



Development and validation of a triplex real-time PCR assay for the simultaneous detection of three mustard species and three celery varieties in food



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ABSTRACT

The paper presents a triplex real-time PCR assay allowing the simultaneous detection of three mustard species (white, black and brown mustard) and three celery varieties (celery roots, celery stalks and leaf celery) in foodstuffs. The triplex assay does not show cross-reactivity with other *Brassicaceae*. Low cross-reactivities were observed with fenugreek, cumin, ginger, caraway, turmeric, lovage and rye, the ΔC_t values were, however, ≥ 12 compared to positive controls. The triplex assay allows the detection of traces of DNA of the allergenic components in spite of an excess of the other DNA templates. Analysis of extracts from model sausages containing defined concentrations of mustard and celery showed that the triplex assay is applicable to both raw and processed foods. It was found to allow the detection of 1 ppm black/brown mustard and 50 ppm white mustard and celery in raw and brewed sausages with a probability $\geq 95\%$.

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1. Introduction

In spite of an extensive discussion of food allergies in the mass media the general public is not aware of the fact that in Europe mustard and celery allergies are prominent among the spectrum of allergies detected by epidemiologists. Especially in France and Switzerland, where mustard and celery are consumed very frequently, allergies to mustard and celery are rather prevalent. Mustard allergy accounts for about 1–7% of food allergies in France. About 30–40% of food allergic patients living in France or Switzerland are sensitised to celery (EFSA, 2004).

Commercial food products may contain three mustard species belonging to the plant family *Brassicaceae*, white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). Three varieties of celery, a member of the *Apiaceae* family, play a role in food industry: celery roots (*Apium graveolens* var. *rapaceum*), celery stalks (*Apium graveolens* var. *dulce*) and leaf celery (*Apium graveolens* var. *secalinum*).

It has been shown that allergic reactions to white mustard are mainly triggered by a number of allergenic proteins: a 2S seed storage albumin (Sin a 1) (Menéndez-Arias, Moneo, Domínguez, & Rodríguez, 1988), an 11S seed storage globulin (Sin a 2)

(Palomares et al., 2005), a non-specific lipid transfer protein (Sin a 3) and a profilin (Sin a 4) (Sirvent et al., 2009). Allergic symptoms to brown mustard are elicited by a 2S seed storage albumin (Bra j 1) structurally similar to Sin a 1 (Gonzales de la Peña, Menéndez-Arias, Monsalve, & Rodríguez, 1991).

To date, the following allergenic proteins have been identified in celery: a protein homologous to Bet v 1 (Api g 1) (Breiteneder et al., 1995) and two isoforms (Api g 1.01 and Api g 1.02) (Wangorsch, Ballmer-Weber, Rosch, Holzhauser, & Vieths, 2007), a non-specific lipid-transfer protein (Api g 2) (Gadermaier et al., 2011), a profilin similar to Bet v 2 (Api g 4) (Scheurer, Wangorsch, Hausteiner, & Vieths, 2000) and a glycoprotein (Api g 5) (Ganglberger et al., 2000), in previous studies described as cross-reactive carbohydrate determinants (CCD) (Fötisch, Altmann, Hausteiner, & Vieths, 1999).

Several studies indicate that food processing does not affect allergenicity of mustard and celery proteins or decreases allergenicity only to a minor extent because of their rather high resistance to heat and digestive enzymes (Ballmer-Weber et al., 2002; Jankiewicz et al., 1997; Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodríguez, 2012).

Both mustard and celery have been reported to potentially cause anaphylaxis, which is, in addition to other symptoms, characterised by a rapid drop of blood pressure that can be life-threatening. Persons sensitised to mustard and/or celery are

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therefore well advised to strictly avoid being exposed. In order to help excluding mustard and celery from the diet, both foods have to be indicated on the ingredient list of prepacked foodstuffs in many countries including the member states of the European Union (Commission of the European Union., 2007).

Analytical methods suitable to verify correct labelling of allergenic ingredients have to be selective to avoid false positive results. To avoid false negative results, the methods must be sensitive and applicable to raw and highly processed foods. Since foodstuffs, e.g. instant noodles, sauces and sausages, may contain both mustard and celery, a method allowing the simultaneous detection of white, black and brown mustard, celery roots, celery stalks and leaf celery would help saving time and costs and lowering the risk of cross-contamination.

Mustorp et al. published a multiplex quantitative ligation dependent probe amplification method for the detection of eight allergenic foods including mustard and celery (Mustorp, Drømtorp, & Holck, 2011). Köppel et al. presented a hexaplex real-time PCR method allowing the simultaneous detection of hazelnut, peanut, celery, soy, mustard and cashew (Köppel, van Velsen-Zimmerli, & Bucher, 2012). However, in both multiplex assays the mustard system is not specific for mustard but also detects other members of the *Brassicaceae* family, e.g. broccoli, radish and cabbage. Recently, our group developed and validated two duplex real-time PCR assays, one for the simultaneous detection of white mustard and celery (Fuchs, Cichna-Markl, & Hohegger, 2013), the other one for the simultaneous detection of white, black and brown mustard (Palle-Reisch, Cichna-Markl, & Hohegger, 2014). The duplex assay for white mustard and celery does not show cross-reactivity with 64 biological species, including various members of the *Brassicaceae* and *Apiaceae* family. The duplex assay for the simultaneous detection of white, black and brown mustard does not detect other *Brassicaceae*, but it shows low cross-reactivities with cinnamon, cumin, fenugreek, ginger, rye and turmeric. Cross-reactivity does, however, not influence the accuracy of the assay since the Ct (threshold cycle) values obtained for cinnamon, cumin, fenugreek, ginger, rye and turmeric were significantly higher than those obtained for black and brown mustard.

Very recently, Luber et al. published a tetraplex real-time PCR method for the simultaneous determination of soy bean, celery, white mustard and brown mustard (Luber, Demmel, Pankofer, Busch, & Engel, 2015). The tetraplex assay is based on two singleplex real-time PCR assays developed in our group, a singleplex assay for white mustard (Fuchs, Cichna-Markl, & Hohegger, 2010) and a singleplex assay for black and brown mustard (Palle-Reisch, Wolny, Cichna-Markl, & Hohegger, 2013).

In the present study we aimed at combining the primers and probes previously used in singleplex (Fuchs, Cichna-Markl, & Hohegger, 2012; Fuchs et al., 2010; Palle-Reisch et al., 2013) and duplex assays (Fuchs et al., 2013; Palle-Reisch et al., 2014) to a triplex real-time PCR assay in order to be able to simultaneously detect white, black and brown mustard, celery roots, celery stalks and leaf celery. The triplex assay should then be validated with regard to selectivity, limit of detection (LOD) and its applicability to detect the three mustard species and celery varieties in raw and processed foods.

2. Materials and methods

2.1. Chemicals and foods

N-Cetyl-N,N,N-trimethylammoniumbromide (CTAB), ethylenedinitritetraacetic acid disodium salt dihydrate (EDTA), phenol/chloroform/iso-amylalcohol 25/24/1 (v/v/v) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Life

Sciences (Buchs, Switzerland). Proteinase K, chloroform, iso-amyl-alcohol, 2-propanol, ethanol, hydrochloric acid and sodium chloride were delivered from Merck (Darmstadt, Germany). RNase, α -amylase and herring sperm DNA were purchased from Roche (Mannheim, Germany). Ultrapure water (Millipore, Synergy®, Merck, Darmstadt, Germany) was used for extracting DNA and for carrying out PCR reactions. Seeds from white, black and brown mustard, celery roots, celery stalks and leaf celery, pork meat, table salt and foodstuffs were bought at local merchants or supermarkets. Dried spices were provided by Kotányi (Wolkersdorf, Austria).

2.2. Production and brewing of model sausages

For method validation, two sets of model sausages with defined concentrations of mustard and celery as well as a control sausage free of mustard and celery were produced as described previously (Palle-Reisch et al., 2013). Model sausages of set 1 contained 10,000, 5000, 1000, 500, 100, 50, 10, 5 or 1 mg/kg of each white mustard, black mustard and celery roots, sausages of set 2 10,000, 5000, 1000, 500, 100, 50, 10, 5 or 1 mg/kg of each white mustard, brown mustard and celery roots. Aliquots of 3 g were filled in 50 mL beakers and brewed for 15 min at 75–78 °C in a water bath (GFL, Burgwedel, Germany) equipped with an alarm thermometer (Testo GmbH, Vienna, Austria). The brewed sausages were directly used for DNA extraction. Raw sausages were stored at –20 °C.

2.3. DNA extraction

DNA was isolated from plant species, model sausages and commercial food products with the CTAB method according to the protocol described previously (Fuchs et al., 2010). In brief, 3.0 g of the homogenised sample was mixed with 10 mL CTAB extraction buffer, 80 μ L α -amylase and 18 μ L proteinase K solution and incubated (50 °C, overnight). After centrifugation (4000 rpm, 15 min, model 5810 R, Eppendorf, Vienna, Austria) 1 mL of the supernatant was mixed with 600 μ L chloroform/isoamylalcohol (24/1, v/v) and centrifuged (13,200 rpm, 10 min, model 5415 R, Eppendorf). 660 μ L of the aqueous supernatant was blended with 1320 μ L precipitation solution and incubated (room temperature, 60 min). After a further centrifugation step, the precipitate was dissolved by adding 450 μ L 1.2 M NaCl solution, 50 μ L 10-fold RNase buffer and 5 μ L RNase and incubating (56 °C, 10 min). After cooling, the solution was mixed with 500 μ L phenol/chloroform/isoamyl-alcohol (25/24/1, v/v/v) and centrifuged. Next, 400 μ L of the aqueous supernatant was mixed with 400 μ L isopropanol, incubated (–20 °C, 60 min) and centrifuged. The obtained DNA precipitate was washed with 70% ethanol solution. The dried DNA pellet was dissolved in 100 μ L H₂O_{dd} (56 °C, 20 min). The DNA extracts were stored at –20 °C.

After measuring the absorbance of the DNA extracts at 260 nm (A_{260}) and 280 nm (A_{280}) with a nano photometer (Implen, Munich, Germany), the concentration was calculated according to the following equation: c [ng/ μ L] = $A_{260} \times 50 \times$ dilution factor. The ratio of A_{260}/A_{280} was calculated to assess the purity of the extracts.

2.4. Mixtures of DNA extracts

For method validation, a series of mixtures of DNA extracts from the three mustard species and celery varieties were prepared. The composition of the DNA mixtures is given in Table 1.

2.5. Real-time PCR analysis

Real-time PCR was performed with a Rotor-Gene Q instrument using 0.1 mL strip tubes with caps in a 72-well rotor (Qiagen,

Table 1
Composition of DNA mixtures used for validation [ng/PCR tube].

Mixture	White mustard DNA	Black mustard DNA	Brown mustard DNA	Celery roots DNA	Celery stalks DNA	Leaf celery DNA
A	100	100	–	100	–	–
B	100	–	100	100	–	–
C	100	50	50	–	100	–
D	100	50	50	–	–	100
E	95	1.25	1.25	2.5	–	–
F	2.5	47.5	47.5	2.5	–	–
G	2.5	1.25	1.25	95	–	–
H	90	2.5	2.5	5	–	–
I	5	45	45	5	–	–
J	5	2.5	2.5	90	–	–
K	75	6.25	6.25	12.5	–	–
L	12.5	37.5	37.5	12.5	–	–
M	12.5	6.25	6.25	75	–	–
N	45	22.5	22.5	10	–	–
O	10	22.5	22.5	45	–	–
P	45	5	5	45	–	–

Hilden, Germany). White mustard was detected in the green channel (excitation 470 ± 10 nm, detection 510 ± 5 nm), black and brown mustard in the yellow channel (excitation 530 ± 5 nm, detection 557 ± 5 nm) and celery in the red channel (excitation 625 ± 5 nm, detection 660 ± 10 nm). An increase of the fluorescence signal within 40 cycles (Ct value < 40) was considered as a positive result. All DNA extracts, the positive and the no template control were analysed in duplicate.

2.5.1. Primers and probes

The sequences of the primers and probes (white mustard: primer forward_w, primer reverse_w, probe_w; black/brown mustard: primer forward_{b/b}, primer reverse_{b/b}, probe_{b/b}; celery: primer forward_c, primer reverse_c, probe_c) were published recently (Fuchs et al., 2010, 2012; Palle-Reisch et al., 2013). Probe_w was labelled with FAM and TAMRA, probe_{b/b} with YAK and BHQ1 and probe_c with Cy5 and BHQ2. All primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany), with the exception of probe_c which was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.5.2. Optimisation of the triplex real-time PCR assay

The triplex real-time PCR assay was optimised by analysing serially diluted mixtures of DNA extracts from white mustard, black mustard and celery roots (Table 1, mixture A) and white mustard, brown mustard and celery roots (Table 1, mixture B). The DNA concentration of each species ranged from 20 ng/μL to 0.002 pg/μL, which corresponds to 100 ng to 0.01 pg total DNA amount per PCR tube.

Two Master mixes, the TaqMan Universal PCR Master Mix (“TMU Mix”, Applied Biosystems, Foster City, CA, USA) and the QuantiTect Multiplex RT-PCR NR Kit (“QTM Mix”, Qiagen), were tested. When using the TMU Mix, the PCR protocol was as follows: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 60 s at 55 °C. In case of the QTM Mix, the temperature program was: 2 min at 50 °C, 10 or 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 45 s or 75 s at 60 °C.

2.5.3. Optimised triplex real-time PCR assay

Each PCR tube was filled with 12.5 μL QTM Mix, 0.2 μM primer forward_w, 0.3 μM primer reverse_w, 0.15 μM probe_w, 0.2 μM primer forward_{b/b}, 0.2 μM primer reverse_{b/b}, 0.05 μM probe_{b/b}, 0.2 μM primer forward_c, 0.3 μM primer reverse_c, 0.05 μM probe_c, H₂O_{dd} and 5 μL DNA extract (20 ng/μL or 40 ng/μL). The total reaction volume

was 25 μL. The following PCR protocol was applied: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 45 s at 60 °C.

2.5.4. Cross-reactivity experiments

Cross-reactivity experiments were carried out with DNA extracts from members of the *Brassicaceae* and *Apiaceae* family and other plant species commonly used for food production. The DNA extracts were adjusted to 20 ng/μL, corresponding to 100 ng total DNA amount per PCR tube.

2.5.5. Limit of detection, amplification efficiency and reliability

The limit of detection (LOD) of the triplex real-time PCR assay and the amplification efficiency (E) for each allergenic component were determined by analysing serially diluted mixtures of DNA extracts from the three mustard species and celery varieties as well as by analysing DNA extracts from model sausages.

DNA mixtures A–D (Table 1) were serially diluted with water to obtain mixtures containing from 20 ng DNA/μL to 0.002 pg DNA/μL (corresponding to 100 ng–0.01 pg DNA per PCR tube) of white mustard, black and/or brown mustard, and celery roots or celery stalks or leaf celery.

DNA mixtures E–P were serially diluted down to 2 pg/μL with a 20 ng/μL herring sperm DNA solution in order to keep the total DNA amount constant.

DNA extracts (40 ng DNA/μL) were prepared from raw and brewed model sausages containing 10,000–1 mg/kg of each white mustard, black mustard and celery (set 1) or white mustard, brown mustard and celery (set 2).

The amplification efficiencies were calculated from the slopes of the standard curves as follows: $E [\%] = [10^{(-1/(\text{slope}))} - 1] \times 100$.

To evaluate the reliability of the triplex assay near the LODs, DNA extracts (40 ng DNA/μL) from raw and brewed model sausages containing 1, 5, 10 or 50 mg/kg of each allergenic component were analysed in 20 replicates.

Inhibition control reactions contained 5 μL DNA extract (40 ng/μL) from the negatively tested food sample, 12.5 μL QTM Mix, 0.2 μM primer forward_w, 0.3 μM primer reverse_w, 0.15 μM probe_w, 0.2 μM primer forward_{b/b}, 0.2 μM primer reverse_{b/b}, 0.05 μM probe_{b/b}, 0.2 μM primer forward_c, 0.3 μM primer reverse_c, 0.05 μM probe_c, 5 μL of the positive control (mixture of DNA extracts containing 20 ng/μL of each white mustard, brown mustard and celery root DNA) and H₂O_{dd}. The reactions were carried out in duplicates.

3. Results and discussion

3.1. Development and optimisation of the triplex real-time PCR assay

The triplex real-time PCR assay was developed by combining primers and probes from recently published singleplex real-time PCR assays for white mustard (Fuchs et al., 2010), black/brown mustard (Palle-Reisch et al., 2013) and celery (Fuchs et al., 2012), which were already successfully applied in a duplex assay for celery and white mustard (Fuchs et al., 2013) and a duplex assay for white mustard and black/brown mustard (Palle-Reisch et al., 2014). Although in primer and probe design we had paid attention to avoid complementarity in order to lower the probability of formation of heterodimers, we had to test the compatibility of the primers and probes in practice.

First the compatibility of primers and probes for black/brown mustard and celery was tested by analysing serially diluted mixtures of DNA extracts from black or brown mustard and celery roots (1:1, 20 ng/μL to 0.002 pg/μL of each species). Amplification was carried out by using 0.3 μM of primer forward (f), f_{b/b}, 0.3 μM of primer reverse (r), r_{b/b}, 0.05 μM of probe_{b/b} (as in the singleplex assay for black/brown mustard (Palle-Reisch et al., 2013)),

0.2 μM of primer f_c , 0.3 μM of primer r_c , 0.05 μM of probe $_c$ (as in the singleplex assay for celery (Fuchs et al., 2012)) and the TaqMan Universal PCR Master Mix (TMU Mix). Amplification efficiencies were 100.0% for black mustard, 98.3% for brown mustard and 91.3% for celery. Black mustard and brown mustard DNA could be detected down to a concentration of 0.2 and 0.02 $\text{pg}/\mu\text{L}$ (corresponding to 1 and 0.1 pg total DNA amount per PCR tube), respectively, and celery DNA down to 20 $\text{pg}/\mu\text{L}$ (100 pg DNA per PCR tube). Since efficiencies and LODs were similar to those obtained with the singleplex assays we assumed that the primers and probes for black/brown mustard and celery are compatible.

In order to test the compatibility of all three primer pairs and probes, serially diluted mixtures (mixtures A and B, Table 1) of DNA extracts from white mustard, black or brown mustard and celery roots were analysed. The DNA concentration of each allergenic component was in the range from 20 $\text{ng}/\mu\text{L}$ to 0.002 $\text{pg}/\mu\text{L}$. Experiments were carried out with 0.2 μM primer f_w , 0.3 μM primer r_w , 0.15 μM probe $_w$ (as in the singleplex assay for white mustard (Fuchs et al., 2010)), 0.2 μM primer $f_{b/b}$, 0.2 μM primer $r_{b/b}$, 0.05 μM probe $_{b/b}$ (as in the duplex assay for white mustard and black/brown mustard (Palle-Reisch et al., 2014)), 0.2 μM primer f_c , 0.3 μM primer r_c , 0.05 μM probe $_c$ (as in the singleplex assay for celery (Fuchs et al., 2012)) and 5 μL DNA extract mixture. In order to achieve high amplification efficiencies, a PCR kit particularly recommended for multiplexing, the QuantiTect Multiplex RT-PCR NR Kit (QTM Mix), was used. The following PCR protocol was applied: 2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, 45 cycles of 15 s 95 $^{\circ}\text{C}$ and 45 s at 60 $^{\circ}\text{C}$. Amplification efficiencies were found to

be 92.2% for white mustard, 95.7% for black mustard, 101.4% for brown mustard and 97.9% for celery. Applying the QTM Mix instead of the TMU Mix hardly affected the Ct values for black/brown mustard (20 ng DNA/ μL , mean Ct: 17.65/16.16 (QTM Mix), 18.30/17.34 (TMU Mix)) but led to significantly lower Ct values in case of celery (20 ng DNA/ μL , mean Ct: 21.63 (QTM Mix), 24.20 (TMU Mix)). Similar effects had been observed for white mustard (mean Ct: 21.04, QTM Mix) when developing the duplex assay for white mustard and black/brown mustard (20 ng DNA/ μL , mean Ct value: 21.31 (QTM Mix), 23.83 (TMU Mix) (Palle-Reisch et al., 2014)). Modifying the temperature program by extending the annealing time from 45 s to 75 s (as recommended in the manual of the QTM Kit) did not affect the amplification efficiencies for white mustard (93.3%), black mustard (97.7%) and celery roots (98.5%). In contrast, elongating the initial denaturation time from 10 to 15 min (as recommended by the supplier of the QTM Kit) or varying the concentration of the primers or probe for celery (0.2 μM primer f_c , 0.2 μM primer r_c , 0.05 μM probe $_c$; 0.3 μM primer f_c , 0.3 μM primer r_c , 0.05 μM probe $_c$) resulted in higher Ct values and thus lower amplification in case of celery DNA. Thus, all further experiments were carried out with the QTM Mix applying the conditions given in Section 2.5.3.

3.2. Cross-reactivity tests

The triplex real-time PCR assay allows the detection of white mustard (green channel, mean Ct value: 20.91), black and brown mustard (yellow channel, mean Ct values: 17.75 and 16.29, respectively) as well as the three celery varieties (celery roots, celery

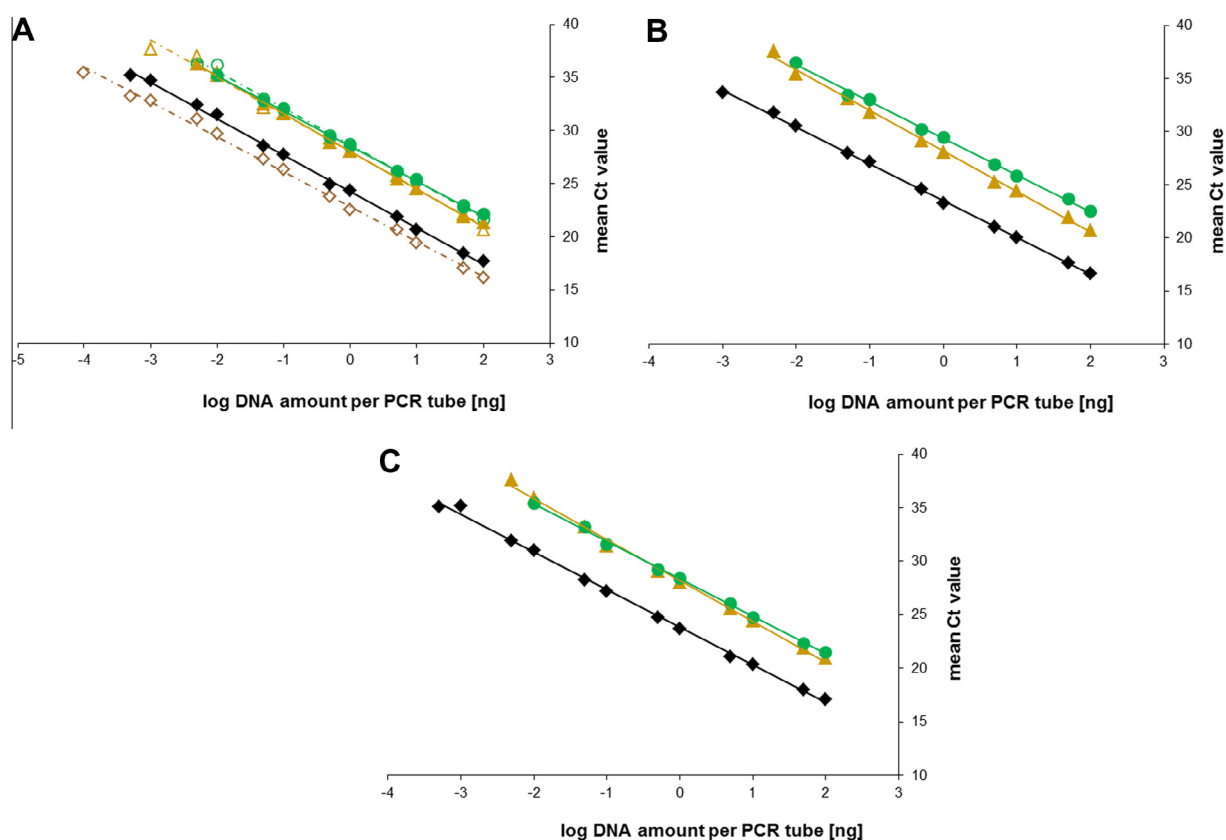


Fig. 1. Standard curves obtained by analysing serially diluted mixtures of DNA extracts. The Ct values are means of two replicates. (A) DNA mixture A: $\blacktriangle$ white mustard: $R^2 = 0.9986$, $y = -3.5488x + 28.039$, $E = 91.3\%$, $\blacklozenge$ black mustard: $R^2 = 0.9985$, $y = -3.4298x + 24.256$, $E = 95.7\%$ and $\bullet$ celery roots: $R^2 = 0.9989$, $y = -3.3007x + 28.551$, $E = 100.9\%$, DNA mixture B: $\triangle$ white mustard: $R^2 = 0.9941$, $y = -3.5031x + 28.017$, $E = 93.0\%$, $\diamond$ brown mustard: $R^2 = 0.9973$, $y = -3.2882x + 22.819$, $E = 101.4\%$ and $\circ$ celery roots: $R^2 = 0.9975$, $y = -3.4533x + 28.647$, $E = 94.8\%$, (B) DNA mixture C: $\blacktriangle$ white mustard: $R^2 = 0.9972$, $y = -3.8035x + 28.173$, $E = 83.2\%$, $\blacklozenge$ black/brown mustard: $R^2 = 0.9992$, $y = -3.4736x + 23.5$, $E = 94.0\%$ and $\bullet$ celery stalks: $R^2 = 0.9980$, $y = -3.4554x + 29.359$, $E = 94.7\%$, (C) DNA mixture D: $\blacktriangle$ white mustard: $R^2 = 0.9963$, $y = -3.8179x + 28.21$, $E = 82.8\%$, $\blacklozenge$ black mustard/brown: $R^2 = 0.9974$, $y = -3.5295x + 23.826$, $E = 92.0\%$ and $\bullet$ leaf celery: $R^2 = 0.9986$, $y = -3.5044x + 28.362$, $E = 92.9\%$.

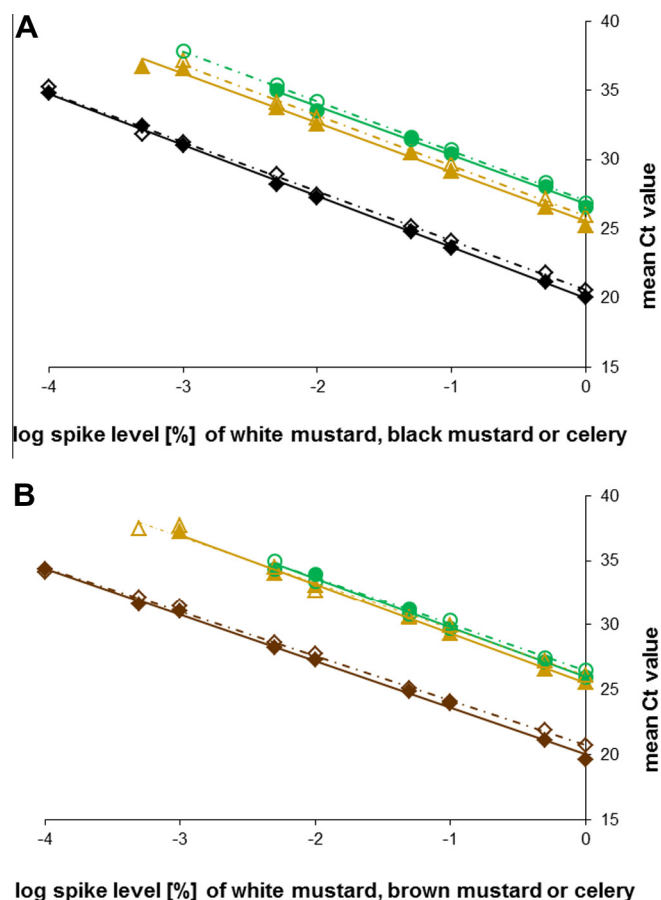


Fig. 2. Standard curves obtained by analysing DNA extracts (40 ng/ μ L) from raw and brewed model sausages containing from 10,000 to 1 mg/kg of each potentially allergenic component. The Ct values are means of two replicates. (A) Model sausages of set 1: raw: $\triangle$ white mustard: $R^2 = 0.9976$, $y = -3.6434x + 25.938$, $E = 88.1\%$, $\diamond$ black mustard: $R^2 = 0.9974$, $y = -3.559x + 20.573$, $E = 91.0\%$ and $\circ$ celery roots: $R^2 = 0.9984$, $y = -3.6078x + 27.017$, $E = 89.3\%$. Model sausages of set 1: brewed: $\triangle$ white mustard: $R^2 = 0.9947$, $y = -3.5458x + 25.549$, $E = 91.4\%$, $\diamond$ black mustard: $R^2 = 0.9990$, $y = -3.6987x + 19.965$, $E = 86.4\%$ and $\bullet$ celery roots: $R^2 = 0.9951$, $y = -3.5327x + 26.821$, $E = 91.9\%$. (B) Model sausages of set 2: raw: $\triangle$ white mustard: $R^2 = 0.9885$, $y = -3.5912x + 26.113$, $E = 89.9\%$, $\diamond$ brown mustard: $R^2 = 0.9980$, $y = -3.4237x + 20.775$, $E = 95.9\%$ and $\circ$ celery roots: $R^2 = 0.9941$, $y = -3.6077x + 26.433$, $E = 89.3\%$. Model sausages of set 2: brewed: $\triangle$ white mustard: $R^2 = 0.9983$, $y = -3.8277x + 25.515$, $E = 82.5\%$, $\diamond$ brown mustard: $R^2 = 0.9979$, $y = -3.5946x + 20.021$, $E = 89.8\%$ and $\bullet$ celery roots: $R^2 = 0.9936$, $y = -3.7958x + 26.022$, $E = 83.4\%$.

stalks and leaf celery; red channel, mean Ct values: 22.15, 22.10 and 21.49, respectively).

Although the selectivity of the primers and probes had already been investigated in the singleplex and duplex assays published previously (Fuchs et al., 2010, 2012, 2013; Palle-Reisch et al., 2013, 2014), DNA extracts from ten common *Brassicaceae* species (broccoli, cauliflower, Chinese cabbage, horseradish, kohlrabi, pak choi, radish, rapeseed, turnip, white cabbage), ten members of the *Apiaceae* (anise, caraway, carrot, coriander, cumin, dill, fennel, lovage, parsley, parsnip) and further plant species (apple, black pepper, cardamom, cinnamon, fenugreek, flaxseed, ginger, marjoram, oregano, rice, rye, sage, summer savory, tarragon, turmeric) were tested for cross-reactivity. No cross-reactivity was observed for the ten *Brassicaceae* species tested. Ginger (mean Ct value: 36.10) resulted in an increase of the fluorescence signal in the green channel. In the yellow channel fenugreek (mean Ct value: 29.83), cumin (mean Ct value: 30.36), ginger (mean Ct value: 32.33), caraway (mean Ct value: 35.09), turmeric (mean

Ct value: 36.17), lovage (mean Ct value: 37.43) and rye (mean Ct value: 39.45) yielded Ct values <40. Cross-reactivities with these species have already been observed in the singleplex assay for black/brown mustard and the duplex assay for white and black/brown mustard (Palle-Reisch et al., 2013, 2014). Since the Ct values were at least 12 cycles higher than those obtained for the positive controls (white, black and brown mustard) and these plant species commonly occur only in traces in mustard containing foodstuffs, the possibility of causing a false positive result is very low. Thus in our opinion these low cross-reactivities do not limit the applicability of the triplex real-time PCR assay in practice.

3.3. LOD, amplification efficiency and reliability

In order to determine the limits of detection (LODs) and the amplification efficiencies (E) of the triplex real-time PCR assay, serially diluted mixtures of DNA extracts from the three mustard species and celery varieties were analysed. The standard curves obtained by plotting the Ct values against the logarithm of the initial DNA amount are shown in Fig. 1. When analysing serially diluted mixtures A and B (Table 1) LODs were found to be 1 and 0.2 pg/ μ L (5 and 1 pg DNA per PCR tube) for white mustard DNA, 0.1 pg/ μ L (0.5 pg DNA per PCR tube) for black mustard DNA, 0.02 pg/ μ L (0.1 pg DNA per PCR tube) for brown mustard DNA and 2 and 1 pg/ μ L (10 and 5 pg DNA per PCR tube) for celery roots DNA, respectively (Fig. 1A). The slopes of the standard curves were in the range from -3.2882 to -3.5488 , indicating amplification efficiencies of 91.3% and 93.0% for white mustard DNA, 95.7% for black mustard DNA, 101.4% for brown mustard DNA and 100.9% and 94.8% for celery roots DNA. Satisfying LODs and amplification efficiencies were also obtained when mixtures containing DNA from white mustard, black/brown mustard and celery stalks or leaf celery (DNA mixtures C and D, Table 1) were analysed. Celery stalks DNA (Fig. 1B) and leaf celery DNA (Fig. 1C) could be detected down to 2 pg/ μ L (corresponding to 10 pg DNA per PCR tube). Amplification efficiencies for celery stalks and leaf celery DNA were found to be 94.7% and 92.9%, respectively. The LODs and amplification efficiencies achieved with the triplex real-time PCR assay are close to those obtained with the singleplex real-time PCR assays (white mustard assay: LOD: 1 pg DNA/ μ L, $E = 91.8\%$ (Fuchs et al., 2010); black/brown mustard assay: LOD: 0.02 pg DNA/ μ L, $E = 100.6\%/86.2\%$ (Palle-Reisch et al., 2013); celery assay: LOD: 2 pg DNA/ μ L, E (celery roots) = 93.9%, E (celery stalks) = 92.9%, E (leaf celery) = 87.4% (Fuchs et al., 2012)).

In order to investigate if the triplex real-time PCR assay also allows efficient amplification when the DNA templates are present in different ratios, DNA mixtures E–P (Table 1) were analysed. These mixtures were serially diluted down to 2 pg DNA/ μ L of each allergenic component with a 20 ng/ μ L herring sperm DNA solution in order to keep the total DNA amount per PCR tube constant. In general, satisfying amplification efficiencies were obtained for all templates independent if they were present in excess or in deficiency. Analysis of serial dilutions of DNA mixture E, for example, resulted in amplification efficiencies of 94.2% for white mustard, 100.0% for black/brown mustard and 114.5% for celery roots. For serially diluted DNA mixture G amplification efficiencies for white mustard, black/brown mustard and celery roots were found to be 88.8%, 85.0% and 91.6%, respectively.

In another series of experiments the LODs and amplification efficiencies of the triplex assay were investigated by analysing two sets of model sausages containing defined amounts (10,000, 5000, 1000, 500, 100, 50, 10, 5, 1 mg/kg) of either white mustard, black mustard or celery roots (model sausage set 1) or white mustard, brown mustard or celery roots (model sausage set 2). In order to investigate any influence of food processing on the detectability

Table 2

Reliability of the triplex real-time PCR assay near the LOD. DNA extracts from raw and brewed model sausages, set 1 (A) and set 2 (B), were analysed in 20 replicates, applying 200 ng total DNA amount per PCR tube.

(A)															
Spike level (mg/kg)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
Model sausage set 1	White mustard					Black mustard					Celery				
Raw															
50	34.52	34.71	34.09	0.54	1.57	28.97	28.82	28.89	0.19	0.66	34.61	36.08	35.10	0.80	2.29
	33.78	33.76				28.74	28.69				35.78	33.88			
	34.70	33.95				28.79	29.05				–	35.24			
	34.54	34.13				28.90	29.02				34.93	35.33			
	33.42	33.70				28.95	29.43				35.23	35.88			
	33.62	34.20				29.07	28.98				35.70	34.85			
	33.63	33.76				29.02	28.69				34.04	34.86			
	35.53	33.82				28.75	28.97				33.92	34.83			
	33.43	33.95				28.54	28.77				36.19	34.78			
	34.07	34.67				28.91	28.76				36.66	34.13			
10	39.11	37.58	36.83	1.08	2.93	30.54	31.81	31.40	0.42	1.32	–	–	n.c.	n.c.	n.c.
	37.05	38.75				32.50	31.46				–	36.05			
	36.38	35.60				31.39	31.29				36.68	36.88			
	37.04	36.76				31.87	31.02				–	37.14			
	36.92	36.37				31.93	31.20				–	36.73			
	–	–				31.11	31.39				37.83	37.32			
	35.08	36.14				31.12	31.61				–	–			
	35.83	35.57				31.54	31.10				–	–			
	37.40	–				31.05	31.44				36.96	36.92			
	37.69	36.88				31.24	31.37				37.42	37.43			
5	36.89	–	n.c.	n.c.	n.c.	31.98	32.71	32.44	0.34	1.06	36.08	–	n.c.	n.c.	n.c.
	–	37.34				32.06	33.02				36.21	–			
	36.95	–				32.59	32.28				37.10	–			
	37.43	37.50				31.68	32.30				37.22	–			
	–	–				32.65	32.43				–	36.24			
	–	36.42				32.80	32.48				36.13	–			
	37.87	36.53				32.63	31.98				–	–			
	35.41	–				33.02	32.43				36.10	36.31			
	36.14	–				32.26	32.35				–	–			
	–	37.71				32.55	32.53				36.76	36.07			
1	–	–	n.c.	n.c.	n.c.	32.85	35.59	35.04	1.53	4.37	–	–	n.c.	n.c.	n.c.
	38.30	–				34.03	–				–	–			
	–	–				34.97	38.41				–	36.35			
	38.04	36.55				35.06	35.05				–	–			
	–	37.50				36.00	34.24				–	–			
	–	–				34.20	35.04				–	–			
	37.02	–				33.39	35.25				–	–			
	37.01	–				39.23	34.61				–	–			
	–	–				34.96	33.90				36.10	–			
	36.83	–				34.51	34.39				–	–			
Brewed															
50	33.56	32.99	34.11	0.70	2.06	28.17	28.33	28.36	0.14	0.49	35.76	34.44	34.84	0.93	2.67
	33.63	34.86				28.32	28.26				35.12	35.36			
	34.14	34.76				28.26	28.53				34.66	34.27			
	36.20	34.48				28.40	28.53				35.22	34.98			
	33.56	34.64				28.61	28.58				34.42	34.41			
	33.87	33.27				28.27	28.38				33.53	37.45			
	34.53	33.79				28.23	28.28				36.28	34.06			
	33.63	33.75				28.05	28.40				35.39	33.49			
	34.17	34.16				28.39	28.33				34.53	34.49			
	34.29	33.91				28.47	28.31				34.87	34.01			
10	36.64	36.25	36.68	0.90	2.45	31.29	31.35	31.11	0.26	0.84	–	–	n.c.	n.c.	n.c.
	36.01	36.90				31.03	30.82				–	37.23			
	38.00	37.66				31.11	31.50				–	–			
	36.63	37.90				31.01	31.16				35.31	–			
	37.58	35.75				30.96	30.78				–	–			
	–	35.21				31.28	31.04				36.92	–			
	36.83	36.07				31.23	31.05				–	–			
	36.22	36.91				31.26	30.60				36.46	–			
	37.98	35.74				31.30	31.69				–	37.53			
	37.46	35.26				30.81	30.94				–	–			
5	–	–	n.c.	n.c.	n.c.	32.07	31.89	32.16	0.44	1.38	36.71	–	n.c.	n.c.	n.c.
	36.19	36.98				33.23	32.04				–	–			
	37.75	–				32.33	32.04				–	–			
	37.63	–				32.65	32.32				–	–			
	36.47	–				31.81	32.37				37.20	–			
	39.13	37.76				31.72	31.69				–	–			

(continued on next page)

Table 2 (continued)

(A)															
Spike level (mg/kg)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
Model sausage set 1	White mustard					Black mustard					Celery				
1	36.78	–				31.41	32.63				–	–			
	37.77	–				32.50	32.05				–	38.33			
	–	36.76				32.07	31.90				–	–			
	–	38.10				32.78	31.65				–	–			
	38.15	–	n.c.	n.c.	n.c.	33.52	34.85	34.73	0.79	2.27	–	–	n.c.	n.c.	n.c.
	–	37.78				34.62	35.15				–	–			
	–	–				36.60	33.97				–	–			
	–	–				34.61	34.66				–	–			
	–	37.41				35.18	34.85				–	–			
	–	–				34.14	33.22				–	36.68			
	–	–				34.65	35.05				–	–			
	–	–				34.54	35.08				–	–			
	38.04	–				34.91	34.03				–	–			
	–	–				34.66	36.27				–	–			
(B)															
Spike level (mg/kg)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
Model sausage set 2	White mustard					Brown mustard					Celery				
Raw															
50	34.28	34.17	34.48	0.63	1.84	29.13	29.25	28.98	0.20	0.70	34.98	34.25	34.61	0.68	1.97
	34.71	34.00				29.28	28.52				34.70	33.91			
	35.03	34.47				28.98	29.08				34.35	34.07			
	33.97	34.27				29.11	28.97				33.99	34.95			
	34.01	34.02				28.93	29.09				33.06	35.86			
	35.45	34.36				28.64	28.93				34.37	33.82			
	34.42	33.75				28.88	29.32				35.05	35.34			
	34.05	36.31				28.72	28.93				35.63	34.94			
	35.04	35.12				29.02	28.97				34.29	35.33			
	34.28	33.85				28.95	28.80				34.55	34.78			
10	37.48	37.24	36.82	1.01	2.73	31.91	32.01	31.55	0.39	1.22	–	35.87	36.33	0.83	2.30
	35.83	35.26				31.28	30.98				35.79	35.63			
	36.42	35.82				31.63	32.00				35.35	35.69			
	36.36	–				31.35	30.97				36.07	36.30			
	38.28	36.10				31.27	31.66				–	35.22			
	37.31	36.69				31.08	31.53				37.29	36.96			
	36.66	38.41				32.00	31.56				–	36.09			
	38.64	38.09				31.51	31.90				–	36.05			
	37.11	35.68				31.72	31.00				37.08	38.29			
	36.04	36.14				31.42	32.27				36.36	37.29			
5	37.12	–	37.49	0.81	2.17	32.09	32.39	32.35	0.33	1.02	–	–	n.c.	n.c.	n.c.
	–	–				32.79	31.89				–	–			
	39.55	38.10				32.29	32.33				–	36.89			
	36.47	37.42				32.52	32.75				–	37.49			
	37.62	–				32.67	31.79				35.85	37.34			
	–	37.98				32.14	32.10				37.74	–			
	37.63	37.10				32.21	32.10				–	–			
	37.63	–				32.86	32.37				–	–			
	36.43	36.90				32.21	33.05				36.67	37.11			
	38.14	36.80				32.14	32.37				36.71	–			
1	–	–	n.c.	n.c.	n.c.	34.05	33.82	34.87	0.79	2.28	–	–	n.c.	n.c.	n.c.
	–	–				34.08	34.80				–	–			
	–	–				35.03	36.44				–	37.09			
	–	–				36.22	35.84				–	–			
	–	–				35.43	35.27				36.94	–			
	–	–				33.87	33.95				–	–			
	37.74	–				34.35	34.80				–	–			
	–	–				34.62	35.42				–	–			
	37.98	–				35.71	34.64				–	–			
	–	–				34.96	34.10				–	–			
Brewed															
50	33.90	33.99	34.37	0.56	1.64	28.11	27.89	28.26	0.16	0.58	35.23	33.67	34.39	0.69	2.00
	33.43	35.69				28.26	28.55				35.41	34.54			
	34.10	35.19				28.41	28.14				33.81	34.38			
	34.84	33.98				28.26	28.27				34.85	33.98			
	34.15	33.74				28.26	28.43				33.25	35.87			
	33.96	34.82				28.26	28.08				34.78	34.03			
	33.79	34.18				28.15	28.07				34.28	34.68			
	34.96	34.56				28.31	28.48				34.58	34.50			

Table 2 (continued)

(B)													
Spike level (mg/kg)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value
Model sausage set 2	White mustard					Brown mustard					Celery		
10	34.12	34.50	37.10	1.24	3.34	28.16	28.47	30.85	0.29	0.95	33.55	33.92	36.42
	34.76	34.75				28.40	28.30				33.48	34.91	
	35.80	37.43				30.61	30.66				35.80	37.22	
	35.38	–				30.95	31.15				36.31	–	
	39.11	39.02				30.78	30.67				–	36.95	
	37.62	36.68				30.71	31.54				37.13	–	
	38.05	–				30.81	30.52				36.34	35.81	
	36.99	39.69				31.01	31.21				37.08	37.05	
	36.16	37.24				30.51	30.99				36.91	–	
	35.81	36.01				31.06	30.87				35.29	–	
5	37.27	36.23	n.c.	n.c.	n.c.	30.72	31.24	31.68	0.62	1.96	–	37.08	n.c.
	36.09	37.18				30.65	30.36				35.71	35.23	
	–	38.37				31.89	31.26				–	–	
	38.71	–				31.39	32.11				–	39.57	
	–	36.85				31.07	31.71				37.15	36.98	
	–	–				31.17	31.54				–	–	
	36.39	37.20				31.85	31.98				35.13	36.81	
	–	35.72				32.73	31.18				37.06	35.55	
	38.13	37.73				31.73	32.12				–	–	
	36.84	–				31.66	31.60				–	–	
1	–	–	n.c.	n.c.	n.c.	31.71	32.99	34.28	0.77	2.24	35.48	–	n.c.
	35.94	34.94				31.96	30.03				37.12	–	
	–	–				34.07	34.53				–	36.73	
	–	–				33.40	34.01				–	–	
	–	–				34.47	33.60				–	–	
	–	37.07				33.64	35.48				–	37.29	
	–	–				33.87	33.92				–	–	
	–	–				33.64	34.18				–	36.19	
	–	37.47				36.11	35.41				–	36.78	
	–	–				34.31	34.92				–	–	
	–	–				33.70	34.78				–	–	
	36.93	–				34.46	33.03				–	–	

–: no increase of the fluorescence signal within 40 cycles.

n.c.: not calculated.

of mustard and celery, not only DNA extracts from raw but also from brewed sausages were analysed. The DNA extracts were adjusted to 40 ng/μL (corresponding to 200 ng DNA per PCR tube). The standard curves obtained are shown in Fig. 2A (set 1) and 2B (set 2). In raw sausages of set 1, the LODs were found to be 10 mg/kg for white mustard and celery and 1 mg/kg for black mustard. Amplification efficiencies, calculated from the slopes of the standard curves, were found to be 88.1% for white mustard, 91.0% for black mustard and 89.3% for celery. Similar LODs and amplification efficiencies were obtained in case of the brewed model sausages of set 1. When analysing model sausages of set 2, LODs for white mustard, brown mustard and celery were found to be 5 mg/kg, 1 mg/kg and 50 mg/kg in raw and 10 mg/kg, 1 mg/kg and 50 mg/kg in brewed sausages, respectively.

In order to investigate the reliability of the triplex assay near the LODs, DNA extracts from raw and brewed model sausages of both sets, containing 50, 10, 5 or 1 mg/kg of each allergenic component, were adjusted to 40 ng/μL (corresponding to 200 ng DNA per PCR tube) and analysed in 20 replicates. The Ct values are given in Table 2A (set 1) and 2B (set 2). Fig. 3 shows the percentages of replicates yielding an increase of the fluorescence signal within 40 cycles. The figure indicates that the triplex real-time PCR assay allows the detection of 1 mg/kg black/brown mustard in raw and brewed sausages with a probability ≥95%. It can be seen that the triplex assay is not as sensitive for white mustard and celery (which is in agreement with the singleplex and duplex assays published previously). Fifty mg/kg white mustard and celery can,

however, be detected with a probability of at least 95% in both raw and processed sausages.

3.4. Analysing commercial foodstuffs

Twenty-three commercial foodstuffs were analysed with the triplex real-time PCR assay to verify implementation of existing legislation on food allergen labelling. Some food products were declared to contain or possibly contain mustard and/or celery whereas other products did not have any information regarding the presence of mustard and celery. According to the ingredient list, twelve products potentially contained both mustard and celery.

DNA of high purity (A_{260}/A_{280} ratios between 1.8 and 2.0) could be extracted from 15 out of 23 of the products. In general, DNA concentrations ranged from 100 to 1200 ng/μL. However, extracts with very low DNA concentrations (≤ 13.5 ng/μL) were obtained from products containing high water, salt and/or fat content, such as dressings, spice mixes or sauces. After adjusting the DNA concentration to 40 ng/μL (200 ng DNA per PCR tube) the extracts were analysed with the triplex assay (extracts with concentrations <40 ng/μL were used without further dilution). The results are summarised in Table 3. Mustard was detected in seven out of eight products declared to contain mustard. Five of these products yielded an increase of the fluorescence signal in the green and the yellow channel, indicating that they contained both white and black/brown mustard. In meat spread 1 only white mustard

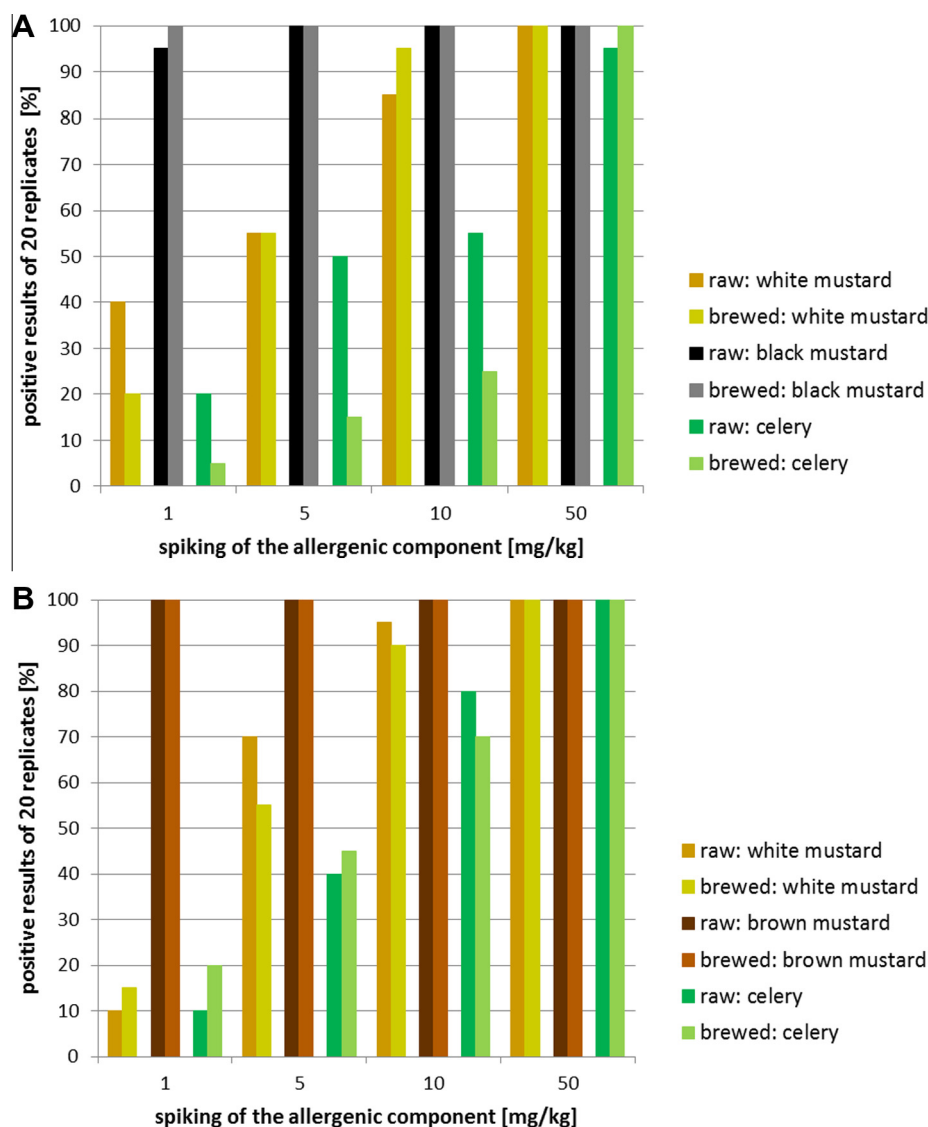


Fig. 3. Reliability of the triplex assay near the LODs by analysing DNA extracts from raw and brewed model sausages: (A) model sausages of set 1, (B) model sausages of set 2.

was detected, whereas the American sauce gave positive signal only for black/brown mustard. In one sample (mayonnaise) declared to contain mustard neither white nor black/brown mustard was detected.

In two out of nine foodstuffs labelled to potentially contain mustard (Mediterranean vegetable pan and spice mix for fish 2), black/brown mustard was detected.

Five of the six products that did not have any hint on the presence of mustard were labelled correctly, since none of the three mustard species was detected. However, one product (spice mix for fish 3), was found to contain white as well as black/brown mustard although mustard was not declared at all.

Only for two out of six products that were declared to contain celery (spaghetti Bolognese and meat spread 2), an increase of the fluorescence signal was observed in the red channel. We assume that in the other four products (fried noodles curry, meat spread 1, meat spread 3 and vegetable aspic) the celery concentration was below 50 ppm and thus below the LOD of the triplex assay. Two out of ten foodstuffs that potentially contain celery yielded positive results. Celery was not detected in the seven products that did not have any hint on the presence of celery.

Inhibition control experiments showed that none of the negative PCR results was caused by polymerase inhibition.

4. Concluding remarks

The triplex real-time PCR assay allows the detection of three mustard species, white mustard (*S. alba*), black mustard (*B. nigra*) and brown mustard (*B. juncea*), and three celery varieties, celery roots (*A. g. var. rapaceum*), celery stalks (*A. g. var. dulce*) and leaf celery (*A. g. var. secalinum*) in food. White mustard is detected in the green channel, black/brown mustard in the yellow channel and the three celery varieties in the red channel.

Cross-reactivity tests with ten *Brassicaceae*, ten *Apiaceae* and further plant species showed that the triplex assay does not detect other *Brassicaceae* species playing a role in food industry, e.g. broccoli, cabbage, cauliflower and rapeseed. Low cross-reactivities were found with fenugreek, cumin, ginger, caraway, turmeric, lovage and rye. These species yielded an increase of the fluorescence signal in the yellow channel, however, at rather high Ct values ($\Delta Ct \geq 12$, compared to black/brown mustard DNA).

Table 3

Analysis of commercial foodstuffs. Declaration: + contains mustard/celery, ± may contain mustard/celery, – mustard/celery not declared; total DNA amount: 200 ng per PCR tube.

Foodstuff	Declaration	PCR result (mean Ct value)		Declaration	PCR result (mean Ct value)
	Mustard	Green channel	Yellow channel	Celery	Red channel
Fried noodles curry	+	+ (37.12)	+ (36.08)	+	– (IC: 22.16)
Meat spread 1	+	+ (35.78)	– (IC: 16.56)	+	– (IC: 21.70)
Vegetarian nuggets	+	+ (30.32)	+ (24.61)	±	– (IC: 22.05)
American sauce	+	– (IC: 21.01) ¹	+ (34.9) ¹	–	– (IC: 21.93) ¹
Yoghurt dressing garlic	+	+ (35.54) ²	+ (32.49) ²	–	– (IC: 22.00) ²
Yoghurt dressing Italian herbs	+	+ (35.46) ³	+ (32.43) ³	–	– (IC: 22.22) ³
Sausage (salanettis)	+	+ (32.68)	+ (35.19)	–	– (IC: 21.90)
Mayonnaise	+	– (IC: 20.97) ⁴	– (IC: 16.33) ⁴	–	– (IC: 21.95) ⁴
Spaghetti Bolognese	±	– (IC: 20.83)	– (IC: 16.33)	+	+ (29.18)
Fried noodles	±	– (IC: 21.11)	– (IC: 16.44)	±	– (IC: 22.10)
Fried noodles chicken	±	– (IC: 21.00)	– (IC: 16.47)	±	+ (38.73)
Fried noodles duck 1	±	– (IC: 21.13)	– (IC: 16.28)	±	– (IC: 22.08)
Mediterranean vegetable pan	±	– (IC: 21.02)	+ (37.11)	±	– (IC: 22.15)
Pasta ai funghi	±	– (IC: 21.25)	– (IC: 16.61)	±	– (IC: 21.97)
Sausage (brat maxe)	±	– (IC: 20.86)	– (IC: 16.52)	±	– (IC: 21.84)
Spice mix for fish 1	±	– (IC: 21.03) ⁵	– (IC: 16.43) ⁵	±	– (IC: 22.10) ⁵
Spice mix for fish 2	±	– (IC: 21.13) ⁶	+ (37.12) ⁶	±	– (IC: 22.05) ⁶
Meat spread 2	–	– (IC: 21.17)	– (IC: 16.29)	+	+ (36.54)
Meat spread 3	–	– (IC: 21.18)	– (IC: 16.68)	+	– (IC: 22.23)
Vegetable aspic	–	– (IC: 21.05)	– (IC: 16.41)	+	– (IC: 21.88)
Fried noodles duck 2	–	– (IC: 21.14)	– (IC: 16.43)	±	+ (36.24)
Aspic	–	– (IC: 20.86)	– (IC: 16.62)	–	– (IC: 21.97)
Spice mix for fish 3	–	+ (35.47) ⁷	+ (33.25) ⁷	–	– (IC: 21.78) ⁷

PCR result:

+: increase of the fluorescence signal within 40 cycles.

–: no increase of the fluorescence signal within 40 cycles.

IC: inhibition control.

¹2.5, ²4.5, ³10.0, ⁴22.5, ⁵67.5 ng DNA per PCR tube.

Cross-reactivities with the species mentioned above will therefore not affect the accuracy of the triplex assay.

Results obtained by analysing a variety of mixtures of extracts from the three mustard species and celery varieties indicate that the triplex assay allows the detection of traces of DNA of the allergenic components in spite of an excess of the other templates. Analysis of extracts from model sausages containing defined concentrations of mustard and celery showed that the triplex assay is applicable to both raw and processed foods.

In our opinion the triplex real-time PCR assay is highly suitable for being applied in routine analysis since it is selective, sensitive, applicable to raw and processed foods and, due to multiplexing, offers the possibility to save time and costs.

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