.1.2. Oligonucleotides

The employed primers and probes are listed in Table 1. To enable a simultaneous detection, specific fluorescence labels were used: The probe of the celery-specific system was labeled with FAM, the probe of the brown mustard system was labeled with ROX, and a HEX-labeled probe was used for white mustard. BBQ was used as a quencher. The probe of the soy bean specific real-time PCR was labeled with ATTO 425 and quenched with DDQI. The lectin probe was obtained by biomers.net GmbH (Ulm, Germany), the other primers and probes were purchased from TIB MOLBIOL GmbH (Berlin, Germany).

2.2. Methods

2.2.1. DNA extraction and standard addition

4.00 g of homogenized boiled sausage was weighed into a 50 mL Falcon-tube. 20 mL CTAB buffer [20 g/L cetyltrimethylammoniumbromide (CTAB), 1.4 mol/L sodium chloride, 0.1 mol/L tris(hydroxymethyl)-aminomethane (TRIS) (Trizma base), 0.02 mol/L disodium ethylenediaminetetraacetate (Na₂-EDTA) (99+%; pH 8.0)] and 60 µL proteinase K (recombinant, Roche Diagnostics GmbH, Mannheim, Germany) was added and the mixture was incubated over night at 65 °C under shaking. In parallel, 4.00 g of the analytes (celery seeds, soy flour, white and brown mustard seeds) were treated in the same way. After centrifugation at 7000 g for 10 min, the lysate mixtures of the boiled sausages and the analytes were prepared. The supernatants of the analytes were

Table 1
Oligonucleotides for the detection of celery, soy bean, white and brown mustard.

System	Function/Name	DNA-sequence	Length	Concentration in the reaction mix	Reference
White mustard	Forward primer/MADS D F	5'-TGAAAACTCTCTCCCTCTAGG-3'	24 bp	0.100 µM	(Fuchs et al., 2010)
	Reverse primer/MADS D R	5'-ACAAATGCACACAAGACAGATATAGA-3'	28 bp	0.100 µM	
	Probe/MADS D pr	5'-HEX-TACATGATGCTTACCTCGC-BBQ-3'	19 bp	0.120 µM	
Brown mustard	Forward primer/11f	5'-GTTGAGCCGAGGGTCATAATTTC-3'	23 bp	0.300 µM	(Palle-Reisch et al., 2013)
	Reverse primer/11r	5'-TCGACTTAGGCATCTTACCG-3'	21 bp	0.300 µM	
	Probe/11pr	5'-ROX-CGAGAGTCCGAATACTGGGCTGGGGTC-BBQ-3'	27 bp	0.050 µM	
Celery	Forward primer/Cel-MDH-iF	5'-CGATGAGCGTGTACTGAGTC-3'	20 bp	0.300 µM	(Hupfer et al., 2007)
	Reverse primer/Cel-MDH-iR	5'-AATAGGAACATAACATTATCATACCAAC-3'	29 bp	0.300 µM	
	Probe/Cel-MDH-probe	5'-6FAM-AACAGATAACGCTGACTCATCACCCG-BBQ-3'	27 bp	0.200 µM	
Soy bean	Forward primer/Lectin-F	5'-TCCACCCCATCCACATTT-3'	19 bp	0.900 µM	(BVL L08.00-59, 2013)
	Reverse primer/Lectin-R	5'-GGCATAGAAGGTGAAGTTGAAGGA-3'	24 bp	0.900 µM	
	Probe/Lectin probe	5'-ATTO425-AACCGGTAGCGTTGCCAGCTTCG-DDQI-3'	23 bp	0.200 µM	

mixed in an equal ratio and diluted with 1xTE buffer (1x Tris EDTA buffer, AppliChem GmbH, Darmstadt, Germany) to obtain lysate mixtures of the analytes with 1, 0.1, 0.01 and 0.001%. Those mixtures were used for the standard addition as well as for the preparation of spiked lysates with concentrations of 1, 5, 10, 20, 40, 100 and 400 mg of each analyte per kg sausage. The materials were spiked by pipetting after lysis, to overcome the inaccuracy of spiking trace amounts by weighing.

The standard addition was performed by adding 40 μL of the diluted lysate mixtures (1%, 0.1%, 0.01% and 0.001%) of the analytes to four aliquots (400 μL each) of the spiked lysates for the validation or to four aliquots (400 μL each) of the sample lysates for the analysis of the commercial foods. The standard addition of the diluted lysate mixtures results in aliquots 1.1, 1.2, 1.3 and 1.4 per sample material with final analyte concentrations of $x + 0.0909$, $x + 0.00909$, $x + 0.000909$ and $x + 0.0000909\%$, whereby x is the initial analyte concentration of the sample material or the spiked lysate, respectively.

400 μL ReadyRed chloroform/isoamylalcohol (MP Biomedicals Europe, Illkirch, France) was added to the mixtures. After mixing for 30 s and subsequent centrifugation at 17,000 g for 10 min, 375 μL of the clear supernatant was added to 400 μL cold isopropanol ($\geq 99\%$, SIGMA-ALDRICH GmbH, Steinheim, Germany); the mixture was gently shaken and incubated for 30 min at 7 °C. After centrifugation at 17000 g for 10 min, the isopropanol was discarded and 500 μL ethanol (70%) was added to the residual pellet. The sample was shaken in an overhead shaker and centrifuged again at 17000 g for 5 min. After removing the ethanol, the pellet was dried in a vacuum centrifuge (Eppendorf, Hamburg, Germany) for 10 min (setting: V–Al, 30 °C) and dissolved in 100 μL 1xTE buffer. To reveal potential cross-contamination during the extraction process, an extraction blank, that is without sample material, but with all reagents, was co-extracted for each extraction series.

2.2.2. DNA purification and normalization

The dissolved DNA was purified using the QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the DNA was eluted in 25 μL elution buffer.

After the purification of the DNA extracts, the DNA concentration was measured using the fluorometrical intercalating dye Quant-iT PicoGreen dsDNA reagent (Invitrogen, Karlsruhe, Germany). λ -DNA (Invitrogen, Karlsruhe, Germany) was used for the generation of a standard curve. The fluorescence was measured by a GENious plus reader (Tecan, Männedorf, Switzerland) at a wavelength of 520 nm. The calculation of the DNA concentration of the samples was performed using the resulting linear equation of the standard curve. To overcome differences in the extraction yields between individual extractions, the DNA extracts were normalized with 1xTE buffer to a concentration of 20 ng/ μL and stored at –20 °C until use.

2.2.3. Tetraplex real-time PCR

The reaction mixture consisted of 20 μL mastermix and 5 μL DNA template. The concentrations of the oligonucleotides were adopted from the original publications, except for white mustard, for which the concentrations from the original publication were reduced to obtain an efficiency in a range similar to the other real-time PCR systems combined in the tetraplex approach (Table 1). Each reaction was carried out in a well of a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, Foster City, USA). All extracts were tested in duplicate. Additionally, every plate contained six standards in duplicate, an extraction blank value and a no template control (NTC). After sealing with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, USA), the plate was centrifuged for 1 min at 100 rcf (Universal 320, Andreas Hettich

GmbH & Co.KG, Tuttlingen, Germany). The real-time PCR was performed using a Stratagene MX3005P cycler (Agilent Technologies, Waldbronn, Germany). The temperature program was as follows: Polymerase activation at 95 °C for 15 min, followed by 45 cycles with 94 °C for 30 s and 60 °C for 60 s. After each cycle, the fluorescence intensities were measured.

The software MxPro – Mx3005P v4.10 (Applied Biosystems, Foster City, USA) was used for data analysis. The thresholds and Ct values were determined automatically by the software using the settings “adaptive baseline” and “amplification based threshold”.

2.2.4. Validation

The quantification via combination of tetraplex real-time PCR and standard addition was validated with lysate mixtures of boiled sausage and standard materials (celery, soy bean, white and brown mustard). After the standard addition, the DNA was extracted from each combination of lysate mixtures and analyzed by means of real-time PCR. In every run, a standard curve was obtained by analyzing six serial dilutions of a mixture containing DNA from all of the standard materials. The efficiency calculated from the slope of the standard curve was used in the calculation of the allergen contents.

2.2.4.1. Recovery. Two extractions were carried out for the concentration levels of the lysate mixtures of 400, 100 and 40 mg/kg, respectively. In the real-time PCR, for each concentration level two extractions were performed and eleven replicates were analyzed. The experimentally determined analyte concentrations in the lysate mixtures were compared to the adjusted concentrations.

2.2.4.2. Repeatability. The DNA of lysate mixtures of boiled sausages mixed with lysate of standard material with concentrations of 400, 100 and 40 mg/kg was extracted twice. On the first day, each level of standard addition of each extract was measured five times by means of real-time PCR. On the second day, the real-time PCR analysis was repeated with three replicates (ENGL, 2011). For each analyte concentration, the RSD_r was calculated from the quotient of the determined standard deviations and the means, weighted by the sample size (Luber et al., 2014).

2.2.4.3. Robustness. Two extractions were carried out for the concentration levels of the lysate mixtures of 400, 100 and 40 mg/kg, respectively. In the real-time PCR, five replicates were measured for each level of standard addition and extraction. A second operator pipetted three further replicates for the analysis by means of real-time PCR. The means of the concentrations determined by the first and the second operator were calculated. The quotient of the difference of these means and the mean of all values determined by both operators was calculated and results in the relative deviation of the means.

2.2.4.4. Limit of quantification. Two extractions were carried out for the concentration levels of the lysate mixtures of 20, 10, 5 and 1 mg/kg, respectively. In the real-time PCR, five replicates were measured for each level of standard addition and extraction. For each concentration level and analyte, the deviation of the values for a confidence interval of 95% was determined and plotted against the respective concentration. The LOQ is then the intersection point of the curve with the straight line of $y = 30\%$. The equation of the curve was used for the calculation of the LOQ by inserting 0.3 into the equation as value y (Waiblinger, Gutmann, Hädrich, & Pietsch, 2001).

3. Results and discussion

3.1. Principle of the quantification approach by tetraplex real-time PCR

The tetraplex real-time PCR is composed of four real-time PCR systems for the detection of celery, soy bean, white and brown mustard. The system for white mustard is based on the mRNA gene for the MADS D protein (Fuchs et al., 2010). The brown mustard system detects the *Brassica nigra* partial RT gene for reverse transcriptase from gypsy-like retroelement 13G42-26 (Palle-Reisch et al., 2013). The celery system is based on the mannitol dehydrogenase gene of celery (Hupfer et al., 2007). The soy bean system is based on the lectin gene of soy bean (Federal Office of Consumer Protection and Food Safety (BVL), 2013). This tetraplex real-time PCR setup was combined with a standard addition method to achieve the simultaneous quantification of the four allergenic foods. The standard addition as described by Eugster (2010) was modified as follows: (i) The DNA was extracted with a CTAB-based extraction method and purified with the QIAquick purification kit. (ii) The concentrations of the added lysates of the standard material to the four sample aliquots were modified to match the lower concentration range of interest from 10 to 10,000 mg/kg (iii) In contrast to the published method, where an efficiency of 90% was assumed (Eugster, 2010), the efficiency was determined for each analyte in each analysis series and used in the calculation of the quantitative results.

The functionality of the tetraplex real-time PCR was demonstrated by a series of experiments. At first, a serial dilution (ranging from 100 to 0.01 ng per reaction) of the DNA from brown mustard was analyzed by means of singleplex real-time PCR. In subsequent steps, DNA from white mustard, celery and soy bean was added to the dilution series and the resulting mixtures were analyzed by means of the respective duplex, triplex and tetraplex real-time PCR-systems. As shown in Fig. 1, the sequential addition of further analytes had no negative influence on the efficiencies of the real-time PCR systems for brown mustard, celery and soy bean; only the efficiencies of the white mustard system varied from 85 to 102%, depending on the number of real-time PCR systems

combined. The particularly low efficiency of 85% determined for white mustard in the triplex-system may result from the fact that no ct value for the lowest concentration of analyte DNA (0.01 ng/reaction) could be determined under these experimental conditions and that this affected the calculation of the efficiency from the linear equation of the dilution series. Apart from this exception, all efficiencies were in the range of 90–110%, which is required by the ENGL working group (ENGL, 2008) for quantitative real-time PCR. The described experiments only cover one of the possible orders in the course of the sequential addition from singleplex to tetraplex real-time PCR. Therefore, further experiments would be necessary to reveal the potential influence of the combination of real-time PCR systems and analytes on the slight fluctuations of the calculated efficiencies for each of the four real-time PCR systems used in singleplex, duplex, triplex and tetraplex, respectively. For the tetraplex real-time PCR system, actually used for the simultaneous detection and quantification of the four analytes, the efficiencies for the four analytes ranged from 90.5 to 96.6%. In addition, the calibration curves of the tetraplex-system showed sufficient linearities over the whole dynamic range ($R^2 \geq 0.98$). Therefore, the tetraplex-system was considered suitable for the quantification of the allergenic foods celery, soy bean, white mustard and brown mustard.

3.2. Validation of the quantification method

3.2.1. Recovery

According to the Working Group of the European Network of Genetically Modified Organism Laboratories (ENGL, 2008), the recovery is defined as the compliance of the average value determined by means of a series of test results and an acknowledged reference value. For GMO analysis, it shall be in the range of 75–125% of the accepted reference value over the whole dynamic range. The AOAC Food Allergens Analytical Community considered recoveries between 50 and 150% to be acceptable for quantitative food allergen ELISA methods (Abbott et al., 2010). As no performance criteria for the quantification of food allergens by means of real-time PCR have been established up to the present, both criteria were applied to the validation of the tetraplex real-time PCR.

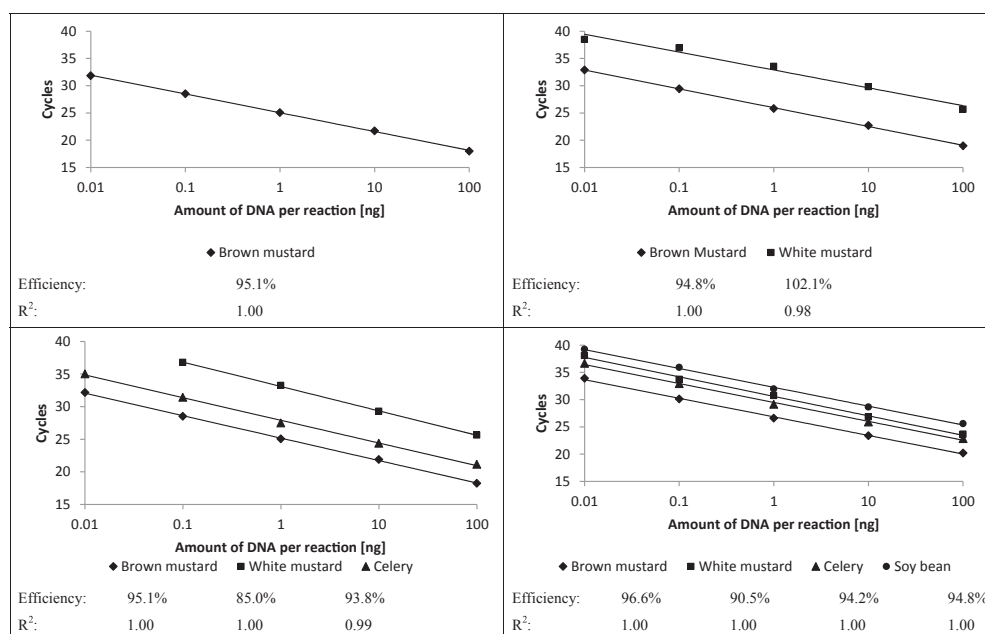


Fig. 1. Calibration curves of brown and white mustard, celery and soy bean by means of singleplex, duplex, triplex and tetraplex real-time PCR, respectively, from dilution series of 100 to 0.01 ng DNA per reaction.

Taking the standard deviations into consideration, the observed recoveries are comparable to the requirements of the AOAC International; even the mean recovery at the concentration level of 400 mg soy bean per kg sample (165%) does not differ significantly ($\alpha = 0.05$) from 150% (Fig. 2). The recoveries obtained in the tetraplex system are in the same order of magnitude as those reported for commercial singleplex quantification kits available for soy bean (77–102%) (CONGEN, 2013a) and celery (82–116%) (CONGEN, 2013b). For 7 of the 12 samples the recovery rates were within the acceptance criteria of the ENGL working group. However, it has to be kept in mind that these acceptance criteria are inferred from the analysis of GMO, where the concentration range of interest (decision level: 0.9%) is higher. For the trace analysis of allergens, these criteria may therefore be used as a starting point of reference, but a strict compliance cannot be required.

3.2.2. Repeatability

The ENGL working group describes the repeatability as the relative standard deviation of test results obtained under repeatability conditions (ENGL, 2008). Repeatability conditions are summarized as conditions where test results are achieved with the same method, on identical test items, in the same laboratory, by the same operator, with the use of the same equipment within short intervals of time. The relative repeatability standard deviation (RSD_r) shall be $\leq 25\%$.

For soy bean the RSD_r of the tetraplex real-time PCR determined for the concentration levels 40–400 mg/kg analyte in sausage lysate ranged from 11.9 to 17.7% (Fig. 3). They are in the same order of magnitude as those reported for the available commercial singleplex quantification kit (12.1–22.1%, for 4–400 mg/kg analyte in cornmeal) (CONGEN, 2013a). The repeatability of the tetraplex real-time PCR for celery was determined to be 10.5% for 40 mg/kg analyte in sausage and 18.1% for 400 mg/kg. In comparison, the validation reports of the commercial quantification kit state 15% for 40 mg/kg celery in cornmeal and 14% for 400 mg/kg (CONGEN, 2013b). In general, the RSD_r of the white mustard system were significantly higher than those for the other analytes. For the concentration levels of 40 and 100 mg white mustard per kg sample the RSD_r exceeded the threshold of 25% established by the ENGL working group. The RSD_r of all three concentration levels of brown mustard were the lowest. A comparison with other methods is not possible, as no data of quantitative real-time PCR methods for brown mustard are available so far.

3.2.3. Robustness

The robustness of a method is expressed by its capacity to remain unaffected by small deviations from the applied experimental conditions, e.g. different thermal cycler model or operator.

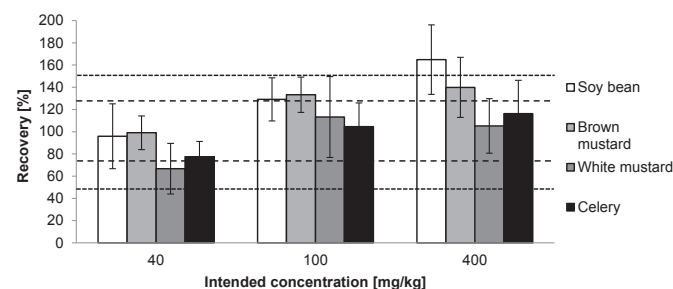


Fig. 2. Recoveries of soy bean, celery, brown and white mustard spiked in concentrations of 40, 100 and 400 mg/kg to lysate mixtures of boiled sausage (dotted lines: Acceptance criteria of the AOAC International; dashed lines: Acceptance criteria of the ENGL working group).

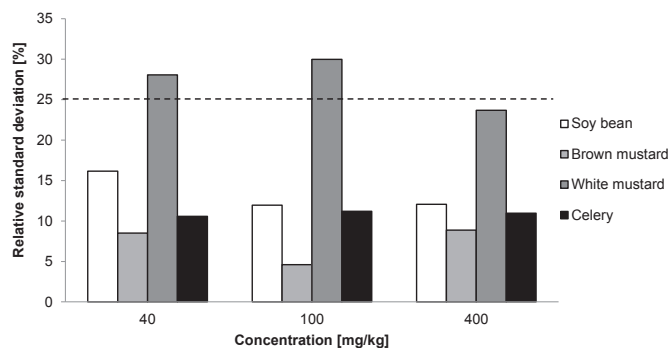


Fig. 3. Relative standard deviations under repeatability conditions of the determined concentrations of soy bean, celery, brown and white mustard in lysate mixtures of boiled sausages (dashed line: Acceptance criteria of the ENGL working group).

According to the European Network of Genetically Modified Organism Laboratories Working Group, the obtained deviation from the test result shall be within $\pm 30\%$ (ENGL, 2008).

The relative deviations of the means of the different analyte concentrations are depicted in Fig. 4 and ranged from 3% (soy bean 100 mg/kg) to 29% (white mustard 100 mg/kg). The threshold value of 30% established by the ENGL working group could be fulfilled for every analyte concentration.

3.2.4. Limit of quantification

The limit of quantification (LOQ) corresponds to the lowest concentration or amount of analyte in a sample, its quantification being reliable with an acceptable level of accuracy and precision (ENGL, 2008). The LOQ has been defined as the concentration for which the deviation of the values is below 30% for a confidence interval (CI) of 95% (Waiblinger et al., 2001).

The LOQ of soy bean was determined to be 8.5 mg/kg and the resulting LOQ of brown mustard was 2.6 mg/kg. The determination of the LOQ of white mustard yielded a concentration of 36.8 mg/kg, whereas the LOQ of celery was 3.7 mg/kg. As for the commercial singleplex real-time PCR systems, the sensitivities for the tetraplex real-time PCR system were in the low mg/kg range as relevant to the detection of food allergens (CONGEN, 2013a; CONGEN, 2013b; Poms, Klein, & Anklam, 2004). The LOQs of the commercial real-time PCR systems for the detection of celery and soy bean were determined theoretically from standard curves of dilution series with a known concentration and may therefore vary depending on the matrix (CONGEN, 2013a).

The LOQ of white mustard was approximately tenfold higher than those of the other three analytes. However, the LOQ of white

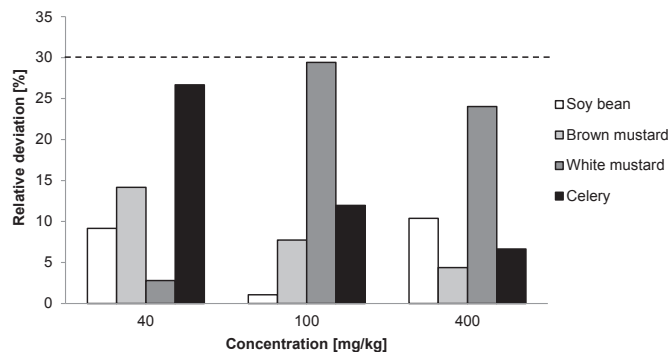


Fig. 4. Relative deviation of the means of the concentrations of soy bean, celery, brown and white mustard for the determination of the robustness of the method (dashed line: Acceptance criteria of the ENGL working group).

mustard is also still in the range of the upper limits of 10–100 mg/kg of the allergenic food or 1–10 mg/kg of the protein fraction, that are considered by the “food allergy” working group of the German Society for Allergology and Clinical Immunology and of the Association of German Allergologists to protect most allergic consumers from severe allergic reactions (Vieths et al., 2006).

The validation data of the tetraplex real-time PCR show that the modified standard addition method by Eugster (2010) is applicable for the quantification of soy bean, celery, white and brown mustard using boiled sausages employed as sample material. In comparison with the commercial quantification kits for soy bean and celery, the validation parameters are within the same range, except for the LOQs that are lower for the commercial quantification kits. However, the quantification by means of these two kits is matrix-dependent. It is based on allergen-containing cornmeal as reference material which does not correspond to the matrix of most foods under consideration; therefore, the transferability of the cornmeal-based results would need to be confirmed before the kits can be applied to other matrices.

The recovery rates, the relative repeatability standard deviations, the relative deviations of the means and the limits of quantification show differences between the respective analytes. They may result from the presence of four different real-time PCR systems in one chemical reaction which can lead to mutual influence of the systems. Additionally, the target sequences of the respective real-time PCR systems may be present in different copy numbers in the genome which influences the sensitivity of the real-time PCR. A further reason may be the different efficiency of the amplification associated with the secondary structure of the respective target sequences and the related annealing temperatures. The secondary structure results from the amount of the nucleobases guanine and cytosine and therefore varies for every real-time PCR system (Yakovckuk, Protozananova, & Frank-Kamenetskii, 2006). The differences between the concentration levels result from the low concentration range in which the quantification is performed. At this low concentration, only a few DNA copies per target are present which leads to an increase of the coefficient of variation and, therefore, a higher variability of the values (Horwitz, 1982).

3.3. Screening and quantification of soy bean, celery, white and brown mustard in commercial food samples

The validated tetraplex real-time PCR was applied to the analysis of commercially available samples which were either labeled with one or more of the analytes as ingredients or with a “may contain traces of ...” statement (“may contain traces of celery, soy bean and mustard”/“may contain traces of mustard”). The samples were screened for the allergens soy bean, celery, brown and white

mustard using qualitative real-time PCR. The detected allergenic ingredients were then quantified using the developed combination of real-time PCR and standard addition. For the qualitative analysis, the sample DNA was extracted once and the tetraplex real-time PCR was performed in triplicates (Table 2). The subsequent quantification by means of standard addition was achieved from three DNA extractions. For each extraction, two replicate series were analyzed by means of tetraplex real-time PCR. The serially diluted DNA standards were also measured in duplicate. The concentrations were calculated separately for each replicate series. As example, Table 3 shows the results for the quantification of celery in a commercially available food sample. Four aliquots of the sample material are spiked with different dilutions of the supernatant of celery resulting from incubation with CTAB buffer and Proteinase K. From the ct values of the four aliquots the amount of celery in the sample is determined by approximation calculation as described by Eugster (2010). This results in 6 values (2 replicates from 3 extractions) out of which the mean and standard deviations are calculated. In advance, the Cochran's C test was applied to test the variance homogeneity of the three extractions depending on each individual analyte (Kromidas, 2000).

The screening results of the sample material “mixed spices for fried noodles Thai style” are shown in Table 2. On this product curry has been labeled as an ingredient, containing celery and mustard, which were confirmed by the qualitative analysis with mean ct (ct_{mean}) values of 30.85 (white mustard), 36.75 (brown mustard) and 32.58 (celery). Although soy beans were labeled as components of powdered soy sauce, soy bean could not be detected. The quantitative analysis resulted in amounts of 4005 ± 2148 mg/kg white mustard and of 1411 ± 637 mg/kg celery (Table 4). The amounts of 3 ± 1 mg/kg quantified for brown mustard confirmed the assumed traces indicated by the high ct values ($ct_{\text{mean}} = 36.75$) of the qualitative analysis.

The sample “fried noodles with chicken” was screened positive for all four analytes, which is in accordance with the labeling indicating celery, mustard and powdered soy sauce as ingredients. The subsequent quantitative analysis determined the concentration of white mustard to be 1034 ± 57 mg/kg and of celery to be 338 ± 69 mg/kg. No quantification of soy bean was possible due to the fact that no increase of fluorescence for at least two PCRs within one replicate series could be measured and, therefore, the standard addition procedure could not be applied. The amount of brown mustard was below the LOQ of 2.6 mg/kg.

The precautionary labeling “may contain traces of celery, soy bean and mustard” has been used on the product “mashed potatoes with meatballs”. The screening of the sample material detected the presence of celery and mustard, but not of soy bean. The content of celery was determined to be 67 ± 6 mg/kg and that of white mustard 136 ± 31 mg/kg.

Table 2
Qualitative analyses of commercially available food samples by means of tetraplex real-time PCR.

Food sample	Qualitative analyses of PCR triplicates for each analyte [ct values]			
	Soy bean	White mustard	Brown mustard	Celery
Mixed spices for fried noodles Thai style	no Ct	30.71	37.21	32.62
	no Ct	30.90	36.87	32.44
	no Ct	30.95	36.18	32.69
Fried noodles with chicken	40.73	30.75	36.96	32.33
	37.99	30.53	38.52	32.11
	37.97	30.69	37.73	31.88
Mashed potatoes with meatballs	no Ct	31.44	36.69	30.76
	no Ct	31.61	38.21	31.05
	no Ct	31.87	36.99	30.64
Noodles Asian style	35.45	38.99	40.85	31.21
	34.56	no Ct	37.88	31.14
	35.01	no Ct	38.53	31.19

Table 3

Ct values for the quantitative analysis of celery in fried noodles with chicken by means of tetraplex real-time PCR and standard addition.

	Ct values				Resulting amount [mg/kg]	Mean $\pm$ standard deviation [mg/kg]
	Aliquot 1.1	Aliquot 1.2	Aliquot 1.3	Aliquot 1.4		
Extract 1						338 $\pm$ 69
Replicate a	32.66	34.12	35.01	34.84	261	
Replicate b	32.89	33.94	34.44	34.95	415	
Extract 2						
Replicate a	33.10	34.85	34.99	35.51	263	
Replicate b	33.22	34.58	34.82	35.27	373	
Extract 3						
Replicate a	33.03	34.75	34.87	35.04	313	
Replicate b	33.06	35.34	34.43	34.45	405	
No Template Control:	No Ct	Parameters of the standard curve: Efficiency = 98.40% $R^2 = 0.997$				
Extraction Blank:	No Ct					

Soy sauce and celery were listed as ingredients of the sample material “noodles Asian style”. In addition, the labeling “may contain traces of mustard” was used. The qualitative analysis of the sample material led to positive results for the presence of the labeled ingredients soy bean and celery. The precautionary labeling of mustard could be confirmed for brown mustard ($ct_{\text{mean}} = 39.09$), but not for white mustard (only one of three results is positive) by qualitative analysis. The following quantification resulted in 134 ± 19 mg/kg for soy bean and 616 ± 219 mg/kg for celery. The content of brown mustard in the sample material was below the LOQ.

Celery has been listed on three of the analyzed commercial products as ingredient and on one product as trace amount. It was detected and quantified in all four sample materials, whereby the lowest amount was determined in the product with the precautionary labeling “may contain traces of celery, soy bean and mustard”.

Two of the investigated commercial samples contain mustard as ingredients, which could be confirmed by qualitative and quantitative analysis of white mustard. Assumed traces due to precautionary labeling could be qualitatively and quantitatively verified for the analysis of white mustard in one of two samples. The presence of brown mustard was determined for all sample materials by qualitative analysis. In contrast to the positive screening results, no quantification of brown mustard was achieved except for “mixed spices for fried noodles Thai style” with a low

concentration of 3 mg/kg. The positive screening results of brown mustard may be explained by the high sensitivity of the real-time PCR system that shows the lowest LOQ of all systems. Nevertheless, the results of the qualitative analysis are in good accordance with those of the quantification: The sample “mixed spices for fried noodles Thai style” results in a mean ct value of 36.75 and can be quantified to be 3 mg/kg which is above the method's LOQ of 2.6 mg/kg. All other samples resulted in mean ct values above 36.75 for the qualitative analysis that indicate the presence of brown mustard in very low concentrations which appear to be below the LOQ of the employed tetraplex approach.

Although soy bean was labeled on all products as ingredient or at least as trace amount (“mashed potatoes with meatballs”) the amount of soy bean could be quantified only for one sample material (“noodles Asian style”). This might be due to the fact that powdered soy sauce, a highly processed ingredient, has been used. As a result of the production process that includes a fermentation step of the soy beans and a subsequent spray drying, the DNA might be degraded and thus no detection by real-time PCR might be possible.

4. Conclusion

The results of the commercially available samples demonstrate that the developed tetraplex real-time PCR system in combination with standard addition method is applicable for the quantitative

Table 4

Quantitative analysis of soy bean, celery, white and brown mustard in commercially available food samples by means of tetraplex real-time PCR and standard addition.

Food sample	Labeling	Analyzed analyte	Mean ($n = 6$) [mg/kg]	Standard deviation [mg/kg]
Mixed spices for fried noodles Thai style	Ingredients: curry (containing celery and mustard) and powdered soy sauce (soy beans, wheat)	Soy bean	— ^a	— ^a
		White mustard	4005	2148
		Brown mustard	3	1
		Celery	1411	637
Fried noodles with chicken	Ingredients: spices (containing celery and mustard) and powdered soy sauce (soy beans, wheat)	Soy bean	— ^c	— ^c
		White mustard	1034 ^b	57 ^b
		Brown mustard	<LOQ	<LOQ
		Celery	338	69
Mashed potatoes with meatballs	May contain traces of celery, soy bean and mustard	Soy bean	— ^a	— ^a
		White mustard	136 ^b	31 ^b
		Brown mustard	<LOQ	<LOQ
		Celery	67 ^b	5 ^b
Noodles Asian style	Ingredients: powdered soy sauce [soy sauce (water, soy beans, wheat, salt)] and celery. May contain traces of mustard	Soy bean	134 ^b	19 ^b
		White mustard	— ^a or ^c	— ^a or ^c
		Brown mustard	<LOQ	<LOQ
		Celery	616	219

^a Not quantified due to negative screening results.

^b $n = 4$; one DNA extract ($n = 2$) was eliminated as an outlier according to Cochran's test ($P = 95\%$).

^c Not quantified due no increase in fluorescence for at least two PCRs within one replicate series.

analysis of traces of allergens in foods. The range of efficiencies determined for the four analytes in the tetraplex-system and the elaborated validation data demonstrate the suitability of the method for practical purposes. The variability in recovery rates as observed for soy bean can be taken into account depending on the concentration range determined for the analyte. The method should be a valuable tool for food manufacturers and food safety authorities. The advantage of a multiplex real-time PCR system is the simultaneous analysis of several analytes. Compared to singleplex real-time PCR systems it is time-saving which is important for routine analysis of allergens. In addition, less DNA extract is being used when several analytes are quantified simultaneously. This is advantageous if only small amounts of sample material are available.

Acknowledgment

The authors thank Ulrich Waiblinger from the CVUA Freiburg for providing the boiled sausage material and Marzena Maggipinto, Karola Grünwald and Daniela Sebah for technical assistance. This study was financially supported by the Bavarian State Ministry of the Environment and Public Health.

References

- Abbott, M., Hayward, S., Ross, W., Godefroy, S. B., Ulberth, F., Van Hengel, A. J., et al. (2010). Appendix M: validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. *Journal of AOAC International*, 93, 442–450.
- Boye, J. I., Danquah, A. O., Thang, C. L., & Zhao, X. (2012). Food allergens. In *Food biochemistry and food processing* (pp. 798–819). Wiley-Blackwell.
- Burks, A. W., Tang, M., Sicherer, S., Muraro, A., Eigenmann, P. A., Ebisawa, M., et al. (2012). ICON: food allergy. *Journal of Allergy and Clinical Immunology*, 129(4), 906–920.
- CONGEN. (2013a). *Quantification of soya using SureFood® ALLERGEN QUANT Soya*. Art.-Nr. S3201. Version 1.0. Berlin, Germany: Congen Biotechnologie GmbH.
- CONGEN. (2013b). *Quantifizierung von Sellerie mit SureFood® ALLERGEN QUANT Celery*. Art.-Nr. S3205. Version 3.4D. Berlin, Germany: Congen Biotechnologie GmbH.
- CONGEN. (2013c). *Nachweis von Senf mit SureFood® ALLERGEN Mustard*. Art.-Nr. S3109 Version 2.4D. Berlin, Germany: Congen Biotechnologie GmbH.
- ENGL European Network of Genetically Modified Organism Laboratories Working Group (ENGL working group). (2008). *Definition of Minimum performance requirements for analytical methods of GMO testing*.
- ENGL European Network of Genetically Modified Organism Laboratories Working Group (ENGL working group). (2011). *Verification of analytical methods for GMO testing when implementing interlaboratory validated methods*.
- Eugster, A. (2010). Alternative quantitative Bestimmung von Allergenen in Lebensmitteln mittels Real-Time-PCR. *Deutsche Lebensmittel-Rundschau*, 106, 434–438.
- European Parliament and Council of the European Union. (2011). Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Official Journal of the European Union*, L 304/18.
- Federal Office of Consumer Protection and Food Safety (BVL). (2013). *Official Collection of analysis methods according to § 64 of the German food and Feed Code (Lebensmittel- und Futtermittelgesetzbuch, LFGB). Food testing – Detection and determination of mustard (Sinapis alba) and soy bean (Glycine max) in boiled sausages with real-time PCR*. L 08.00–59.
- Fuchs, M., Cichna-Markl, M., & Hohegger, R. (2010). Development and validation of a real-time PCR method for the detection of White Mustard (*Sinapis alba*) in foods. *Journal of Agricultural and Food Chemistry*, 58(21), 11193–11200.
- Fuchs, M., Cichna-Markl, M., & Hohegger, R. (2012). Development and validation of a novel real-time PCR method for the detection of celery (*Apium graveolens*) in food. *Food Chemistry*, 130(1), 189–195.
- Gryson, N., Messens, K., & Dewettinck, K. (2008). PCR detection of soy ingredients in bread. *European Food Research and Technology*, 227(2), 345–351.
- Hirao, T., Hiramoto, M., Imai, S., & Kato, H. (2006). A novel PCR method for quantification of Buckwheat by using a unique internal standard material. *Journal of Food Protection*, 69(10), 2478–2486.
- Horwitz, W. (1982). Evaluation of analytical methods used for regulation of foods and drugs. *Analytical Chemistry*, 54(1), 67A–76A.
- Hupfer, C., Waiblinger, H.-U., & Busch, U. (2007). Development and validation of a real-time PCR detection method for celery in food. *European Food Research and Technology*, 225(3–4), 329–335.
- Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., & Waiblinger, H.-U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *European Food Research and Technology*, 230(3), 367–374.
- Köppel, R., Velsen-Zimmerli, F., & Bucher, T. (2012). Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *European Food Research and Technology*, 235(5), 843–852.
- Kromidas, S. (2000). *Handbuch Validierung in der Analytik*.
- Luber, F., Demmel, A., Herbert, D., Hosken, A., Hupfer, C., Huber, I., et al. (2014). Comparative assessment of DNA-based approaches for the quantification of food allergens. *Food Chemistry*, 160, 104–111.
- Mustorp, S., Engdahl-Axelsson, C., Svensson, U., & Holck, A. (2008). Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *European Food Research and Technology*, 226(4), 771–778.
- Palle-Reisch, M., Wolny, M., Cichna-Markl, M., & Hohegger, R. (2013). Development and validation of a real-time PCR method for the simultaneous detection of black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) in food. *Food Chemistry*, 138(1), 348–355.
- Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: a review. *Food Addit Contam*, 21(1), 1–31.
- Richter, K., Rubin, D., & Lampen, A. (2012). Current aspects in risk assessment of allergen traces in foodstuffs. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz*, 55(3), 394–401.
- Sicherer, S. H. (2011). Epidemiology of food allergy. *Journal of Allergy and Clinical Immunology*, 127(3), 594–602.
- Vieths, S., Holzhauser, T., Erdmann, S., Fuchs, T., Henzgen, M., Klein-Tebbe, J., et al. (2006). Neue Deklarationspflicht für Nahrungsmittelallergene in Lebensmitteln. Stellungnahme der Arbeitsgruppe Nahrungsmittelallergie der Deutschen Gesellschaft für Allergologie und des Ärzteverbandes Deutscher Allergologie. *Allergologie*, 29, 403–409.
- Waiblinger, H. U., Gutmann, M., Hädrich, J., & Pietsch, K. (2001). Validierung der Real-time PCR zur Quantifizierung von gentechnisch veränderter Soja. *Deutsche Lebensmittel-Rundschau*, 97, 121–125.
- Yakovckuk, P., Protozananova, E., & Frank-Kamenetskii, M. D. (2006). Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. *Nucleic Acid Research*, 34(2), 564–574.